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IN VITRO-IN VIVO CORRELATIONS OF DRUG RELEASE FOR BIODEGRADABLE POLYMER STENT

Wang Xintong

SCHOOL OF MATERIALS SCIENCE & ENGINEERING

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Abstract

The feasibility of inhibition effect of control-released rapamycin from biodegradable polymer matrices on human smooth muscle cells and in vivo neointimal hyperplasia was investigated in this study. Results obtained enable us to understand more of the impact of rapamycin on smooth muscle cells and on in vivo restenosis for future modifications of rapamycin release profiles.

A degradation controlled release kinetics was observed for rapamycin release in amorphous polymer PLGA 53/47 and PLGA 50/50. Higher rapamycin loading resulted in a faster release in buffer 7.4 due to the plasticization and base-catalysis mechanisms. The addition of plasticizer polyethylene glycol accelerated rapamycin release to a linear profile. But a higher concentration of PEG as 10 wt% did not show a faster release than 5 wt% PEG indicating that the hydrophilic PEG has limited plasticization effect on hydrophobic rapamycin release. Long and complex end group such as lauryl ester in PLGA 50/50 greatly hindered the polymer degradation and rapamycin release although the polymer is of a comparatively lower molecular weight, which suggests the polymer structure determines the drug release rate to a large degree. Polymer coating virtually slower rapamycin release since an extra step of diffusion had to be fulfilled for release. Thus rapamycin release was modified to several different release profiles successfully.

Rapamycin release in cell culture conditions exhibited a significantly faster profile than in buffer system. Under cell culture conditions, however, plentiful FKBP-12 in proliferating smooth muscle cells formed tight complex with a considerable amount of rapamycin to inhibit the function of mTOR. Lipoproteins and albumins in serum also play an important

Abstract

role in enhancing the solubility of rapamycin in cell culture medium. Results of this study are instructive in prediction of release profiles in cell culture or even *in vivo* studies.

Results of BrdU assay indicated the inhibition of rapamycin to DNA synthesis of proliferating human smooth muscle cells, which represented the inhibition to cell proliferation. Rapamycin also inhibited the cell growth to 50% as proved by cell metabolic activity study. The influence of rapamycin in cell proliferation and growth was cytostatic rather than cytotoxic. However, when inhibition effect from rapamycin was removed, cells still maintained the ability to re-enter the cell cycle and proliferate, which was investigated in cell recovery study. Interestingly, cells inhibited by rapamycin of high concentration still managed to proliferate although in a very low level, this is probably because cells still maintained a set of mechanisms when these pathways were inhibited and continued to proliferate. Rapamycin inhibited cell attachment to polymer films. The elimination of smooth muscle cell attachment enables unhindered rapamycin release and avoids the hyperplasia in stents.

Bi-layered PLLA/PLGA stents with and without rapamycin were successfully inserted into rabbit femoral arteries for 2 weeks. Histochemical stainings show a significant neointimal thickening but a mild inflammatory reaction induced by control polymer stent. The new intima was rich in fibrin and collagen with smooth muscle cells buried in the matrix. Control-released rapamycin greatly reduced the neointimal hyperplasia, inhibited cell proliferation in intima and prevented the inflammatory response to rapamycin-eluting stents. The 2% rapamycin-loaded stent has a higher release rate and resulted in more inhibition effects than 1% rapamycin-loaded stent. In the two-week *in vivo* study, it is apparent that a higher the rapamycin loading and a faster release rate will result in more inhibition effects.

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Table of Contents

Abstract	i
Acknowledgements	iii
List of Figures	ix
List of Tables	xiii
Chapter 1 Introduction	1
1.1 Background	1
1.2 Problem statement	3
1.3 Objective	4
1.4 Scope	5
1.5 Overview	7
Chapter 2 Literature Review	8
2.1 Introduction to intravascular stents	8
2.2 Current status of metallic stents	11
2.2.1 Bare metallic stents	11
2.2.2 Non drug eluting coated metallic stents	13
2.2.3 Drug-eluting coated metallic stents	15
2.3 Polymeric stents	16
2.3.1 Non biodegradable polymer stents	18
2.3.2 Biodegradable non drug-eluting polymer stents	19
2.3.3 Drug-eluting biodegradable polymer stents	21

Table of Contents

2.3.4 Limitations of polymer stents	22
2.4 The ideal stent design	23
2.4.1 Mechanical properties	23
2.4.2 Biocompatibility	24
2.4.3 Deployment	24
2.4.4 Expandability	24
2.4.5 Drug-loading ability	24
2.4.6 Biodegradability	25
2.5 Biocompatible polymers	25
2.5.1 Biocompatibility of polymers	25
2.5.2 Biodegradable polymers	29
2.5.3 Biodegradation of polymers	32
2.6 Restenosis	35
2.6.1 Definition of restenosis	35
2.6.2 Difference between atherosclerosis and restenosis	36
2.6.3 In-stent restenosis	39
2.6.4 Drugs used during stenting	39
2.6.5 Radiotherapy for ISR	44
2.7 Controlled local drug delivery	45
2.7.1 Controlled release devices	45
2.7.2 Drug release kinetics	46
2.7.3 Introduction to in vitro-in vivo correlations	52
2.8 Inhibition effect of sirolimus on cell growth and proliferation	54
2.8.1 Cell cycle	54
2.8.2 mTOR (mammalian Target of Rapamycin)	56

2.8.3 Effect of mTOR on cell growth and proliferation	56
2.8.4 Inhibition effects of sirolimus on mTOR signaling pathways	57
2.9 Summary	58
 Chapter 3 Materials and Experimental Details	 60
3.1 Materials	60
3.1.1 Polymers	61
3.1.2 Reagents and drug	62
3.1.3 Cell line and culture medium	62
3.2. Sample preparation	63
3.2.1 Polymer film preparation	63
3.2.2 Polymer films for in vitro drug release study	67
3.2.3 Polymer film sterilization for cell culture studies	68
3.2.4 Cell culture	69
3.2.5 Polymer film and stent preparations for in vivo studies	71
3.2.6 <i>In vivo</i> studies	73
3.3 Equipment and testing techniques	74
3.3.1 High Performance Liquid Chromatography for drug release study	74
3.3.2 Gel Permeation Chromatography for degradation study	77
3.3.3 Modulated Differentiation Scanning Chromatography for study of polymer thermal properties	77
3.3.4 WST-8 colorimetric assay for Cell growth study	78
3.3.5 BrdU colorimetric assay for DNA synthesis study	80
3.3.6 Scanning Electron Microscope for polymer degradation and cell attachment study	82
3.3.7 Cell Growth Recovery Study	84

Table of Contents

3.3.8 Water uptake and mass loss study	84
 Chapter 4 In vitro Rapamycin Release Study	 86
4.1 Introduction	86
4.2 Screening of materials	87
4.2.1 Degradation study	87
4.2.2 Thermal properties	100
4.3 Drug release study	104
4.3.1 Different drug loadings	104
4.3.2 Plasticization effect	106
4.3.3 Different polymers	109
4.3.4 Polymer coating effect	112
4.4 Summary	113
 Chapter 5 Drug Release Study in Cell Culture	 114
5.1 Introduction	114
5.2 Degradation study	114
5.3 Drug release study	116
5.3.1 Rapamycin release by percentage	116
5.3.2 Rapamycin release by amount	122
5.4 Summary	124
 Chapter 6 Effect of Rapamycin on Cell Growth and Proliferation	 125
6.1 Introduction	125
6.2 Control-released rapamycin inhibits DNA synthesis in SMCs	126

6.3 Effect of rapamycin on cell growth	131
6.4 Cell growth recovery study	136
6.5 Cell attachment to drug-eluting polymer film	142
6.6 Summary	149
 Chapter 7 In vivo Inhibition Effect on Neointimal Hyperplasia by Rapamycin	 150
7.1 Introduction	150
7.2 Stent expansion evaluation	151
7.3 Histochemical and immunohistological studies	154
7.3.1 Morphology and inflammatory studies with Hematoxylin and Eosin staining	154
7.3.2 Fibrin deposition study with Masson thichrome and anti α -actin staining	158
7.3.3 Cell proliferation study with Anti Ki-67 staining	162
7.4 Summary	164
 Chapter 8 Conclusion and Recommendations	 165
8.1 Conclusions	165
8.2 Recommendations	168
 References	 172

List of Figures

Figure 2.1	Eukaryotic cell cycle	55
Figure 3.1	Structure of rapamycin	61
Figure 3.2	Multi-layer polymer films. (a) Double-layer polymer film; (b) Triple-layer polymer film	65
Figure 3.3	Automatic film applicator AG-2150 from GARDNER, Germany	65
Figure 3.4	Demonstration of polymer sample in 96-well plate	67
Figure 3.5	Demonstration of polymer sample in 96-well plate	71
Figure 3.6	A: HPLC, B: GPC	75
Figure 3.7	HPLC graphs of the calibration	76
Figure 3.8	Calibration curve of rapamycin	76
Figure 3.9	WST-8 mechanisms	79
Figure 4.1	GPC graphs of degradation studies. (a): PLGA 53/47 (Sample A in Table 3.3); (b): PLGA 53/47 with 5% PEG 4000 (Sample C); (c): PLGA 50/50 (Sample E); (d): PLGA 53/47 coated with 50/50 (Sample F)	90
Figure 4.2	Molecular weight of PLGA 53/47 with and without PEG 4000 and PLGA 50/50	91
Figure 4.3	Molecular weight losses of PLGA 53/47 and PLGA 50/50	93
Figure 4.4	Water uptake of PLGA 53/47 and plasticized PLGA 53/47 with PEG	94
Figure 4.5	Mass losses of PLGA 53/47 and plasticized PLGA 53/47 with PEG	94
Figure 4.6	Surfaces morphologies of PLGA 53/47 and plasticized PLGA 53/47 with PEG without immersion and after immersion for 7 days. (a): Sample A at day 0; (b): Sample A at day 7; (c): Sample C at day 0; (d): Sample C at day 7; (e): Sample D at day 0; (f): Sample D at day 7	95

List of Figures

Figure 4.7	Surface morphologies of PLGA 53/47 and plasticized PLGA 53/47 with PEG with immersion of 14 and 21 days. (a): Sample A at day 14; (b): Sample A at day 21; (c): Sample C at day 14; (d): Sample C at day 21; (e): Sample D at day 14; (f): Sample D at day 21	96
Figure 4.8	Cross sections of PLGA 53/47 and plasticized PLGA 53/47 with PEG without immersion and after immersion for 14 days. (a): Sample A at day 0; (b): Sample A at day 14; (c): Sample C at day 0; (d): Sample C at day 14; (e): Sample D at day 0; (f): Sample D at day 14	98
Figure 4.9	Chemical structure of PLGA 50/50 with lauryl ester end group	99
Figure 4.10	Glass transition temperatures of PLGA 53/47 and PLGA 50/50 during immersion	101
Figure 4.11	Glass transition temperature of plasticized PLGA 53/47	102
Figure 4.12	Glass transition temperature of PLGA 53/47 coated with PLGA 50/50	103
Figure 4.13	Rapamycin of different drug loadings released from PLGA 53/47	104
Figure 4.14	The release rate constant of PLGA 53/47 with 1% and 2 % of rapamycin	106
Figure 4.15	Rapamycin release from PLGA 53/47 with and without addition of 5 wt% PEG	107
Figure 4.16	Rapamycin release profiles from PLGA 53/47 plasticized by 5 wt% and 10 wt% PEG	108
Figure 4.17	Rapamycin release constant of PLGA 53/47 with 5 wt% and 10 wt% PEG	109
Figure 4.18	Rapamycin release profiles of PLGA 53/47 and PLGA 50/50	111
Figure 4.19	Release constant of PLGA 53/47 and PLGA 50/50	111
Figure 4.20	Rapamycin release profiles of PLGA 53/47 with blank PLGA 50/50 coating	113
Figure 5.1	Degradation study of PLGA 53/47 in buffer and in cell culture medium	115
Figure 5.2	Compare rapamycin release from PLGA 53/47 (1% rapa) in cell culture medium with in buffer 7.4	117

Figure 5.3	Compare rapamycin release from PLGA 53/47 (2% rapa) in cell culture medium with in buffer 7.4	117
Figure 5.4	Compare rapamycin release from PLGA 53/47 (1% rapa, 5% PEG) in cell culture medium with in buffer 7.4	118
Figure 5.5	Rapamycin release in cell culture medium	119
Figure 5.6	Rapamycin release profile by amount	122
Figure 6.1	BrdU study for DNA synthesis in SMCs	127
Figure 6.2	DNA synthesis by percentage of control samples	130
Figure 6.3	Cell growth study by WST-8 assay	132
Figure 6.4	Cell growth by percentage of control samples	134
Figure 6.5	Cell recovery after rapamycin treatment for 7 days	137
Figure 6.6	Cell recovery after rapamycin treatment for 14 days	139
Figure 6.7	Cell recovery comparison between rapamycin treatment of day 7 and day 14	141
Figure 6.8	Different growth curves due to different treatments	142
Figure 6.9	Cell attachment to PLGA 5347 film at day1. (a): Cell attachment to film with 1 wt% rapamycin; (b): Cell attachment to control film without rapamycin	144
Figure 6.10	Cell attachment to PLGA 5347 film at day3. (a): Cell attachment to film with 1 wt% rapamycin; (b): Cell attachment to control film without rapamycin	145
Figure 6.11	Cell attachment to PLGA 5347 film at day7. (a): Cell attachment to film with 1 wt% rapamycin; (b): Cell attachment to control film without rapamycin	147
Figure 6.12	(a) hCASMCM on cell culture plastic by inverted microscope x 400 (Passage 4); (b) hCASMCM on control PLGA 5347 film by SEM (x 500) 5 days after cell seeding	148
Figure 7.1	Stent self-expansion with and without sterilization	152
Figure 7.2	Stent dimension after insertion (a): Stent in rabbit femoral artery; (b): Stent taken out from artery. Outer diameter of fully expanded stent is around 2 mm, with marker at two ends; (c): length of fully expanded stent is about 9 mm.	153

List of Figures

Figure 7.3	Morphology of femoral artery after stenting for 2 weeks with H&E staining. (a): artery with 1% rapamycin-loaded stent (Magnification $\times 50$), (b): artery with 2% rapamycin-loaded stent (Magnification $\times 50$), (c): artery with control stent (Magnification $\times 50$). <i>Abbreviations:</i> I: intima, L: lumen, M: media layer, S: stent, T: thrombus.	156
Figure 7.4	Inflammatory reactions after 2 weeks (stained with H&E). (a): tissue with control stent. L: location of the lumen, F: fibrin, M: macrophage. Arrow shows the monocytes while the arrowhead points to the lymphocyte. The polymorphonuclear cells are marked in a circle. (b): tissue with 1% stent. L: location of the lumen, S: stent material. Arrow shows polymorphonuclear cells, while the arrowhead shows the lymphocyte. Monocytes are marked in a circle. (Magnification $\times 400$)	157
Figure 7.5	Angiogenesis in neointima. The arrows point to capillaries. <i>Abbreviations:</i> A: arteriole, V: venule, F: fibrin. (Magnification $\times 200$)	158
Figure 7.6	Masson trichrome staining of femoral artery with stenting for 2 weeks. From (a) to (d): femoral artery with control stent (Magnification: $\times 50$, $\times 100$, $\times 200$ respectively), (e): femoral artery with 2% rapamycin-loaded stent (Magnification: $\times 200$). <i>Abbreviations:</i> A: adventitia, I: intima, M: media, S: stent. <i>Symbols:</i> * collagen, ** fibrin. Arrow in (b) shows the internal elastic lamina; arrow in (c) points to new arteriole in the intima; arrowhead in (e) shows the fibrin deposition around stent.	160
Figure 7.7	Anti α -actin staining after stenting for 2 weeks (Magnification: $\times 400$). (a): the media layer; (b): the intima layer. <i>Abbreviation:</i> A: Adventitia, M: media.	161
Figure 7.8	Anti Ki-67 staining of femoral artery with stenting for 2 weeks. (a): artery with the control stent (Magnification $\times 200$), (b): artery with 1% rapamycin-loaded stent (Magnification $\times 400$), (c): artery with 2% rapamycin-loaded stent (Magnification $\times 400$). <i>Abbreviations:</i> I: intima, M: media, S: stent.	163

List of Tables

Table 3.1	Properties of polymers used	60
Table 3.2	Supplements in cell culture medium	62
Table 3.3	Polymer film combinations in buffer release study	68
Table 3.4	Polymer films used in cell culture study	69
Table 3.5	Polymer films used in in vivo study	72
Table 5.1	Rapamycin release by percentage	119
Table 5.2	Rapamycin release by amount	124

Chapter 1

Introduction

1.1 Background

Percutaneous Transluminal Cardiovascular Angioplasty (PTCA) has become a main therapeutic method to treat cardiovascular occlusion, especially for coronary occlusion. However, the consequent re-occlusion (restenosis) of blood vessel, caused by inadvertent injury to the vessel wall, greatly limits its use¹. To overcome this short coming, cardiovascular stents have been developed to support the targeted vessel, keep it patent and thus avoid restenosis. As a result, coronary stents have received considerable attention, as they have been shown to significantly reduce restenosis to acceptable levels. To date, there has been vast development in the design and material used for coronary stents^{2,3}.

Metallic coronary stents are currently available in market, but they do not eliminate the problem of restenosis, especially in vessels of relatively smaller diameters. With an increase in the usage of metallic stents, statistics, thus far, have shown that in-stent restenosis (ISR) occurs in 10-50% of such cases. It is estimated that about 150,000 patients were influenced by ISR in 1999 in the U.S.^{4,5} Unlike angioplasty, the effect of stent injury is more chronic, involving a greater degree of injury and foreign body responses followed by inflammation, and so leading to a more pronounced neointimal lesion.

To improve the drawbacks of PTCA and metallic stents, polymer coated metallic and drug-eluting stents with various effective anti-thrombotic and anti-proliferative agents have been developed, which have been shown to reduce ISR. The study by *Sousa et al.* in human coronary arteries with a sirolimus-coated BX velocity stent showed that there was no in-stent or edge re-stenosis observed ⁶. Drachman et al. reported that poly (lactide-co- ϵ -caprolactone) (PLA/PCL) -coated paclitaxel-releasing stent showed a reduction of 50-60% ISR in a rabbit model ⁷. The results of drug-eluting stents in animals and human clinical trials were very encouraging. However, the main drawback for these coated metal stents were their thin drug containing coating, which is not able to sustain the necessary long-term (up to 3-6 months) drug delivery as well as a well controlled drug release rate. Drug loading on these coatings had also to be small in order to avoid adverse embrittlement and delamination of the biodegradable polymer coating. If the whole stent has to be made to release drugs, then the total drug loading would have to be increased significantly. This became one of the key reasons to develop a totally biodegradable polymeric drug eluting stent.

The first biodegradable stent, using poly (L-lactide) (PLLA), was developed during the 1980s by Stack and Clark of Duke University, U.S. ⁸ It was a milestone in the development of a biodegradable coronary stent, although there was no drug incorporated. The first successful clinical trial for biodegradable stent was carried out by Tamai and Igaki, who designed a zigzag helical coil pattern made of poly-L-lactide monopolymer with a thickness of 0.17 mm. The restenosis rate per lesion was shown to be only 10.5% after six months' implantation ⁹.

Polymer materials have also been shown to possess better biocompatibility and drug release control than metal or inorganic stents. Many biopolymers have shown themselves to be compatible with high drug capacity and good control over drug release. Their ability to degrade and be resorbed completely without negative responses also gives a unique advantage over metal stents. For these reasons, more and more medical devices are being made from polymeric materials instead of metallic and inorganic materials.

1.2 Problem statement

Rapamycin is a potential anti-proliferative drug which was chosen as the candidate drug to be used in a biodegradable stent. Although rapamycin has been incorporated into coated metallic stents and has been tested in a few animal and clinical studies, few reports on the release kinetics of rapamycin are available. Due to the high hydrophobicity of rapamycin, its solubility in aqueous solutions is extremely low. According to published report, the solubility of rapamycin in water is 2.6 $\mu\text{g/ml}$ ¹⁰, however, preliminary drug solubility studies has shown that the solubility of rapamycin in water was only about 0.02 $\mu\text{g/ml}$. The low solubility brings problems in monitoring in vitro rapamycin release. Another issue that occurs in the study of rapamycin release is the instability of this drug. The $T_{1/2}$ of rapamycin in 7.4 phosphate buffer solution (PBS) at 37 °C is 13 hours because of hydrolytic degradation ¹¹. Low solubility and instability of rapamycin in aqueous solutions make the direct monitoring of rapamycin release by HPLC to be very difficult.

Some researchers reported the inhibition effect of rapamycin on the proliferation of smooth muscle cells (SMC) ¹². However, the gap of research is that there are few studies reporting on the effects of sustained released rapamycin on the inhibition of human

coronary artery smooth muscle cells (hcaSMCs). Till now, there has also been no report on the recovery of hcaSMCs after rapamycin inhibition. In addition, there is also no report on the effect of rapamycin on SMC attachment to drug-eluting biodegradable polymers.

Understanding of the rapamycin release kinetics from biodegradable polymers like PLGA and the controlled-released rapamycin on the inhibition of hcaSMCs is the prerequisite for the design of drug-eluting polymer stent. Thus, an in-depth investigation on the rapamycin release kinetics from biodegradable polymers and its inhibition of hcaSMC is absolutely necessary.

1.3 Objective

The main objectives of this work are to firstly, study the rapamycin release kinetics from polymer PLGAs, and secondly, study the effects of control-released rapamycin on hcaSMC. In meeting these objectives, the work undertaken in this study was thus divided into the following three parts:

The first part is to study the material properties, and to design and monitor rapamycin release mechanisms from biodegradable PLGAs. In this part, a thorough investigation in rapamycin release will be conducted. Rapamycin release kinetics will be studied in different polymers, with plasticization and coating effect, to see how these modifications will influence rapamycin release. Thermal and degradation properties of the polymers will also be studied to understand and support the results of in vitro release kinetics.

The second part is to choose some drug-release types and apply them in cell culture systems. The effects of rapamycin released with chosen release profiles on hcaSMC proliferation will be studied. Meanwhile, the effects of control-released rapamycin on cell viability will be investigated to monitor the cell viability change. The hcaSMC recovery after treatment of rapamycin and the interaction between hcaSMC and the polymer film will also be studied.

The third part is to study the release kinetics of rapamycin under cell culture conditions. The results of this part will be compared to release profiles in buffer. Here, a correlation between in buffer release and in cell culture release will be established.

The fourth part is to understand how the rapamycin-eluting stents of different release rates are able to inhibit neointimal hyperplasia, inflammatory response and cell proliferation. A correlation between in vitro inhibition on hcaSMCs and in vivo inhibition on neointimal thickening and inflammatory reaction will be established.

1.4 Scope

In this work, lactic/glycolic acid polymers PLGA and PLLA will be used because of their good biocompatibility and biodegradability. PLLA was used only as a supporting layer, because of its good mechanical strength, as required of coronary stents. Its degradation and characterization would therefore not be of concern in this study. PLGA, on the other hand, would be used as the drug eluting polymer, where its controlled drug release mechanism and profiles would be studied in-depth.

Only, rapamycin, a hydrophobic drug, will be chosen as the model drug in this study. Rapamycin has been proven to be effective in the inhibition of SMC proliferation and migration both in animal and human cell lines^{12, 13}.

Since a matrix-type will give more mechanical strength and be easier to handle the drug release profile, the prototype of our biodegradable coronary stent is matrix-type. Polymer samples to be used in this study are restricted to thin films with certain fixed thickness to simulate the matrix-type coronary stent.

Physiochemical properties to be tested in this study are thermal and degradation properties as glass transition temperature (T_g) and molecular weight (M_w). These will be studied simultaneously with rapamycin release study since the drug release rate and changes in polymer behavior will be highly associated to these polymer properties.

The hcaSMCs are to be used in cell culture study since hcaSMCs is the key reason to re-stenosis formation. Though some study reported that fibroblasts formation may be more significant in re-stenosis¹⁴, but SMCs play a more significant transitional role between early inflammation response and later fibrin deposition.

The PLLA/PLGA bilayer stents will be inserted into the rabbit femoral artery, since it is a well-established model in studying in vivo biocompatibilities and restenosis of coronary artery stent. The effects of different rapamycin releasing rates on femoral artery restenosis will only be studied up to 2 weeks.

1.5 Overview

A critical literature review is reported in Chapter 2, which will cover the introduction of stents and biodegradable polymers, mechanisms of restenosis, candidate drugs, drug release kinetics and the interaction between rapamycin and SMC. Chapter 3 will report on the materials and experimental details. The in vitro rapamycin release results will be reported in Chapter 4. Chapter 5 reports on the result of rapamycin release study in cell culture. Effects of rapamycin on hcaSMCs will be reported in Chapter 6. Effects of control released rapamycin on neointimal hyperplasia in rabbits will be reported in Chapter 7. Conclusions and recommendations for future work will be discussed in Chapter 8.

Chapter 2

Literature Review

This chapter is presented in four main sections. The first section, section 2.1 presents a critical review on the status of intravascular stents complete with the advantages and disadvantages of these stents. Reviews of metallic stents and polymeric stents with their perennial problems are outlined in sections 2.2 and 2.3 respectively. After that, Section 2.4 discusses the ideal design for coronary stent. Biopolymers are among the best candidates for biocompatible stents, their classification and properties are discussed in section 2.5. Section 2.6 highlights restenosis in coronary stents as well as drugs using in stenting to prevent restenosis. The concepts of local drug delivery and the use of polymeric devices for controlled drug release are reviewed in section 2.7. Following that, detailed mechanisms that rapamycin inhibits smooth muscle cells are introduced in section 2.8. Finally section 2.9 presents a summary of biodegradable drug-eluting polymeric stent.

2.1 Introduction to intravascular stents

After the introduction by Gruentzig³, Percutaneous Transluminal Cardiovascular Angioplasty (PTCA) has been used worldwide to treat the blood vessel blockage caused by atherosclerosis or vascular injury. PTCA is a technique used to dilate the areas of arterial occlusion with a catheter which has an inflatable small sausage-shaped balloon at the tip. Angioplasty has gained universal acceptance as it has been shown to be the safest

method to treat vascular narrowing, with fatalities during a procedure less than 1%. However, it still has considerable drawbacks which limit its application to a large degree. The main shortcoming is restenosis, which means the blood vessel's re-narrowing after the process of PTCA. Restenosis occurs in 30-50 % of cases within the first few months after successful intervention¹⁰. It is widely accepted that the restenosis after angioplasty can be best thought as a balance between intimal formation and arterial remodeling, not intimal formation or vessel re-modeling alone as in the current paradigm. The mechanism of restenosis is not completely understood yet, though from reports related to the pathological aspect of restenosis, we can divide its causes into two categories.

- i. First, due to the irregular shape of the occlusion, the balloon cannot expand evenly throughout the blockage, resulting in an opening that is not smooth and thus turbulence in blood flow. This, together with the rough interior vascular surface, can increase the risk of complete arterial blockage, caused mainly by fibrin sediment, platelet aggregation, smooth muscle cell (SMC) proliferation, migration and excessive extra-cellular matrix production¹¹. The latter is considered an intimal formation, as it is a process of neointimal hyperplasia in response to balloon injury¹². Platelet and medial cells secrete growth factors (GF) and other stimuli to activate immunocytes and the immune system. These signal compounds can also activate the smooth muscle cell and endothelial cell proliferation and migration to initiate the healing process of the injury site.
- ii. Secondly, the blood vessel can recoil elastically after the dilation of balloon, thus reducing the vessel lumen as it remodels itself. Some reports state that in stenotic arteries, increased shear stress at the lesion site because of the increased velocity of flow could initiate compensatory enlargement. This increased internal elastic lamina

area subsequently could minimize the effect of the elastic recoil re-narrowing¹³, which occurred more in nonrestenotic than restenotic arteries¹⁴. However, this phenomenon is both hard to predict and control.

Although repeated and prolonged dilation may restore vessel patency in most instances, 1-4 percent of patients undergoing PTCA will require emergent coronary artery bypass grafting (CABG)¹⁵. After 2 decades of experience with balloon angioplasty in coronary arteries and despite the extensive investigation into a variety of therapies and techniques, the 30- 50 percent incidence of restenosis has not changed. Restenosis seems independent of devices, techniques and skills of operators. Systemic administered agents have also failed in preventing restenosis.

Considering the limitations of PTCA, Dotter² introduced metallic stent into peripheral arteries and Gruentzig³ in coronary arteries to permanently support the vessel and keep it patent. This breakthrough device has greatly increased safety of angioplasty and has led to marked reduction of emergent brachytherapy and bypass surgeries. Intra-vascular stents give the promise of solving both problems of acute occlusion and late restenosis. Blood flow can be restored with the stent as a scaffold, which is tacked to the luminal wall at the injury and plaque sites, thus keeping the lumen patent and reducing smooth muscle cell exposure. Stenting also reduces SMC trauma and discourages thrombus formation due to the laminar flow pattern established by the stent, when compared to the rough surface after angioplasty only. Intravascular stents in tubular or coiled configuration have achieved considerable success and stents with various designs and materials have been developed.

Thus bare stents were successful in a remarkable reduction of restenosis, as demonstrated by the Belgium Netherland Stent Trial (BENESTENT) ¹⁶ and the Stent Restenosis Study (STRESS) ¹⁷ trials. Martin Höher also found that after six months, a significantly lower restenosis and re-occlusion rate (32% and 3%) compared with the PTCA group (64% and 24%); restenosis analysis according to treatment was 72% (PTCA) versus 29% (Stent) ¹⁸. Long-term results of stent implantation for chronic iliac artery occlusions showed that the primary patency rates were 84% at 1 year, 81% at 2 years, 78% at 3 years ¹⁹. Also a three-year follow-up after Palmaz-Schatz metallic stenting showed a comparable result of 66% freedom from stent-site revascularization ²⁰. In another report, after 4 years, no difference in mortality (2.7% vs 2.4%) and nonfatal MI (Myocardial infarction) (2.2% vs 2.8%) were found between stent and angioplasty group. However, the requirement for further revascularization procedures of the target lesions was significantly reduced in the stent group (12% vs 25% in angioplasty group) ²¹.

2.2 Current status of metallic stents

2.2.1 Bare metallic stents

Since Sigwart *et al.* ²² first introduced a self-expandable, stainless steel stent into human coronary arteries in the mid 1980s, quite a few designs of metallic stents have been tested in an attempt to prevent occlusion and restenosis after cardiovascular angioplasty. Early animal and clinical trials with these metallic stents showed excellent effect in preventing or minimizing complications and stenosis after PTCA. The primary implant success rate is now above 90% ²³. An 8-year outcome after Palmaz-Schatz stent implantation showed that the 8-year event-free survival (freedom from death, freedom from death/MI/target-

stented site revascularization and freedom from death/MI/any coronary revascularization) was 86%, 62% and 47% respectively²⁴. The results are encouraging, since the survival rate reached 86% in a total of 426 patients even after 8 years. Three-year results from Klugherz's work also showed the absolute survival and event-free survival to be 88% and 56% respectively²⁵. In another report by *Choussat et al.*, the survival rate when using the less-shortening Wallstent® in 6,534 consecutive patients was about 81% 3 years after implantation²⁶. However, it should be noticed in Choussat report that before stent discharge, there was 0.7% death rate and 4.2% MI, while after stent discharge, 13.9 % of patients died and 11.1% suffered an MI. This finding indicated the need for a biodegradable stent to avoid the problems due to metallic stent discharge.

In summary, there is no doubt that intravascular metallic stents offer the promise for solving the problem of acute occlusion and temporary restenosis after PTCA, and play an irreplaceable role in the forefront of cardiovascular intervention. However, stents actually increase the amount of late luminal narrowing due to intimal formation and later in-stent restenosis. The overall rate of stent restenosis remains unacceptably high (about 30%) as a result of 56% - 75% cross-sectional lumen loss²⁷. Listed below are some of the key disadvantages of metallic stents:

- i. Although the physical presence may prevent the vessel elastic recoil and force the lesion to expand outward, it probably represents a nidus for ongoing smooth muscle cell proliferation and migration as well as platelet aggregation and thrombus formation.
- ii. All biological intravascular surfaces are negatively charged, while metals and alloys used for most intravascular devices are electropositive in electrolytic solutions. This

difference of net electrical charge causes a large amount of thrombosis mediators, platelets and immunocytes to adhere to stents as an initial response of biological defense.

- iii. Another surface property that imparts metal reactivity with blood is free surface energy. Most clean metals have higher critical surface tensions than the 20-30 dynes/cm required for thromboresistance, and are therefore thrombogenic.
- iv. Metal corrosion may occur with the release of toxic products (eg. nickel). Toxic products released by metals may influence the cell growth, cause abnormal tissue response and even flow with blood through body and lead more serious systemic side effects.
- v. The ferromagnetic properties of these metals may make radiologic diagnostic tests less useful or even dangerous.

In addition, the flexibility and rigidity in the interaction with lumen wall may also influence stent biocompatibility and thrombogenicity. Flexibility mismatch between the metallic stent and the compliant vessel wall may cause sufficient trauma to actually induce the restenosis. And this mismatch could result in aneurysm formation.

2.2.2 Non drug eluting coated metallic stents

To resolve the above problems, polymer coatings, with and without drug elution capabilities, have been employed to reduce the stent thrombogenicity and enhance

biocompatibility. The Gianturco self-expanding metallic stent, covered by Nylon mesh as a support for neointimal encasement, was introduced by Yoshioka *et al.*²⁸ and Roeren *et al.*²⁹ reported a Palmatz-silicone coated stent, which was implanted into the aorta of rabbits and showed good biocompatibility, without inflammation and foreign body reactions. As a widely used biomaterial, polyurethane was also considered for the stent coating. In the study of amphiphilic polyurethane-coated metallic tube stents implanted in porcine coronary arteries, it was shown that the biocompatible polyurethane decreased acute thrombotic reaction in the porcine model³⁰. Another development on polymer-coated stent is the diamond-like carbon coated stent, which was wrapped with a thin layer of carbon (approximately 50 nm) and showed good hemo- and biocompatibility. Schwartz and his colleagues developed a natural material, fibrin, coated tantalum stent^{31, 32}. The native compound was chosen because this polymer deposits naturally at the site of injury, is readily available, bioabsorbable and will not be recognized by the body as a foreign material and hence minimize the immunological response and supply excellent compatibility.

However, pure polymer-coated stents cannot eliminate restenosis. Another concern with polymer-coated stent is that the polymer coated surface may be damaged by the balloon during deployment. Questions still arise about the effect of stent on endothelialization and long-term patency although it is sure that a polymer coating can reduce the thrombogenicity of metallic stent³³. The reduction of acute thrombosis can be taken as the main advantage of these coated stents. The continual hyperplasia, leading to occlusion, is still the most potential problem.

2.2.3 Drug-eluting coated metallic stents

Since systemic drug therapies to inhibit in-stent restenosis have proved disappointing³⁴⁻³⁶, recent research has generated tremendous enthusiasm for localized drug delivery by polymer-coated metallic stents in the prevention of restenosis. Local delivery has the theoretical advantage of avoiding adverse systemic effects while maintaining sustained release and high local concentrations of drug. It is an attractive method that addresses the major components of post-angioplasty restenosis, negative remodeling, and intimal hyperplasia, while potentially minimizing the risk of systemic toxicity by anti-coagulants and anti-proliferative agents.

The first clinical anti-proliferative drug-eluting stent study was performed using the polymeric sleeve paclitaxel-coated stent. Quantitative angioplasty revealed a post-stent deployment minimum lumen diameter (MLD) of 2.9 mm in both paclitaxel-eluting stent group and control group and after 6 months, MLD for bare and drug-coated stent groups were 0.9 and 2.2 mm respectively³⁷. In another trial, 238 patients were randomized to a single rapamycin coated stent or the bare metal Bx Velocity® stent and after 6 months, a major adverse cardiac event occurred in only 3% of the patients treated with a rapamycin coated stent vs. 27% of the patients in the bare metal control group. Target lesion re-vascularization occurred in none of the patients in the rapamycin coated stent group and in 22% of the patients in the bare metal control group. Angiographic analysis demonstrated minimal late lumen loss for the rapamycin coated stent group (0.01 mm) as compared with the bare stent (0.80 mm)³³. Another report about the difference between local and systemic drug delivery was on heparin. Despite intravenous administering of heparin for several days, it seemed to have no obvious impact on restenosis³⁶, but when

applied locally for a prolonged period of time, it significantly reduced the smooth muscle proliferative response³⁸. Some other clinical studies with drug-coated stents are currently ongoing. With the completion of these studies, more could be understood about the long-term outcome of drug-eluting polymer stent.

Apart from the disadvantages of metallic and nondrug-eluting polymer-coated stents, the drug-eluting polymer-coated stent has its own shortcomings. The thin polymer coating may only be able to carry a limited amount of drug, thus making it difficult to maintain high local drug concentration for an extended period of time. With such thin coatings (10 microns), it is difficult to exercise meaningful control over drug release. The drug release kinetics and the exact influence on intimal formation is thus difficult to be ascertained, which also make the in vitro-in vivo correlations difficult.

2.3 Polymeric stents

In addition to metallic and polymer-coated metallic stents, stents completely made of polymeric materials are being tested as an alternative to metal stents since polymers have been widely used in cardiovascular devices. There are many reasons to fabricate stents composed completely of polymer materials:

- i. Significant progress has been achieved in increasing the level of blood compatibility of polymers.
- ii. By selecting suitable monomer units, polymerization procedures and processing techniques, the properties (e.g. surface characteristics and mechanical strength) of

the stents can be controlled in an attempt to produce a synthetic polymer with smooth surfaces. This would significantly reduce the activation of blood coagulation³⁹. It would also result in a better fit of the stent against the arterial wall which would decrease subsequent neointimal hyperplasia caused by acute and chronic vessel injury⁴⁰.

iii. Polymer stents can incorporate or bind drugs for later local controlled delivery at the targeted site that would inhibit thrombus formation and neointimal proliferation⁴¹. It has been estimated that a polymer stent can deliver a drug dose to the targeted in the coronary artery that is 10 times higher than systemic administration⁴². Local administration of various drugs including urokinase,⁴³ heparin,⁴⁴ taxol,⁴⁵ hirudin⁴⁶ and peptide⁴⁷ are being investigated to prevent thrombosis and restenosis.

iv. Polymer stents may have better flexibility which permits them to be delivered in tortuous arteries more easily and reduce the risk of damage to the lumen wall⁴⁸.

Polymer stents composed of polymeric materials like Dacron[®] and Teflon[®] have been implanted into biliary tract successfully⁴⁹. Straight polyethylene stents were also tested for treatment of distal malignant biliary obstructions⁵⁰. On the other hand, bioresorbable urethral and ureteral stents based on biodegradable polymers such as poly (L-lactide) and its copolymers were also attractive because of their good biocompatibility and biodegradability. According to the stability of the material under physiological conditions, polymer stents can be further divided into biostable polymer stents and biodegradable stents.

2.3.1 Non biodegradable polymer stents

The principle of this stent design is to maintain luminal integrity of the coronary vessel, in a similar manner to that achieved with current metallic stents, and remain biologically permanent following coverage with endothelium. A flexible, self-expanding polyethylene terephthalate (PET) stent and a delivery system for implantation in porcine coronary arteries were developed and tested by Murphy and co-workers⁵¹⁻⁵³. The polymer stent was deployed in the coronary arteries of 11 Yucatan swine by withdrawal of an outer polyethylene delivery sheath, thus allowing the PET stent to self-expand to a preformed configuration. The study showed that percutaneous deployment of polymeric stents in the coronary arteries was technically feasible and PET possesses the necessary mechanical properties for use as a stent.

However, the PET stent led to an intense inflammatory and neointimal proliferative response that resulted in vessel occlusion. Histological examination of the stented site indicated no evidence that the dissection of the vessel wall had occurred at the time of initial deployment. The research may be useful for the selection of materials used as an intravascular device. The authors stated that, although polymer stents have major potential advantages over metallic stents and can be configured in an appropriate design for intracoronary delivery, significant biomaterial problems such as neointimal proliferation, need to be addressed before clinical test can begin.

The same problem was also demonstrated by another study using PET stent. According to Van Der Giessen's result, the mechanical features of the polymer stent were similar to a

stainless steel stent, but the problem was still the late occlusion and the difficulty for deployment^{54, 55}.

2.3.2 Biodegradable non drug eluting polymer stents

Biodegradable polymer stents have the potential to remain in situ for a predicted period of time keeping the vessel wall patent and then degrading to non-toxic substances gradually. Therefore, after completion of their functions and resorption of the stents, the vessel wall can preserve its normal function and surgical procedures to remove the stent are avoided. The degradation rates of these stents are usually controlled by varying the degree of polymerization and / or crystallization and other processing methods.

Following the first biodegradable PLLA stent by *Stack et al.* in the 1980s⁸, other biodegradable coronary stents have been developed. The Igaki-Tamai™ stent⁹ is the first biodegradable stent used in clinical trials. It is made of poly-L-lactic acid monopolymer and has a zigzag filament type helical coil design. The thickness of the stent filament strut is 0.17 mm and the length of the stent is 12 mm. The stent is pre-mounted for balloon-expansion, and also has self-expanding capability and two radiopaque gold markers. It has a complete biodegradable period of between 1- 1.5 years. Human trial result showed that the stenosis rate per lesion was 10.5% and the rate per patient was only 6.7% after 6 months' implantation. Neither early stent recoil nor major clinical events (death, myocardial infarction and acute thrombosis) occurred in the 15 patients (19 lesions) within 30 days.

The results were promising, but some limitations in this human trial should be pointed out. First is the relatively small patient population (15 patients and 19 lesions). Second, the stent had to be dye-heated for self expansion to 80°C initially and, according to the report, the temperature at the stenting site was around 50°C, which may cause damage to the vessel wall. Third, the lumen size in this trial was larger than 3 mm, with no reports for performance in smaller vessels. Although it has the mechanical strength which is substantially important to stent and the biodegradable and bioabsorbable properties, it is difficult to incorporate therapeutic agents into this kind of stent due to the design. Thus, it is improbable that the restenosis can be completely eliminated by such a stent.

*Agrawal et al.*⁵⁶ reported another biodegradable intravascular stent made of PLLA. This stent showed good mechanical properties and the in vivo test demonstrated that the stent was deployable, could be endothelialized and did not induce a significant inflammatory and thrombotic occlusion. Based on mechanical strength and in vivo assessment, it was concluded that PLLA is a very strong candidate for use as a material for bioabsorbable polymeric stent.

Another promising biodegradable polymer used for stents is polycaprolactone. Polycaprolactone was employed to make a heat-labile balloon-expandable plastic stent⁵⁷. Since polycaprolactone is a thermo-elastomer which is soft from 52-70 centigrade, the stent may be heated directly to the necessary temperature leading to the expansion of the stent so that it can be embedded in the vessel wall to prevent migration and provide support to overcome the forces of vascular spasm. However, the heating method could also cause damage to the vessel wall as well as necrosis. The mechanical properties may not be adequate since the stent can be embedded into the vessel wall.

In addition to the above, a patent on multilayered biodegradable stent was claimed⁵⁸. The stent is made of various polymers such as PLLA, polyglycolic acid, polycaprolactone, polyorthoesters or polyanhydrides. The unique feature of the device is that one layer addresses the structural requirements of the stent and additional layers control the release of different drugs at predictable rates. The different layers are laminated to one another by using heat or solvents. However, the major concern with this type of stent may be that it is difficult to deploy and fix within the vessel wall.

Another patent on biodegradable drug delivery stent was claimed by Summers et al in 1999⁵⁹. The stent is of a double-helix shape with two strand loops and is composed of three or more layers with one or more supporting layers made of PLLA/PCL mixture, two water soluble agent-eluting layers of a mixture of polyethelene glycol (PEG) and liposome. The main shortcoming of this design is the mechanical properties, especially the collapse strength.

A biodegradable stent was claimed by *Leveen et al.*, which was a helical tube as the skeleton, and monofilament strands which zig zag back and forth between two adjacent loops of the tubular helix⁶⁰. And the balloon will be inserted into the helical tube to expand the stent. However, the difficulty to expand such a stent may be underestimated.

2.3.3 Drug-eluting biodegradable polymer stents

As mentioned above, polymer stents have the potential to act as drug delivery systems. Polymeric materials, especially biodegradable polymers, have been widely used for controlled drug release. Therefore, it is reasonable to design a biodegradable polymer

stent not only offering a physical barrier to the vessel wall, but also presenting a pharmacological approach in preventing thrombosis and intimal proliferation.

Localized delivery of effective agents can reduce neointimal hyperplasia. In addition, anticoagulant molecules (such as heparin) can be bound to the stent through the functional groups of the material. Drugs and peptide used to inhibit thrombosis could be delivered with the programmed degradation of the stent to maintain high local drug concentration for a desired period of time.

Biodegradable polymer stent provides a possibility for combining physical treatment with pharmaceutical therapy leading to new generations of stents. The general principle of all intravascular stents is to oppose the elastic recoil of vascular stenosis and provide internal support. Thus, for polymer stents, which are intrinsically weaker than metallic and polymer-coated metallic stents, the mechanical properties of stents should be tested first. Drugs or peptide contained within polymers can be in a dissolved or dispersed state, or chemically bonded to the polymer side chains. It is expected that chemical incorporation may further enhance the extended release compared with physical entrapment⁶¹. In particular, as a method for local drug delivery, polymer stent can incorporate more drugs and control the drug release rate to the pharmaceutically optimum level which is most effective for inhibiting in-stent restenosis. If the problem of thrombosis and restenosis could be overcome by drug-eluting polymer stent, this new method may gain attention and could replace all other kinds of stents currently available in the market.

2.3.4 Limitations of polymer stents

Polymer stents still have some key issues that should be addressed. The most important ones are material composition, stent design, blood and tissue compatibility and its in vivo performance. The material of the stent strongly affects the mechanical properties and the propensity to thrombosis and to endothelialization. The strength of polymer is intrinsically lower than that of metals and alloys, which means greater bulk may be needed for polymer stents compared with metallic stents to give the approximate mechanical performance. Thus single polymer stent may not provide enough support against the vessel wall. For biodegradable polymer stents, one concern is that they must maintain appropriate mechanical properties for a certain period before losing their strength. The bulk limitation of polymer stents may be increased in small vessels (lumen diameter < 2.5 mm, while the risk of aneurismal formation and rupture would be considered in large vessels. The last limitation is the lack of radio-opacity, which will hinder accurate deployment of stent.

2.4 The ideal stent design

After discussion of current metallic and polymeric stents, the introduction to ideal stent design is given in this section. As a summary, to design an ideal stent, it is necessary to consider the following characteristics:

2.4.1 Mechanical properties

A stent must possess the physical properties to perform its basic function to support the lumen and keep it patent. However, the stent cannot be too rigid, it must maintain sufficient flexibility in tortuous blood vessel to avoid complications and injuries.

2.4.2 Biocompatibility

The stent itself and its breakdown or degraded products must be nontoxic and biocompatible to tissue to avoid serious foreign body responses and following inflammation and thrombosis which finally results in in-stent restenosis.

2.4.3 Deployment

It is convenient that metallic and polymer-coated metallic stents can be placed accurately with the aid of X-ray. However, polymer stents lack this radiopacity and hence are not traceable. This makes it difficult to assess the right placement and may cause the 'escape' of stent to other parts of lumen which in turn block the vessel, induce thrombosis and is extremely hard to be removed. So the metallic markers mounted on two sides of balloon are employed to solve this problem during stent deployment.

2.4.4 Expandability

Interventional devices that do not expand properly in the lesion site to the pre-determined diameter will add the risk of stent migration and embolization when under-sizing; when over-sizing, it will lead to the injury of vessel and subsequent inflammation and intimal hyperplasia.

2.4.5 Drug-loading ability

Drugs should be delivered locally in a concentration sufficient to inhibit the restenosis but without inducing tissue injury and serious side effect. The way to deliver drug locally is to incorporate the drugs into the stent. One of the main drawbacks for metallic stents is lack of drug-loading ability. The only difference between polymer-coated drug-eluting and bare metallic stents is that a high concentration of locally administered drug can be incorporated into coated stent. This characteristic greatly enhances the success after stent implantation.

2.4.6 Biodegradability

Ideally the stent should be biodegradable after the site has recovered. This is to prevent a second intervention for removal of the redundant stent. Leaving the stent in the body permanently may not be the best option considering long-term complications that may arise.

2.5 Biocompatible polymers

According to the considerations for ideal stent design outlined in section 2.4, biocompatible polymers are among the best candidate materials since they have excellent biocompatibility, good mechanical strength, easy expandability and most importantly, the drug loading ability. A detailed review of polymers is presented in this section.

2.5.1 Biocompatibility of polymers

The biocompatibility of an implanted material or prosthetic device is a dynamic and two-way process that involves the time dependent effects of the host on the material and the material on the host. The implantation of any synthetic material initiates a wound healing mechanism that is characterized by the inflammatory response. However, the inflammatory response itself involves complex and highly regulated interactions between specific cells and various molecular mediators. An understanding of these interactions that occurs following biomaterial implantation has been the prerequisite to determine a material's biocompatibility.

(a) The inflammatory response

As a consequence of the polymers' chemical inertia, risks of general non-tolerance may be caused exclusively by impurities (monomers, catalyst rests, etc.) or by the presence of various additives. Regardless of the place where a foreign body response is introduced, it causes a tissular reaction around it. The tissular reaction represents a minimal inflammatory process, which is developed in the five classical phases ⁶²:

- i. Initial Phase of Inflammatory Induction: Implantation of a biomaterial induces simultaneous or stepwise release of various mediators of cellular and interstitial origin.
- ii. The Hyperemy and blood stasis stage: In this stage, local hyperemy, vasodilation of arteriole and constriction of veinlet occur, lead to capillary stasis and more permeation of capillary walls.

- iii. Plasmatic exudation stage: Exudation of water, salts and proteins appears, which results in alterations in permeability, which leads to formation of a fibrin network around the inflammatory center of the biomaterial implant.
- iv. Cellular infiltration stage: Polynuclear cells, and platelet origin cross the capillary walls. The intensity of the leukocytes and the proteolytic reaction correlated directly with "irritating" properties of the biomaterial.
- v. The repair stage: The polynuclear afflux diminishes, monocytes and lymphocytes infiltrate at the meantime to form macrophages or fibroblasts which induce tissue repair.

(b) Response of blood to biomaterials

It is well known that the future of biomaterials within the cardiovascular system depends on understanding their interaction with blood, especially those leading to the resistance to clot formation. Blood is a suspension of cells (erythrocytes, leukocytes and platelets) in plasma, an aqueous solution containing a variety of organic and inorganic molecules. Among blood cells, platelets are of enormous significance in blood compatibility. The injury to a vessel involves disruption of the endothelium and initiates a series of events allowing platelets to adhere to the damaged surface, as follows:

- i. Once initiation of the inflammatory response occurs upon implantation of a synthetic material, increased vascular permeability at the wound site leads to the exudation of specific plasma proteins that adsorb to the polymer surface, it is with this adsorbed protein layer that the cells actually interact.

- ii. Platelets interact with these proteins such as collagen, and the stimulated plate releases a number of substances, principally adenosine diphosphate (ADP), which causes the platelets to aggregate and form a platelet mass.
- iii. A series of events called coagulation cascade in the plasma proteins is activated, which leads to the formation of a thrombus. The coagulation cascade may be initiated by a glycoprotein associated with a phospholipid (extrinsic pathway) or by the exposure of a non-endothelial surface like biomaterials (intrinsic pathway), which is probably because of the negatively charged surfaces of biomaterials. In both cases, there is a localized conversion of inactive molecules to proteolytic enzymes, that comes together with the conversion of Factor X to Xa and culminates in the conversion of prothrombin to thrombin, which catalyzes the polymerization of fibrinogen to insoluble fibrin, finally causes the clot.

Considering the important role of protein adhesion upon biomaterial surface in the process of blood-material interactions, ways to minimize the surface influence should be considered when choosing biomaterials for cardiovascular devices.

(c) Other considerations

Other than inflammatory response and hemocompatibility issues, thrombi or emboli caused by materials should be considered for new medical device. Impairment of cell function and carcinogenesis for long term usage will also be examined. Allergic reaction may bring serious problem for biomaterials⁶².

2.5.2 Biodegradable polymers

The general criteria for selecting a biodegradable and bioresorbable polymer for use as a biomaterial is to match the mechanical properties and the time of degradation to the needs of the application. The ideal polymer for a particular application would be configured so that it has the mechanical properties that match the application, remaining sufficiently strong until the surrounding tissue has healed. But on the other hand, it should be easily processed into the final product form. It does not invoke an inflammatory or toxic response. It should be metabolized in the body after fulfilling its purpose, leaving no trace. The polymer must demonstrate acceptable shelf life and can be easily sterilized.

Currently, only devices made from homopolymers or copolymers of glycolide, lactide, caprolactone, p-dioxanone, and trimethylene carbonate have been cleared for marketing by FDA. A number of other polymers, however, are being investigated for use as materials for biodegradable devices. Most of the commercially available biodegradable devices are polyesters composed of homopolymers or copolymers of glycolide and lactide. There are also devices made from copolymers of trimethylene carbonate and ϵ - caprolactone, and a suture product made from polydioxanone.

- i. Polyesters: Polylactide, polyglycolide and copolymers of lactide/glycolide (PLGA), are among the most commonly used biomaterials for drug delivery and tissue engineering. The interest in these materials has resulted from several characteristics: (a) they break down to naturally occurring metabolites, (b) materials with a variety of useful properties can be obtained by copolymerization of the two monomers, (c) development of successful suture materials based on these polymers has led to a

great deal of experience with these materials in humans, so that their safety is now well documented.

ii. Polyglycolide (PGA): Polyglycolide is the simplest linear aliphatic polyester. PGA was used to develop the first totally synthetic absorbable suture, marketed as Dexon in the 1960s by Davis and Geck, Inc. (Danbury, CT). Glycolide monomer is synthesized from the dimerization of glycolic acid. Ring-opening polymerization yields high-molecular-weight materials, with approximately 1-3% residual monomer present. PGA is highly crystalline (45-55%), with a high melting point (220-225 °C) and a glass-transition temperature of 35-40 °C. Because of its high degree of crystallization, it is not soluble in most organic solvents; the exceptions are highly fluorinated organics such as hexafluoroisopropanol. Fibers from PGA exhibit high strength and modulus and are too stiff to be used as sutures except in the form of braided material. Sutures of PGA lose about 50% of their strength after 2 weeks and 100% at 4 weeks, and are completely absorbed in 4-6 months. Glycolide has been copolymerized with other monomers to reduce the stiffness of the resulting fibers.

iii. Polylactide (PLA): Lactide is the cyclic dimer of lactic acid that exists as two optical isomers, D and L. L-lactide is the naturally occurring isomer, and D, L-lactide is the synthetic blend of D-lactide and L-lactide. The homopolymer of L-lactide (PLLA) is a semicrystalline polymer. These types of materials exhibit high tensile strength and low elongation, and consequently have a high modulus that makes them more suitable for load-bearing applications such as in orthopedic fixation and sutures. Poly (D, L-lactide) (PDLLA) is an amorphous polymer exhibiting a random distribution of both isomeric forms of lactic acid, and

accordingly is unable to arrange into an organized crystalline structure. This material has lower tensile strength, higher elongation, and a much more rapid degradation time, making it more attractive as a drug delivery system. Poly(L-lactide) is about 37% crystalline, with a melting point of 175-178°C and a glass-transition temperature of 60- 65°C. The degradation time of PLLA is much slower than that of PDLA, requiring more than 2 years to be completely absorbed. Copolymers of L-lactide and D, L-lactide have been prepared to disrupt the crystallinity of L-lactide and accelerate the degradation process.

- iv. Poly (lactide-co-glycolide): Using the polyglycolide and poly (L-lactide) properties as a starting point, it is possible to copolymerize the two monomers to extend the range of homopolymer properties. Copolymers of glycolide with both L-lactide and D, L-lactide have been developed for both device and drug delivery applications. It is important to note that there is no linear relationship between the copolymer composition and the mechanical and degradation properties of the materials. For example, a copolymer of 50% glycolide and 50% D, L-lactide degrades faster than either homopolymer. Copolymers of L-lactide with 25-70% glycolide are amorphous due to the disruption of the regularity of the polymer chain by the other monomer.

2.5.3 Biodegradation of polymers

(a) Biodegradation mechanisms

Biodegradation reactions may consist of three mechanisms:

- hydrolysis under the reaction of water or enzyme
- phagocytic activity of macrophages;
- lytic activity of foreign body giant cells (FBGCs).⁶³

Typically, normal biodegradation of polymers refers to the procedure of hydrolysis. From a chemical standpoint, absorbable devices are thought to undergo five general stages of degradation. These stages are not discrete and may overlap⁶⁴. Stages are described below:

- i. First, hydration of the implant begins when the device is placed in the body. During this stage, the device absorbs water from the surrounding environment. Hydration of the amorphous segments of the polymer occurs faster than with the crystalline segments. In a three dimensional polymer matrix, water penetrates deeply into the interior areas of hydrophilic polymers eventually resulting in a fragmentation degradation mechanism as opposed to the surface erosion mechanism observed with more hydrophobic polymer matrices.
- ii. Second, de-polymerization or chemical cleavage of the polymer backbone occurs, which results in a reduction in mechanical properties. In this process, water reacts with the polymer in a hydrolytic fashion resulting in cleavage of covalent chemical bonds with a reduction in average molecular weight and physical strength. The degradation of the amorphous zones occurs first, then followed by the degradation

of the more crystalline zones, however, the mass of the polymer remain essentially unchanged and there are few foreign body cells observed at the tissue-implant interface.

- iii. Third, loss of mass integrity occurs when the implant has essentially no cohesive strength and begins to fragment into pieces of low molecular weight polymers due to the chemical cleavage of the back bone covalent bonds.
- iv. Fourth, absorption occurs when further hydrolysis causes the fragment size to be suitable for assimilation by phagocytes or when further hydrolysis simply leads to soluble monomeric anions which dissolve into the intercellular fluid. It is during this stage that the implant undergoes an observed loss in mass which corresponds to the physical absorption process.
- v. The final stage of degradation is elimination. For PLA, PGA and their copolymer, PLA is converted into carbon dioxide and pyruvate, pyruvate then enters the Krebs cycle via the acetylation of coenzyme A. During this stage, the majority of the elimination of PLA degradation products occurs through respiration (carbon dioxide) with only minor elimination via urine or feces. Final hydrolysis of PGA results in glycolate, some of which is excreted directly in the urine. Some glycolate may also be oxidized to glyoxylate which is then converted to glycine, serine, and pyruvate which then can enter the Krebs cycle as before with lactate⁹².

(b) Factors in biodegradation

The biodegradation rate of a polymer mainly depends on the intrinsic properties of the polymer, including the wettability (hydrophilicity versus hydrophobicity), the molecular weight, the degree of crystallinity and the glass transition temperature:

- i. Polymer composition: The intrinsic composition is one of the main causes that determine the stability of the polymer backbone, the permeability of water into the polymer matrix because of branch groups' properties and the degradation mechanisms (bulk or erosion), thus influences biodegradability.
- ii. Molecular weight: Biodegradability increases as molecular weight decreases. One reason is that the low molecular weight polymers are more hydrophilic than the corresponding high molecular weight polymers because they have a higher percentage of end groups (including carboxylic end groups and hydroxy end groups). This increased hydrophilicity permits a more rapid influx of water into the polymer matrix and leads to higher water content in the matrix, which speeds biodegradation. Another contributing factor may be that low molecular weight polymers possess more carboxylic end groups that catalyze the biodegradation.
- iii. Crystallinity: For water-dependent biodegradation, the penetration of water into the polymer or the permeability of the polymer to water greatly determines the rate of biodegradation. Water will readily penetrate amorphous regions, but the semi-crystalline nature of a polymer will tend to limit water accessibility. Therefore, semi-crystalline polymers degrade more slowly than amorphous polymers.
- iv. Glass Transition Temperature: The glass transition temperature reflects polymer chain mobility, which determines the ease of water diffusion and chemical attack. If

the glass transition temperature is lower than the biodegradation temperature (e.g., body temperature), the individual molecules possess more mobility to confirm with chemical and/or possibly enzymatic attack, which results in faster biodegradation. On the other hand, if the glass transition temperature is higher than the biodegradation temperature, the individual molecules possess less mobility and are less likely to confirm with chemical and/or possibly enzymatic attack, which results in slower biodegradation.

- v. Other parameters: Additives, impurities, nature and amount of drugs incorporated into the polymer matrix, and intermediate degradation products may influence the biodegradation of polymers.

2.6 Restenosis

As discussed previously, restenosis is the main drawback to coronary stent. The concept of restenosis, its pathological mechanisms and drugs using in therapy are reviewed in this section.

2.6.1 Definition of restenosis

Restenosis, defined as “the arterial healing response after injury incurred during transluminal coronary revascularization”¹ has been the principal drawback of coronary angioplasty since its inception nearly 25 years ago. Restenosis following percutaneous coronary intervention is very different from coronary atherosclerotic disease. Classical atherosclerotic coronary disease is characterized by a plaque containing varying amounts

of lipid, calcification, and fibrosis. Atherosclerotic coronary disease is associated with the presence of hyperlipidemia, systemic arterial hypertension, tobacco use, diabetes, family history of coronary disease and obesity.

2.6.2 Difference between atherosclerosis and restonosis

Atherosclerosis is a complex process with interactions among monocytes, macrophages, activators, platelets, smooth muscle cells and other molecules, but commonly is regarded as the result of a dynamic interaction between blood elements, disturbed flow, and the vessel wall, involving several pathological processes ⁶⁵.

- i. Shear stress resulting from blood flow within the vessel plays a particular important role in the endothelial dysfunction. Low-density Lipoprotein (delivering cholesterol) is another major cause of injury to the endothelium and underlying smooth muscle and becomes especially so after oxidation⁶⁶. Endothelium has a critical role in vascular haemostasis. It modulates regulation of the plasma lipoproteins, adhesion of leukocytes, release of prothrombotic and antithrombotic factors, growth factors and vasoactive substance ^{67, 68}.
- ii. Hemodynamic factors apparently make some area lesion prone. In these areas, inflammation with increased endothelial permeability and endothelial dysfunction lead to increased uptake of macromolecules (lipoprotein, leukocyte adhesion molecules, growth factors and chemotactic factors which cause proliferation of monocytes/macrophages and smooth muscle cells) from blood into the lesion sites.

- iii. Simultaneously or subsequently, intimal smooth muscle cells proliferate, migrate and synthesize new matrix, resulting in an elevated intimal lesion, namely, the mature atherosclerotic plaque. Whilst initially the artery compensates by dilation (remodeling) at some point it can no longer do so and the lesion protrudes into the lumen to alter the flow of blood.
- iv. Calcification and thrombosis with platelet aggregation and fibrin formation are other factors contributing to the development of atherosclerosis⁶⁹.

Proliferation of smooth muscle cells, matrix synthesis, and lipid accumulation may gradually narrow the arterial lumen and ultimately lead to myocardial ischemia (MI) and anginal pain. Actually, plaque disruption is common but often asymptomatic, and the associated rapid plaque growth is also usually clinically silent^{69, 70}. This kind of disruption is probably responsible for the unpredictable, sudden and nonlinear progression of coronary lesions frequently observed angiographically. Without thrombosis, coronary atherosclerosis is generally benign. It is plaque disruption or fissuring and subsequent thrombosis that makes coronary atherosclerosis dangerous⁶⁵. The resultant thrombotic response, which affects the clinical presentation and outcome, is partly determined by the reactivity of the circulating platelets and the balance between the coagulation and fibrinolytic systems.

In contrast, restenosis is characterized histologically by neointimal formation (the appearance of cells and matrix in the intimal layer of the artery) and concentric compression of the outer layer of the blood vessel. During PTCA, balloon injures the arterial endothelium and most importantly, the media (middle muscular layer) of the blood vessel. The high pressures used in balloon inflation commonly cause a rupture of the medial layer, usually at the junction of the plaque and the more normal segment of the

artery. This causes tears or dissections within the artery, exposing deep layers of the media and the outer arterial layer (adventitia). The endothelial injury that occurs results in a loss of antithrombotic factors such as nitric oxide (NO) and tissue plasminogen activator, and allows platelet adhesion and aggregation. These platelets then degranulate and release pro-coagulant factors, e.g., platelet-derived growth factor (PDGF), thromboxane (TX)_{A2}, adenosine diphosphate (ADP), leading to the formation of a deep intramural thrombus. This is then followed by proliferation and extracellular matrix synthesis by smooth muscle cells that have migrated in response to mechanical stretch injury, growth factor production, and disruption at the site of balloon angioplasty, which ultimately leads to the neointimal hyperplasia.⁷¹

Although the mechanism of arterial injury during angioplasty appears to be widely accepted, there has been some debate over the mechanism of restenosis. In general, most investigators agree that the process of restenosis is associated with the healing response to the arterial injury from the angioplasty. Initial studies in rats suggested that the intimal and medial layers of the vessel were the major sites of the restenosis process. However, these observations appear not to be applicable to clinical findings. Studies on the role of the adventitia in restenosis have suggested that this layer plays the key role in both the proliferation and the concentric compression of the external elastic lamina (negative remodeling) that defines restenosis. These findings have been corroborated by the experiments that demonstrated a beneficial effect of intracoronary radiation when the radiation energy was directed at the adventitia, but not when it was focused on the media⁷².

2.6.3 In-stent restenosis

Histologically, In-Stent Restenosis (ISR) is quite distinct from restenosis after balloon angioplasty. As mentioned above, post-angioplasty restenosis is thought to involve vessel elastic recoil, negative remodeling, thrombus at the site of injury and neointimal (NI) formation. In contrast, intravascular ultrasound studies suggest that stenting virtually eliminates vessel elastic recoil and negative remodeling^{73, 74}, thus in-stent restenosis is largely a result of neointimal formation, which is composed principally of proliferating smooth muscle cell and extracellular matrix. In the pig model of stenting⁷⁵, persistent thrombosis has not played an important role in neointimal formation, although recent observation suggest it may have some influence, particularly in the setting of hyperglycemia^{76, 77}.

A number of variables are known to increase the risk of ISR. Patients with diabetes or a history of prior restenosis have a higher rate of ISR. Patients positive for allergic patch-test reactions to the stent components nickel and molybdenum also appear to have increased rates of ISR⁷⁸, that's one reason why breakdown products of metallic stent may cause severe restenosis. Procedure-related variables such as stent length, number of stents used and dimension characters of lesions in vessel have also been suggested with the ISR rates.

2.6.4 Drugs used during stenting

There has been great interest in the nature of stents themselves and the methods used to implant them as stimuli for ISR. Stent design, composition, length and stent guidance

strategies have been received considerable attention^{79, 80}. However, the currently the most promising method to prevent ISR is using local delivery of antithrombotic and antiproliferative drugs' delivery using polymer-coated stents.

The development of a drug-coated metallic or polymeric stent begins with the identification of suitable pharmacological agents. A precise understanding of the fundamental downstream molecular events responsible for growth factor- or cytokine-mediated smooth muscle cell proliferation and thrombus formation has allowed rational selection of potential therapeutic drugs for local delivery to prevent restenosis. A long list of drugs have been under investigation and rapamycin, paclitaxel, heparin and hirudin are most often used to prevent in-stent restenosis. The current lead investigational agents include rapamycin and taxol (or its derivative paclitaxel).

- i. Rapamycin (Sirolimus): Rapamycin is a potent immunosuppressive agent with anti-inflammation and antiproliferative effects, approved by FDA 1999 for preventing renal transplant rejection. Rapamycin is a microbial product isolated from the actinomycete *Streptomyces hygroscopicus*, was initially discovered as an antifungal agent in the mid-1970s⁸¹. Rapamycin is a white or light-yellow hydrophobic drug that has low stability in aqueous solutions, which allows it to pass through cell membrane easily. Rapamycin is a very potent regulator of cell cycle activation. It has been speculated that an important regulatory element to control and integrate the protein synthesis and degradation (the former is the most energy-consuming process and thus be most highly regulated) is FRAP (FKBP12- Rapamycin-Associated Protein). The complex between rapamycin and its binding protein FKBP 12, a member of the immunophilin family of proteins, inhibits the actions of FRAP⁸². This results in increased levels of cyclin-dependent kinase inhibitors resulting in

inhibition of cell cycle protein phosphorylation and subsequent cell cycle arrest at G1/S transition. A characteristic feature of rapamycin is its ability to inhibit growth factors signaling for both immune and nonimmune cells. This effect includes at least fibroblasts, endothelial cells, hepatocytes and smooth muscle cells.⁸³ This antiproliferative effect of rapamycin renders it a promising compound for the prevention of post-angioplasty and in-stent restenosis. If rapamycin is given as a long-term systemic treatment, the primary adverse effects include hypercholesterolemia and hypertriglyceridemia⁸⁴. Some side effects such as gastrointestinal toxicity and vasculitis were reported in high dose regimens in dog and primate models, however, none of these systemic side effects has been observed after stent-based local delivery so far.

- ii. Paclitaxel: The second compound currently under investigation is paclitaxel, a potent inhibitor of various neoplastic cells that has a novel chemical structure and a unique mechanism of action. Paclitaxel, taxol and other taxol derivatives are microtubule inhibitors that prevent cell migration and proliferation by inducing the microtubules to form long, stable chains. It is derived from the pacific yew tree and has been approved by the FDA for the treatment of ovarian cancer. Paclitaxel's mechanism of action consists of polymerization of tubulin, which results in the formation of abnormally stable and nonfunctional microtubules, thereby inhibiting cellular replication in the G0/G1 and G1/M phases. Previous in vitro studies demonstrated inhibition of migration and proliferation of vascular smooth muscle cells by paclitaxel, and initial promising in vivo studies of systemic and local paclitaxel administration to inhibit intimal growth have been reported²⁷.

Paclitaxel is highly lipophilic and this character promotes a rapid cellular uptake by enabling it to pass easily through the hydrophobic barrier of cell membranes. Another distinct character of paclitaxel is that the unique mode of action supports a long-lasting antiproliferative action even after a brief, single-dose application at very low concentrations, as previously shown in tumor cells.

Although the cardiotoxicity observed in anticancer trials is usually mild and reversible, significant cardiovascular events occurred in small numbers of patients treated with paclitaxel in clinical trials. These included myocardial infarction, atrial fibrillation, mild congestive heart failure, sudden death, hypertension and so on ⁸⁵. However, the systemic concentrations observed in trials with local drug delivery are 100-1, 1000 lower compared to the concentrations observed in patients treated for cancer. So, it is required to understand the effects of rapamycin and paclitaxel of various concentrations in local delivery, which is quite different from the results of the systemic clinical trials.

iii. Actinomycin-D: Actinomycin-D is an antibiotic derived from *Streptomyces parvullus* used for its antineoplastic properties in the treatment of malignancies. The compound inhibits proliferation of cells by forming a stable complex with double-stranded DNA, thus inhibiting DNA-primed RNA synthesis ²⁷. Actinomycin-D is a more potent and cytotoxic antiproliferative agent than rapamycin or paclitaxel.

iv. Heparin: Heparin is used as an anti-thrombotic drug. It is not a single structure, but rather a family of mucopolysaccharide chains of varying composition. Heparin contains the unique five-residue sequence, forms a high-affinity complex with antithrombin III. The formation of antithrombin - heparin complex greatly increases

the rate of inhibition of two principal procoagulant proteases, factor Xa and thrombin. Accelerated inactivation of both the active forms of proteases prevents the subsequent conversion of fibrinogen to fibrin that is crucial for clot formation. Heparin has been shown to reduce neointimal proliferation and restenosis following vascular injury in experimental models.

Heparin is relatively non-toxic. However, parenteral administration precludes its long-term use. It is generally given to postoperative patients and to those with acute infarctions requiring immediate anticoagulant action. Heparin overdose or hypersensitivity may result in excessive bleeding. Protamines, highly positively charged low-molecular-weight proteins, are used as anti-dote for excessive bleeding complications.

v. Hirudin: Hirudin is a small compound that is a direct thrombin inhibitor. The advantage of hirudin over other anticoagulants is its ability to block thrombin at multiple sites, without the need for circulating antithrombin III. Its small size allows hirudin to inhibit clot-bound thrombin and to prevent further thrombus formation. Animal studies with hirudin have demonstrated a reduction in restenosis rates ⁸⁶.

vi. Gene transfer: Gene transfer is defined as the direct introduction of a desirable gene into a local environment, with the aim of increasing the function of that gene to gain pathophysiologic benefit. Gene transfer has been viewed as most suited to local therapy. Studies have shown a generally consistent effect in the order of 30% to 70% inhibition of NI (Neointimal Inflammation) formation by modification of smooth muscle cell proliferation, cell migration and endothelial function ⁸⁷. Some reports in human trials also show it a promising method to prevent restenosis ⁸⁸.

Adenoviruses have the advantage as vectors in that they can produce large amount of highly purified recombinant viruses. But there have been recent concerns that adenoviruses may induce inflammation, particularly when introduced via the intraluminal route and may promote thrombus formation, which is identical with what mentioned above of the development of atherosclerosis. As to the question of how to achieve local delivery, catheter-based delivery devices for vascular gene transfer have been investigated for restenosis, but with limitations ⁸⁸.

2.6.5 Radiotherapy for ISR

Radiation therapy provides a non-pharmacological approach to controlling the response to injury and unfavorable remodeling that occurs after percutaneous cardiovascular intervention. The main effect of vascular radiotherapy is breaking single- and double-stranded DNA and killing actively dividing cells in the media, intima and adventitia. Recent clinical trials have demonstrated a reduction in neointimal hyperplasia after intravascular radiation ⁸⁹. And some other clinical trials also demonstrated the positive effect of brachytherapy in the treatment of restenosis. Radiotherapy (brachytherapy) is the main method to treat acute in-stent restenosis currently.

However, the unusually late stent thrombosis seen in the patients treated with radiation therapy suggests that radiation may impair the ability of the vessel wall to endothelialize the stent struts ⁹⁰. Other findings within six months of coronary radiation therapy include coronary aneurysms and acellular, necrotic areas of tissue ("black holes"). These "black holes" are significant, at least in part, because they contain proteoglycan and other

prothrombotic material. Thus, coronary radiation for ISR appears to show promising early benefit, but, the long-term benefits and associated risks still remain unclear⁹¹.

2.7 Controlled local drug delivery

The basic concept underlying the use of polymer delivery devices is that a sustained drug level can be produced in a certain body compartment by balancing the biological processes of drug metabolism and elimination against the drug-release characteristics of the polymeric delivery system. In many cases, it would be desirable to confine the action of a drug to a particular diseased tissue or organ, thus allowing the drug to exert its therapeutic effects within the organ without causing adverse systemic effects. Localization of drug action can also be used to enhance the efficacy or potency of certain types of agents. As a combination of controlled and localized drug delivery device, biodegradable polymeric stents have the potential to avoid serious side effects and maintain effective drug concentrations at the injury sites.

2.7.1 Controlled release devices

Currently there are two kinds of release devices:

- Capsule-type, in which the active therapeutic agents are encapsulated in a closed compartment surrounded by a polymeric membrane;
- Matrix-type, in which active agents are dispersed homogeneously in a polymer matrix. There are also two types in the matrix system, one is the agents only

disperse in the polymer matrices without being one part of the polymer chain, and the other type is the agents are linked to the polymer molecules and become a section of the polymer chains, and this type is also called 'carrier type'.

2.7.2 Drug release kinetics

The release of drugs from capsule-type delivery can be taken as: the drug molecules inside the outer layer of polymer dissolve into the polymer structure, diffuse through it, and finally partition into the tissue surrounding the drug delivery device. *Chien et al.*⁹² investigated the in vitro and in vivo release of progestins from a silicone capsule and established a theoretical model to derive the following mathematical expression:

$$Q/t = (C_p K D_s D_p) / (K D_s h_p + D_p h_d) \quad (\text{Eq. 2.1})$$

Where Q = cumulative amount of released drug

C_p = solubility of drug in the polymeric membrane

K = partition coefficient of drug between polymeric device and the surrounding tissue fluid

D_s = diffusion coefficient of drug in the surrounding tissue fluid

D_p = diffusion coefficient of drug in the polymeric membrane

h_p = thickness of the polymeric membrane

h_d = thickness of the hydrodynamic diffusion layer

From the equation above, it can be deduced that the controlled release profile of drug from the capsule-type polymeric delivery device follows zero-order kinetics, which represents a constant release rate.

(a) Matrix-type drug delivery systems

When a drug is dispersed uniformly in a polymeric matrix, it is called matrix-type drug delivery system. The solid drug particles on the surface layer of the polymeric device are eluted first; when this layer becomes exhausted, the next layer then begins to deplete. This system can be divided into surface-degrading and bulk-degrading systems. Except for a few surface-degrading systems such as polyorthoesters and polyanhydrides, most systems degrade by a combination of the two mechanisms.

- i. Degradation-controlled matrix systems: In degradation-controlled monolithic systems, the drug is dispersed uniformly throughout the polymer matrix, and diffusion of the drug is slow compared with polymer degradation. For surface degrading systems, drug release is affected by the surface-to-volume ratio and the geometry of the device. Following is the description of surface-degrading systems:

⁹³ If M_t is the amount of drug released from a **sphere** of radius r in time t , then the kinetic expression describing release would be given by:

$$dM_t/dt = k_0 4\pi r^2 \quad (\text{Eq. 2.2})$$

where k_0 is a rate constant. Since the rate-determining erosion process occurring at position r is carried out at an ever decreasing radius, the drug release rate, dM_t/dt decreases continuously. From the mass balance, M_t is also given by:

$$M_t = (4\pi/3)C_0[a^3 - r^3] \quad (\text{Eq. 2.3})$$

where C_0 is the uniform initial concentration of drug dispersed throughout the device and a is the initial radius at time $t=0$. Substitution of Equation (2.3) into Equation (2.2) and differentiation gives the following relationships:

$$r = a - (k_0/C_0)t \quad (\text{Eq. 2.4})$$

Substituting Equation (2.4) into Equation (2.3) yields

$$M_t = (4\pi/3)C_0\{a^3 - [a - (k_0/C_0)t]^3\} \quad (\text{Eq. 2.5})$$

and since $(4\pi/3)C_0 a^3$ is equal to M , which is the cumulative amount of drug released as time approaches infinity, we have

$$M_t/M = 1 - [1 - (k_0t)/(C_0a)]^3 \quad (\text{Eq. 2.6})$$

Similar derivation for a **cylinder** of radius a and length L provides the following equation:

$$M_t/M = 1 - [1 - (k_0t)/(C_0a)]^2 \quad (\text{Eq. 2.7a})$$

and the equivalent expression for a **slab** with a thickness of $2a$ would be

$$\begin{aligned} M_t/M &= 1 - [1 - (k_0t)/(C_0a)]^1 \\ &= k_0t/(C_0a) \end{aligned} \quad (\text{Eq. 2.7b})$$

From equations above, it is apparent that only slab provides a zero-order release.

- ii. Diffusion-controlled matrix systems: A detailed understanding of drug release from a matrix undergoing bulk degradation is difficult because drug release occurs by a combination of diffusion and degradation. If the drug diffuses rapidly from the device relative to the degradation of the polymer, the drug release is governed by the equations derived for simple diffusion-controlled systems. The situation becomes more complex if the drug is released during bulk degradation, since the matrix is gradually loosened by degradation. In this case, the drug permeability through the polymer will increase with time, offsetting the decrease in concentration gradient. The following is a model presented by Baker ⁹⁶ for describing the permeability changing with time. The release of dispersed drug from non-degradable devices is described by the following Higuchi equation ⁹⁶:

$$M_t = A(2DC_s C_0 t)^{1/2} \quad (\text{Eq. 2.8})$$

for $C_0 \gg C_s$

$$M_t = A(2PC_0 t)^{1/2} \quad (\text{Eq. 2.9})$$

where P is the drug permeability ($P = DC_s$). If the matrix is gradually loosened by degradation, the drug permeability will increase with time and such an increase will offset the decline of drug release. If it is assumed that the fractional increase in permeability is inversely proportional to the number of intact (undegraded) bonds in the polymer matrix, then:

$$P/P_0 = (\text{initial number of bonds})/(\text{number of bonds remaining})$$

$$=N/(N-Z) \quad (\text{Eq. 2.10})$$

P_0 = the original permeability

P = the permeability after Z cleavages of polymer chains

If further assuming that bond cleavage follows simple first-order kinetics, then

$$dZ/dt = k(N-Z) \quad (\text{Eq. 2.11})$$

where k is the first-order rate constant. This leads to

$$\ln [N/(N-Z)] = kt \quad (\text{Eq. 2.12})$$

Substitution of Equations (Eq. 2.10) and (Eq. 2.12) into Equation (Eq. 2.9) yields

$$M_t = A (2P_0 e^{kt} C_0 t)^{1/2} \quad (\text{Eq. 2.13})$$

It is noted that drug release was accelerated as a result of the increase in permeability above the initial value by matrix degradation. If the matrix disintegrates before drug depletion, a large burst effect in drug release will be observed.

(b) Parameters influencing drug release

- i. Partition coefficient (K): The release of drugs from both capsule - and matrix-type polymeric drug delivery devices is rate limited by the interfacial partition of the

drug molecules from polymer surface toward an elution medium. The coefficient for this interfacial partition is defined as ⁹²:

Partition coefficient $K = C_s/C_p$

C_s = solubilities of the drug species in the elution solution

C_p = solubilities of the drug species in the polymer structure

- ii. Polymer solubility (C_p): If a polymer is treated as a solid solution, microscopically, then the solution process of a drug crystal in a polymer can be visualized as consisting of two consecutive steps: (1) the dissociation of drug molecules from their crystal lattice, and (2) the solvation of these dissociated drug molecules in the polymer structure. The polymer solubility of a drug species is very much dependent on its chemical structure.
- iii. Solubility (C_s): Solubility can affect the mechanisms and rate profiles of drug release from matrix-type polymeric delivery systems.
- iv. Polymer diffusivity (D_p): This parameter refers to the diffusion of molecules in a polymer structure, which is sensitive both to the physicochemical nature of the polymer material and the physicochemical properties of the diffusant species itself.
- v. Solution diffusivity (D_s): Under the diffusion layer-limiting partition-controlled process, the solution diffusivity (D_s) plays a rate-limiting role in determining the rates of drug release from both capsule- and matrix-type delivery systems.

- vi. Other parameters: Other important parameters affecting the drug release process are drug content, membrane thickness, and thickness of hydrodynamic diffusion layer.

High drug concentration causes change in the packing of polymer chains, which results in a faster permeation of release medium and expedites the release rate. For membrane and multi-layered matrix system, the membrane thickness is a significant parameter for the drug diffusion, with the increasing of thickness, drug diffusion rate will be deduced and it is difficult for drug to diffuse to the outer layer. If the hydrodynamic diffusion layer is too thin, it will be easy for released drug to reach a high concentration and slow down the diffusion rate from polymer matrix to the release medium.

2.7.3 Introduction to *in vitro-in vivo* correlations

A Food and Drug Administration (FDA) interpretation of *in vitro-in vivo* correlation (IVIVC) has been cited as: "To show a relationship between two parameters. Typically a relationship is sought between *in vitro* dissolution rate and *in vivo* input rate. This initial relationship may be expanded to critical formulation parameters and *in vivo* input rate."⁹⁴ As suggested by the definition of IVIVC, physicochemical properties of a dosage form other than dissolution should not be overlooked as an *in vivo* measurement. However, with respect to quality control testing, more weight tends to be placed on the cumulative dissolution of a dosage form over time as an *in vitro* indicator of *in vivo* performance. For controlled-release dosage forms, it is especially desirable to determine the cumulative absorption-time profile. Other endpoints of *in vivo* performance of a dosage form might be investigated, such measurement of drug efficacy. The ultimate goal of an IVIVC should be to establish a meaningful relationship between *in vivo* behavior of a dosage

form and *in vitro* performance of the same dosage form, which would allow *in vitro* data to be used as a surrogate for *in vivo* behavior.

For controlled-release products, an *in vitro-in vivo* correlation should be based on an intra-product comparison. That is, only variations of the drug-release rate for a specific formulation controlled-release mechanism may be considered in the correlation of a given device. Because different controlled-release devices generally employ different controlled-release mechanisms, it may not possible to make a comparison between different controlled-release devices. It must be emphasized that *in vitro-in vivo* correlations for controlled-release formulations should be considered to be device-specific.

The following ways should be utilized if *in vitro-in vivo* correlation for controlled-release dosage forms would be of benefit ⁹⁵:

- i. Identify appropriate dissolution conditions for a formulation which result in data relevant to *in vivo* performance;
- ii. Validate the use of dissolution testing and specifications as a quality control tool for process control, dissolution specifications in quality control ranges may be shown to be relevant to *in vivo* data;
- iii. Predict *in vivo* performance of a formulation based on *in vitro* dissolution data, which may be used in the justification of dissolution specifications and may aid in the design of formulation release-time profiles resulting in optimal plasma or tissue concentration-time profiles.

Logically, a higher degree of correlation may be expected with controlled-release formulations than oral forms, since release from extended-release form tends to become the rate-limiting step in absorption, overcoming permeation rate limitations in fast release. Also, the time-frame allowed to characterize the profile of dissolution of absorption is much longer than with immediate-release dosage forms, which permits a greater degree of accuracy and precision in characterizing the dissolution and absorption profile.

2.8 Inhibition effect of rapamycin on cell proliferation and growth

2.8.1 Cell cycle

Normal intermedia smooth muscle cells in coronary artery are quiescent; when stimulated, they migrate through to the intima. At the same time, the cells are also activated from a passive 'G0' state to a proliferating state, effectively resulting in a continuous growth cycle. Both the migration of the cells and the triggering of the cell proliferation contribute to cause stenosis. To avoid stenosis, the inhibition of SMC migration and proliferation are necessary.

For most of eukaryotic cells, the cell cycle has two phases, mitosis and interphase which includes G1, S and G2 phases. Figure 2.1 shows the eukaryotic cell cycle. Nuclear division and cell division occur in M phase, the mitosis. During mitosis, daughter chromosomes are separated and located into two new cells, called daughter cells, which are the result of cytokinesis. The replication of DNA dominates S phase, which means the synthesis phase. Between M and S phases there are two Gaps, they are G1 and G2 phases separately, as illustrated in Fig. 2.1.

Figure 2.1 Eukaryotic cell cycle

G1 is a preparation phase for the S phase, during this phase, proteins to initiate DNA synthesis and proteins needed in DNA synthesis are to be translated and assembled. In late G1 phase, there are some checkpoints, which examine if there is any damage in cellular DNA, and if the proteins for DNA synthesis are ready. With successful going through these checkpoints, cells progress past G1 into S phase and start the DNA synthesis. The cyclin-dependent kinase (Cdk) is crucial in regulating cell cycle. The combination of Cyclin D to Cdk4 and Cyclin E to Cdk2 activates these two Cdks by forming Cyclin D/Cdk4 and Cyclin E/Cdk2, which in turn phosphorylate retinoblastoma protein (pRb).¹⁰⁹ The pRb binds to E2F transcription factor to inhibit the initiation of transcription. The phosphorylation of pRb release pRb from pRb-E2F complex, thus start the mRNA transcription to translate some proteins essential to DNA synthesis. Rapamycin inhibits mTOR activities in late G1 phase as shown in Fig. 2.1, thus stop the

cell cycle to S phase. The mechanisms regarding rapamycin inhibition effect on mTOR will be discussed in following sections.

2.8.2 mTOR (mammalian Target of Rapamycin)

Mammalian target of rapamycin (mTOR), which is conserved in yeast, plants and mammals as the target of rapamycin – FKBP12 complex, is one of the most important regulatory elements of protein synthesis as a central controller of cell growth by phosphorylating p70 ribosomal S6 kinase (p70S6K) and eukaryotic translation initiation factor eIF4E, and the regulation of cell proliferation is via upregulation of cyclin-dependent kinases as well as the concentration of p27kip1⁹⁸.

2.8.3 Effect of mTOR on cell growth and proliferation

As a central regulator in cell growth and proliferation, mTOR regulate protein synthesis and the initiation of transcription process.

(a) Role of mTOR in cell growth

RNA translation and protein synthesis are crucial to cell growth and important to determine the cellular size. The p70 ribosomal S6 kinase (p70S6K) and 4E-BP1, the binding protein of eukaryotic translation initiation factor eIF4E are two main targets of mTOR in mammalian cells. The mTOR activates S6K1 by phosphorylation and the suppression of phosphatase activities such as protein phosphatase 1 and protein phosphatase 2A (PP2A). S6K1 subsequently phosphorylates S6 protein of the 40S

ribosomal subunit, the activation of S6 in turn increases the translation of mRNA transcripts with polypyrimidine tract (5' TOP), e.g., ribosomal proteins and eEF-2 elongation factor. The mTOR also phosphorylates initiation factor 4E binding protein, 4E-BP1, which releases the mRNA 5'-cap binding protein eIF-4E, the translation initiation factor in eukaryotic cells. This enables the translation of some specific mRNAs necessary for cell growth and proliferation⁹⁹.

(b) Role of mTOR in cell proliferation

Cell proliferation refers to the increase of cell number with DNA synthesis and mitogenesis. The mTOR regulates cell cycle progression by retinoblastoma protein (pRb) and p27kip1. In some cell lines such as T lymphocyte and murine BC3H1, mTOR inhibits the p27kip1, thus allow cyclinE/Cdk2 and cyclinD/Cdk4 phosphorylates retinoblastoma protein (pRb) and relieve from pRb:E2F complex, so start the transcription process⁹⁸. However, in recent study by *Ruediger C. Braun-Dullaeus, Michael J. Mann et al.*,¹⁰⁰ in proliferating rat and human vascular smooth muscle cells, the regulation of transcription is not by suppressing the p27kip1 level, but by upregulation of cyclin/Cdks directly through PI3K (phosphatidylinositol 3-kinase) signaling pathway.

2.8.4 Inhibition effects of rapamycin on mTOR signaling pathways

The functions of Rapamycin in inhibition of cell growth and proliferation are through a tight complex of rapamycin and FKBP12 (FK506 binding protein, 12kDa). Rapamycin: FKBP12 also stabilizes rapamycin in cells and prolongs its half life (from 9.9-17.5 h)¹⁰¹. Rapamycin:FKBP12 complex interacts with the rapamycin: FKBP12 binding domain of

mTOR thus inhibits the mTOR function from mRNA transcription to protein synthesis. Rapamycin inhibits the protein synthesis by inhibition of the phosphorylation of p70 S6 kinase and 4E-BP1, which results in a reduction of cellular protein mass and also prevent some proteins essential to cell proliferation. Rapamycin:FKBP12 reduces the cyclin/Cdks activity and phosphorylation of pRb, inhibits the mRNA transcription leading to cell cycle arrest in late G1 phase thus results to a delay or totally inhibition of cell proliferation.

2.9 Summary

Biodegradable materials such as poly-L-lactic acid (PLLA) and poly-D, L-lactic and glycolic acid (PDLGA) are the best candidate polymer materials for a fully polymeric, completely degradable coronary artery stent. From the point view of drug release, one advantage of this stent is more drug can be loaded into the polymer matrices to support a sustained drug release; the other one is, the release profile is changeable if we vary the polymer combination, addition of plasticizer, the thickness of matrices or use multi-layer to reach a desirable release profile. In this study, the drug release kinetics was investigated for rapamycin in PLGA 53/47 and PLGA 50/50 (Lauryl ester end group).

For coronary artery stent, a sustained release of rapamycin up to 1 month with lower dose may be more effective than a stent of higher dosage but with rapid release. According to the results of an animal study, *Elazer R. Edelman et al.* produced a comparison between a fast release (90% of rapamycin released in 3 days) and slow release (90% of rapamycin released more than 14 days) in 38 of rabbits by coated metallic stents, then measured the deposition of rapamycin in the vessel wall. They found that the slow release of rapamycin enhanced the efficiency to deliver the drug to vessel wall and concluded that a modulated

release might offer a more effective method of enhancing the stent-based drug delivery than increasing dose ¹⁰². As a matter of fact, rapamycin is a fairly potent drug, as in cell studies, a dosage of 25 ng/ml of rapamycin can inhibit the PDGF-induced migration in rat and human smooth muscle cells, as indicated by *Michael Poon and Steven O. Marx* ¹⁰³. *Sassan Hafizi et al.* found that as low as 10 ng/ml of rapamycin can inhibit the PDGF-induced DNA synthesis in human coronary artery smooth muscle cells ¹⁰⁴. *Moon et al.* also reported that low concentration of rapamycin inhibited rat aortic vascular smooth muscle cells ⁹⁷. From these reports we can have the impression that a very low drug loading will be sufficient for local drug delivery. Therefore the release of rapamycin will be concentrated on the sustained release profile rather than the high dosage, this is the exact advantage for polymeric stent, which can offer different rates of sustained release than metallic stent.

Chapter 3

Materials and Experimental Details

3.1 Materials

3.1.1 Polymers

Poly-L-lactic acid (PLLA) and poly (D,L lactic-co-glycolic acid) (PLGA) are biocompatible and bioabsorbable materials, and they are commercially available and have been used for human application. They were chosen as the candidate materials in this study. PLLA IV 6.63 and PLGAs differing in intrinsic viscosity (IV) were used: PLGA 53/47 (53% lactide and 47% glycolide, IV 0.88), PLGA 80/20 (80% lactide and 20% glycolide, IV 5.01) and PLGA 50/50 (50% lactide and 50% glycolide, lauryl ester end group, IV 0.22). PLLA 6.63, PLGA 53/47 and PLGA 80/20 were purchased from PURAC, FarEast, Singapore. PLGA 50/50 was obtained from Absorbable Polymer Technologies, Alabama, USA. **Table 3.1** gives a summary of the properties of polymer used.

Table 3.1 Properties of polymers used

Polymer	T _m (°C)*	T _g (°C)*	Intrinsic Viscosity (dl/g)**	Manufacturer	M _w (g/mol)#
PLLA 6.63	176.2	59.18	6.63	PURAC	1.28 × 10 ⁶
PLGA 53/47	N/A	35.8	0.88	PURAC	7.69 × 10 ⁴
PLGA 80/20	160.8	53.9	5.01	PURAC	8.50 × 10 ⁵
PLGA 50/50	N/A	28.4	0.22	Absorbable Polymer Technologies	1.12 × 10 ⁴

* T_m and T_g were determined by MDSC.

** Inherent viscosity of as-received raw polymer, in chloroform, 25 °C.

Molecular weight as determined by GPC.

3.1.2 Reagents and drug

The following reagents were used during the course of study. HPLC grade Dichloromethane (DCM), Tetrahydrofuran (THF) and Methyl Alcohol (Methanol) were from Merck, Germany. For in vitro studies, phosphate buffer solution (PBS) with a pH 7.4 was used, which was from OHME, Singapore. Dimethyl Sulphoxide (DMSO) was purchased from Aldrich, USA. Polyethylene glycol (PEG) 4000 was used as a plasticizer and purchased from Merck-Schuchardt, Germany. For cell culture work, the following reagents were used. WST-8 was from Nacalai Tesque, Kyoto, Japan. BrdU cell proliferation kit was from Roche, USA. Glutaraldehyde Grade I was from Sigma. 1,1,1,3,3,3-Hexamethyldisilazane 99.9% (HMDS, $C_6H_{19}NSi_2$) was purchased from Sigma-Aldrich, Steinheim, Germany. The drug, rapamycin (sirolimus, $C_{51}H_{79}NO_{13}$, F.W. 914.2, Light sensitive) was chosen as a candidate in this study and was purchased from HANDE, China. Its chemical structure was shown in **Figure 3.1**:

Figure 3.1 Structure of rapamycin

Pentobarbital Sodium was from Shanghai, China. Heparin sodium solution was purchased from Shanghai 1st biochemical and pharmaceutical Corp., China. Aspirin (tablet) was from Kunming Pharmaceutical Corp., China. Warfarin Sodium (3-(α -Acetonylbenzyl)-4-hydroxycoumarin sodium salt (tablet) was bought from Shanghai Pharmaceutical (Group) Co., China. Ampicillin Sodium was from Harbin Pharmaceutical Group, Harbin, China. The anti α -actin staining kit and anti Ki-67 (MIB-1) staining kit were purchased from Gene Tech Co. Ltd., Shanghai, China.

3.1.3 Cell line and culture medium

Human coronary artery smooth muscle cell line (hcaSMC, Passage 3) was purchased from Clonetics, USA. Cell culture medium was Clonetics SmGM[®]-2 BulletKit, which contains one 500 ml bottle of Smooth Muscle Cell Basal Medium with the following growth supplements, as shown in Table 3.2.

Table 3.2 Supplements in cell culture medium

Growth supplements in Smooth muscle cell basal medium	Concentration by volume (ml/500ml)	Concentration (ng/ml)
Human epithelial growth factor (hEGF)	0.5	0.5
Insulin	0.5	5,000
Human fibroblast growth factor (hFGF-B)	1	2
Fetal bovine serum (FBS)	25	
Gentamicin		50,000
Amphotericin B		50

3.2 Sample preparation

3.2.1 Polymer film preparation

(a) Polymer solution preparation

Purchased PLLA and PLGAs are in the form of granules. A solution-casting technique was used to prepare the polymeric films as follows:

The polymer granules were first dissolved in DCM. Different volumes of DCM were chosen to dissolve these polymers due to their different intrinsic viscosities. The amount of PLLA and PLGA granules were calculated based on the film dimension and density of each material used, as from **Eq. 3.1**.

$$M = d \times V \quad (\text{Eq. 3.1})$$

Where M (g) is the mass of polymer granules, d is the density of raw polymer (g/cm³), V is the final volume of dry polymer films, which can be calculated by length × width × height (cm³). Rapamycin was added as the drug. For polymer solutions with rapamycin, the amount of rapamycin was calculated by weight percentage of PLGA polymer (1 and 2 wt% respectively), mixed with the polymer granules, which was then dissolved in a calculated volume of DCM and left to stir overnight.

Rapamycin can be completely dissolved in PLGA solutions since it is hydrophobic and miscible with PLGA. Since rapamycin is light sensitive, it is important that the whole process was done under isolation from light, including the weighing of rapamycin. All bottles containing polymer solutions with rapamycin were carefully wrapped with aluminium foil. Samples with rapamycin were avoided high temperature. All experiments were kept below 40 °C to avoid drug and polymer degradation.

The effect of plasticizing PLGA on drug elution was also studied. PEG was used as a plasticizer in PLGA. PEG 4000 was first dried and grinded in an oven at 60 °C, then the dried PEG powder was weighed at 5 and 10 wt% of the polymer weight immediately. After drying and weighing, PEG 4000 was mixed with PLGA 53/47 and dissolved in DCM. Solution was stirred continuously overnight.

(b) Casting process

A multi-layer film system was used for all experiments in this study. PLLA 6.63 was used as a supporting layer to give strength, otherwise the sample will be mechanically weak. PLGA 53/47 or PLGA 50/50 layer was used as a drug carrier layer with or without rapamycin as shown in **Figure 3.2**.

A film applicator as shown in **Figure 3.3** was used to cast polymer films with accurate thickness ($\pm 10 \mu\text{m}$). The knife scale of applicator was adjusted according to the wet thickness of polymer solution and placed on the glass plate which was put on top of the applicator, then the homogeneously dissolved polymer solution was poured inside the knife, the film was automatically cast by applicator at set speed.

(a)

(b)

Figure 3.2 Multi-layer polymer films. (a) Double-layer polymer film; (b) Triple-layer polymer film

Figure 3.3 Automatic film applicator AG-2150 from GARDNER, Germany

The double-layer film system was prepared by casting the PLLA layer first, after 10 minutes, then PLGA layer was cast on top of a semi-dried PLLA film to form a double-layer system. For the coated triple-layer polymer films (**Figure 3.2 (b)**), after casting the PLGA 53/47 layer for 10 minutes, the PLGA 50/50 layer was cast on top of this PLGA 53/47 layer to make a coating. After film casting, films were placed at room temperature overnight to allow solvent to be evaporated, then transferred into vacuum oven with a temperature of 30°C drying for 7 days. This evaporation process is to ensure that minimal residual solvent remains. TGA results show that DCM left in dried polymer films was about 0.3% by weight. Both control and drug-containing films were prepared by the same method as mentioned above.

The thickness of film is affected by factors such as viscosity of the polymer solution and solvent evaporation rate. If the polymer solution is too viscous, insufficient flow results in non-uniform thickness. If uneven evaporation occurs, the thickness will also be non-uniform due to different shrinkage rate. In this study, the thickness of PLLA and PLGA layer was controlled within $80 \pm 10 \mu\text{m}$ separately as shown in **Figure 3.4**. The thickness of PLGA 50/50 coating layer was around $60 \pm 5 \mu\text{m}$. All polymer films that did not meet this thickness requirement were rejected. The polymer samples studied in buffer, in cell culture and in vivo are listed in **Table 3.3**, **Table 3.4** and **Table 3.5** respectively.

Figure 3.4 Scanning electron microscope picture of double-layer film cross-section

3.2.2 Polymer films for in vitro drug release study

After drying, polymer films with rapamycin and control films were cut into $5\text{mm} \times 5\text{mm}$ pieces. Polymer samples were then immersed in a 2ml release medium (5% DMSO mixed with 95% pH 7.4 phosphate buffer). 1ml of the release medium was renewed every 3 days. At appropriate time points, the samples were taken out from the medium, wiped dry with water-absorbent paper and put in vacuum for drying at 30°C for 5 days, before being used for further characterizations. **Table 3.3** shows five combinations of polymer films produced for the in-vitro drug release study. These combinations (A to F) would be referred to later in Chapter 4, 5 and 6. Their control films do not contain any rapamycin.

Table 3.3 Polymer film combinations in buffer release study

Polymer Combinations	Blank PLLA 6.63 (Thickness: 80 μm)	PLGA 53/47 (Thickness: 80 μm)	PLGA 50/50
A	1 st layer	With 1 wt% rapamycin	Nil
B	1 st layer	With 2 wt% rapamycin	Nil
C	1 st layer	With 1 wt% rapamycin; 5 wt% PEG4000	Nil
D	1 st layer	With 1 wt% rapamycin; 10 wt% PEG4000	Nil
E	1 st layer	With 1 wt% rapamycin	Blank, thickness: 60 μm
F	1 st layer	Nil	With 1% rapamycin, thickness: 80 μm

3.2.3 Polymer film sterilization for cell culture studies

Dried polymer films were cut into small pieces with a dimension of 3mm × 7mm for both drug-loaded and control samples. For cell attachment study, polymer samples were cut into a disc shape with a diameter of 5mm. The samples were then sterilized by filtered 70% ethanol/water for 24 hours, and were washed in filtered phosphate buffer (pH7.4) for another 24 hours. Three kinds of films were used in the cell studies. These are outlined in Table 3.4 below:

Table 3.4 Polymer films used in cell culture study

Films	Blank PLLA 6.63	PLGA 53/47
	(Thickness: 80 μm)	(Thickness: 80 μm)
A	1 st layer	1% Rapamycin
B	1 st layer	2% Rapamycin
C	1 st layer	1% Rapamycin, 5% PEG 4000
AB ₀	1 st layer	No Rapamycin (Control)
C ₀	1 st layer	5% PEG 4000, No Rapamycin (Control)

3.2.4 Cell culture

The cells used were ‘Human Coronary Artery Smooth Muscle Cells’ (hCASMC) purchased from Clonetics (Cambrex), USA. The Cell line was purchased and delivered at Passage 3. The received cells were then grown and suspended in SMC basal medium with 10% FBS, growth factors and 1% penicillin/streptomycin and GA-1000. Cells in passage 5 were used in the study.

Confluent smooth muscle cells were trypsinized by 0.05% Trypsin-EDTA, incubated in CO₂ incubator at 37 °C for 3 minutes. When all cells were trypsinized and floating, after mixing with cell culture medium, the cell suspension solution was centrifuged at 2000 rpm for 6mins. Cell pellet was re-suspended by 1ml complete cell culture medium;

20 μ l of cell suspension solution mixed with 180 μ l of 0.4% trypan blue was observed immediately and the cell number was counted with hemocytometer under inverted optical microscope.

The cell population was then calculated and the cells seeded into 96-well plates with a cell seeding density of 3×10^3 cells/well. 200 μ l of complete culture medium was pipetted into each well and cells were allowed to attach and grow in a CO₂ incubator overnight. After which, the medium was then completely changed to SMC basal medium with 0.5% FBS and antibiotics but without growth factors. Cells were quiescent for 48 hours, then changed back to 200 μ l /well complete culture medium containing 5% FBS and growth factors.

Sterilized polymer samples with and without drug, were carefully put into 96-well plates with cells seeded at the bottom, as demonstrated in **Figure 3.5**. Cells/samples were incubated in CO₂ incubator at 37 °C. Cell culture medium was changed every 2 days. Subsequent tests were carried out at determined time intervals.

Unlike other transformed cell lines, the hcaSMC cell line supplied by Clonetics can be subcultured for 3 passages with a total about 15 cell doublings. The hcaSMC culture showed a very short maintainable life span. In preliminary studies, cells after passage 7 were unable to maintain the productivity and could not grow to confluence. Cells after passage 7 may go apoptosis in culture conditions. So in this study, it is to be highlighted the cells used were strictly controlled at passage 5.

Figure 3.5 Demonstration of polymer sample in 96-well plate

3.2.5 Polymer film and stent preparations for in vivo studies

Dried double layer polymer matrices (**Figure 3.2 (a)**) without sirolimus, with 1 wt% and 2 wt% sirolimus, were prepared separately by the method described above. The polymer compositions are listed in **Table 3.5**. PLGA 80/20 solution with BaSO₄ (1:2 by weight, which was used to locate the stent in artery by angiogram) was cast at a thickness of 40 μm , dried in vacuum oven for 7 days, before being cut into strip of 1 mm $\times$ 4 mm. The double-layer PLLA-PLGA films were cut into 1 mm $\times$ 30 mm strips with PLGA 80/20-BaSO₄ strips stuck to two ends. The strips were coiled onto a rod with an inner diameter of 2 mm. After heating at 40 $^{\circ}\text{C}$ for 1 hour, the stents were re-coiled onto a rod with an inner diameter of 0.55 mm, and the final stent has an outer diameter of about 1 mm. The

stents were then sealed in plastic bag and stored at -20 °C. Stent expansion with and without immersion in 70% ethanol was monitored by digital micrometer (Kenyence, Germany). The expansion ratio was calculated according to **Eq. 3.2**:

$$(OD_{Expanded} - OD_{Initial}) / OD_{Initial}$$

(Eq. 3.2)

Before surgery, the stents were warmed to room temperature and were sterilized in 70% ethanol for 30 minutes.

Table 3.5 Polymer films used in *in vivo* study

Films	Blank PLLA 6.63	PLGA 53/47
	(Thickness: 80 μm)	(Thickness: 80 μm)
A	1 st layer	1% Rapamycin
B	1 st layer	2% Rapamycin
AB ₀	1 st layer	No Rapamycin (Control)

3.2.6 *In vivo* studies

Aspirin (100mg/day) and Warfarin Sodium (0.5mg/day) were fed to rabbits orally 2 days before surgery and continued for another 3 days after that. The rabbits were anesthetized by 3% Pentobarbital Sodium (IV) at a dose of 1ml/kg body weight and were fixed to the operating table. Heparin sodium was given at a dose of 500 IU/rabbit. The hair on surgery site was removed and sterilized with iodine and 70% ethanol. Right femoral artery was separated and exposed very carefully. The distal end of the separated femoral artery was ligated and a small cut was made at proximal end. A 4F artery sheath was inserted from the cut and the stent was delivered into femoral artery by catheter. The stent was ensured to be fully expanded by keeping the catheter in position for 3 minutes before drawing out of the artery. The proximal end of the artery was ligated after stenting and the wound was stitched. Ampicillin sodium (0.5g/rabbit) was injected (SC) at wounded site. A 20 ml 5% glucose solution was given (IV) after surgery. The rabbits were sacrificed under anesthesia with pentobarbital sodium after 2 weeks. The stents and the surrounding tissue were carefully harvested, and were kept in 10% formaldehyde for histochemical and immunohistochemical studies.

The stent and tissue samples were dehydrated with ethanol and embedded in paraffin after fixation in formaldehyde. Slicing was performed with a microtome (Leica) to cut samples to a thickness of 5 μ m and the sections were attached to glass slides using a water bath at 65 °C. Samples on slides were then stained with Hematoxylin and eosin (H&E), Masson, anti α -actin and anti Ki-67 staining kits after removal of paraffin in xylene and re-hydration with ethanol/water solutions. Stained samples were re-dehydrated with ethanol/water solutions and rinsed with xylene then mounted with mountant. Stained

samples were observed under inverted optical microscope and pictures were taken using Image-Pro Plus®.

3.3. Equipment and testing techniques

3.3.1 High Performance Liquid Chromatography for drug release study

Reverse phase High Performance Liquid Chromatography (HPLC) from Agilent was used in the study of rapamycin release as shown in **Figure 3.6 (a)**. Concentrations of Rapamycin were measured at 278 nm using a ZORBAX Eclipse XDB-C18 column with a pore size of 5 μm (Serial No.: PN990967-902, SN: USNH006154).

Rapamycin is a lipophilic macrocyclic lactone, soluble in most organic solvents and virtually insoluble in water. In this study, 5% of DMSO was used to increase the solubility of rapamycin in release medium. HPLC measures the amount of rapamycin remains in the polymer samples. Based on the initial drug loading, the release rapamycin can be calculated by the method of reverse calculation.

Polymer samples were dried as mentioned previously. After which, the samples were dissolved in 1ml DCM overnight and 9ml methanol was added to precipitate the polymer from DCM and Methanol. Solution was filtered (pore size: $\Phi = 0.2 \mu\text{m}$) and injected into HPLC. The injection volume is 20 μl and the mobile phase is a mixture of 85:15 HPLC grade Methanol and distill water at a flow rate of 1ml/min. The column temperature was room temperature around 25 °C. Retention time for rapamycin under these conditions was about 7.8 minutes.

Figure 3.6 (a): HPLC; (b): GPC

The proportion of released rapamycin to the total initial loading was calculated based on the integrated results of residual drug for in buffer and in cell culture samples. Rapamycin release profile by percentage reflects the release proportional to drug loading, which is essential to study of drug release kinetics. However, in cell studies, the amount of rapamycin released into cell culture medium is more meaningful. This was analyzed using a HPLC facility to measure the rapamycin release. The HPLC graphs for the calibration are shown in **Figure 3.7**. Results were plotted using integrated absorbance at 278 nm as Y-axis and rapamycin concentration ($\mu\text{g/ml}$) as X-axis as shown in **Figure 3.8**. The calibration equation was obtained by testing 7 concentration of rapamycin in the solution containing 85% methanol: 15% Distilled water.

Figure 3.7 HPLC graphs of the calibration

Figure 3.8 Calibration curve of rapamycin

Thus the amount of released rapamycin was calculated by a calibration equation (Eq. 3.3).

$$Y = 52.073 \times A \quad (\text{Eq. 3.3})$$

Where Y is the UV absorbance in HPLC, A is the rapamycin concentration in HPLC samples, its unit is $\mu\text{g/ml}$. The equation gives the correlation between integrated UV absorbance result and rapamycin concentration in HPLC sample.

3.3.2 Gel Permeation Chromatography for degradation study

Degradation studies were conducted on the aging of PLGA films (with drug and plasticizer) by Gel Permeation Chromatography (GPC), as shown in Fig. 3.6 (b). Polymer samples taken from immersion buffer were rinsed by distilled water and then dried in vacuum oven for 7 days, after that, each sample was dissolved in 2ml HPLC grade DCM for 3 days. After dissolving, 8ml THF was added and mixed homogenously using

Ultrasonic Cleaner for 20 minutes. Samples were filtered by $\phi 0.22\mu\text{m}$ filters and then filled into 2ml vials sealed and injected into an Agilent series 1100 GPC system for detection. Samples were flushed by a mobile phase of mix-solvent of 80:20 THF and DCM at 35 °C using a flow rate of 1ml/min. The column is PLgel 5 μm MIXED-D (length/I.D. (mm): 300 x 7.5; Serial No.: sm-m0-53-65), and samples were detected by RID (Reflective Index Detector). Average molecular weight was calculated using a series of polystyrene standards that ranged from 640 to 4,843,000 g/mol in their molecular weight.

3.3.3 Modulated Differentiation Scanning Chromatography for study of polymer thermal properties

The glass transition temperature (T_g) is defined as the temperature at which the bulk polymer ceases to be brittle and glassy in character and becomes more rubbery. At this temperature, the molecular rotation around the backbone becomes significantly easier and the mobility of chain segments higher, resulting in major changes in the properties of the polymer.

Fox and Flory used the following equation to explain the molecular weight effect on T_g :

$$T_g = T_g^\infty - K/M_n \quad (\text{Eq. 3.4})$$

Where T_g^∞ is the glass transition temperature that would be obtained from a polymer of infinite molecular weight, while K is a constant related to parameters describing the free volume.

Because the ends of a chain have more free space of motion than the segments in the center of a chain, and can be thought as having more free volume. Low molecular weight chains have more ends per unit volume than long chains, thus have a higher free volume and hence a lower T_g .

Glass transition temperature of PLGA films immersed in buffer for different time period were monitored using a modulated DSC (DSC 2920, TA Instrument, USA), equipped with refrigerated cooling system (RCS). Testing was performed under flowing nitrogen (50ml/min). Samples were prepared and sealed in hermetic aluminum pans at room temperature. Empty pan was used as the reference in the testing.

3.3.4 WST-8 colorimetric assay for Cell growth study

(a) Mechanism

WST-8, [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] is a highly water-soluble disulfonated tetrazolium salt developed and commercialized by researchers in Japan¹⁰⁵.

WST-8 colorimetric kit includes WST-8 and 1-Methoxy PMS (1-Methoxy-5-methylphenazinium methosulfate), the electron intermediary. When WST-8 solution is in contact with cells, 1-Methoxy PMS goes into cell membrane and enters the cytosol, then receives an electron from NADH/NADPH to become a reduced form. Reduced 1-Methoxy PMS transfers electrons to WST-8 at the cell membrane. Reduced WST-8 shows an orange color, while the ready-to-use WST-8 counting kit is a pink color. Mechanism of WST-8 counting kit is shown in **Figure 3.9**:

Figure 3.9 WST-8 mechanisms

(b) Experiment details

The hcaSMCs were seeded in 96-well plates and allowed to attach and grow. Quiescent cells were stimulated by growth factors, the growth was measured by WST-8 cell counting kit. WST-8 measures metabolic activity of viable smooth muscle cells. Cytostatic or quiescent cells show little or no WST-8 color change. The WST-8 results reflect the proportion of actively viable cells.

10 μ l WST-8 cell counting kit was diluted with 100 μ l cell culture medium per well. Polymer samples with rapamycin were taken out from wells with sterilized tweezers for drug release study. After that, the old culture medium was replaced by 100 μ l fresh culture medium with WST-8. Cells were re-incubated in CO₂ incubator at 37 °C for another 2 hours.

After incubation, 96-well plate was taken out from incubator and the cell culture medium appeared in orange color. WST-8 absorbance was measured by an Elx 800 Universal Microplate Reader (BIO-TEK INSTRUMENT, Inc, Germany) at a wavelength of 450 nm, 630 nm was using as a reference wavelength. Cells after WST-8 study were to be used for BrdU colorimetric assay, so during the measurement, the cover of 96-well plate must not be open when outside aseptic cabinet.

3.3.5 BrdU colorimetric assay for DNA synthesis study

Cell Proliferation ELISA, BrdU, a colorimetric immunoassay for the quantification of cell proliferation, based on the measurement of BrdU incorporation during DNA synthesis

was used as a non-radioactive alternative to the ^3H -thymidine incorporation assay in this study.

BrdU (5-bromo-2'-deoxyuridine) is an analogue of thymidine. During S phase of the cell cycle, BrdU can be incorporated into DNA in proliferating cells. After its incorporation into DNA, cells were fixed and denatured, BrdU was detected by immunoassay using anti-BrdU antibody. The color intensity after linking to TMB (Tetramethyl Benzidine) was measured by Elx 800 Universal Microplate Reader (BIO-TEK INSTRUMENT, Inc, Germany) at 630nm/490nm.

Procedure description:

BrdU colorimetric immunoassay was performed after WST-8 assay for the same cell samples. Cells after WST-8 study were washed carefully with 100 μl filtered pH7.4 phosphate buffer solution for thrice. Buffer was removed completely after washing. BrdU labeling solution was diluted to 100 μM by fresh culture medium, and 100 μl diluted BrdU solution was added per well. The plate was re-incubated at 37°C for 2 hours. During this labeling period, the pyrimidine analogue BrdU was incorporated in place of thymidine into DNA of proliferating cells. After incubation, plate was taken out from incubator, the culture medium with BrdU was removed. Smooth muscle cells were fixed and the DNA was denatured by FixDenant at room temperature by 30 minutes. The denaturation of the DNA is necessary to improve the accessibility of the incorporated BrdU for detection by antibody. 100 μl diluted Anti-BrdU-POD working solution was added per well. Anti-BrdU-POD was bound to BrdU incorporated in newly synthesized cellular DNA. Plate was incubated at 25 °C for 90 minutes. Extra anti-BrdU-POD was

removed by washing of wells extensively with washing buffer. The firm immune complexes were detected by subsequent substrate TMB reaction for 30 minutes. Green color appeared gradually in wells with proliferating hcaSMCs due to product of TMB reaction. The TMB product was then quantified by measuring the absorbance at 630nm/490nm using a scanning multiwell spectrophotometer. The developed green color and thereby the absorbance values directly correlate to the amount of DNA synthesis and hereby to the number of proliferating cells in the respective microcultures. BrdU labeling is a quite complicated quantitative assay which needs careful manipulation. Control wells were made in the same plate using cell culture medium without cells, to deduce error due to the binding of antibody to excessive BrdU and 96-well plate.

3.3.6 Scanning Electron Microscope for polymer degradation and cell attachment study

(a) Polymer film degradation

The Scanning Electron Microscope (SEM) JOEL 5310 was used in observation of polymer film degradation during immersion. Polymer samples taken from buffer were rinsed with distilled water and dried in vacuum oven for 5 days. Samples were cut into rectangular shape, immersed into liquid nitrogen with a tweezer for about 5 minutes, then sample was broken by force. The cross-section was coated with gold particle for 20 seconds and observed by SEM.

The Field Emission Scanning Electron Microscope (FESEM) JOEL 6340F was used to observe the hydrolytic degradation of polymer film surfaces. Polymer films were attached onto the holder, and coated with gold for 1 minute, then follow the instructions in operation manual.

(b) Cell attachment study

An SEM was used in the study of cell attachment to rapamycin-eluting PLGA films since the attachment could not be observed by Inverted Optical Microscope directly. Polymer samples were cut into discs with a diameter of 5 mm. After sterilization with 70% ethanol and washing procedures, which are the same as sample sterilization procedures, films were inserted into the wells of 96-well plate. Films were fixed to the bottom of wells firmly to avoid possible invasion of cells to the plate. Fixed films were incubated with 200 μ l fresh culture medium in CO₂ incubator for 1 day to enable protein attachment.

After pre-incubation of films in culture medium, cells were seeded onto prepared films and incubated in CO₂ incubator at 37°C. The medium was changed at day 3 and day 6 to avoid interfering with cell attachment. At day 1, 3 and 7, culture medium was removed. Films were taken out very carefully, put into 24-well plate and fixation procedures were performed.

Polymer samples with cells were washed by 500-1000 μ l PBS for 2 to 3 times. After that, 300 μ l 3% glutaraldehyde in 1 ml PBS was added to fix cells for 1 hour. This step ensures that the cells were immersed into glutaraldehyde solution. After fixation, samples were washed with 500-1000 μ l de-ionized water 5 minutes for 2 to 3 times to remove excessive glutaraidehyde. Dehydration was done using different concentrations of ethanol in water. Samples were put in 50% ethanol for 15mins, then changed to 75% ethanol for 15mins, after that, 95% ethanol were used for 15minutes twice. At last, pure ethanol was added for 30 minutes twice. After dehydration, cells were dried by HMDS 200 μ l per well in a 24 well plate overnight in hood. After sample fixation and drying, films with cells were coated with gold for 10 seconds and observed by SEM JOEL 5310 at 5 KV.

3.3.7 Cell growth recovery study

Cell growth recovery was studied using WST-8 kit. Cells treated with rapamycin were kept free from rapamycin by removing rapamycin-eluting films and washed thoroughly with PBS. Fresh culture medium was added and reincubated in CO₂ incubator. Cells treated with rapamycin for 7 days and 14 days were used as samples for this study. An extra 14 days' culture was continued to monitor the cell growth recovery by WST-8 assay.

3.3.8 Water uptake and mass loss study

(a) Water uptake

Water uptake during immersion in buffer was studied using control film of A, C and E referring to **Table 3.3**. The initial mass of polymer samples were weighed and recorded as m_0 , then samples were placed into pH 7.4 buffer at 37 °C. Samples were taken out from buffer and rinsed with distilled water at certain intervals. Water on polymer surface was wiped carefully by water-absorbent paper. The wet mass was measured immediately as m_w . After that, samples were dried in vacuum oven at 37 °C for 5 days to evaporate all water and the dried mass was weighed as m_d . Mass measurements up to 0.01 mg were recorded when sample weights were stable. Water uptake was calculated by following equation.

$$(m_w - m_d) / m_d \times 100\% \quad (\text{Eq. 3.5})$$

(b) Mass Loss

Mass loss study was done using control film A (Refer to **Table 3.3**). Method was mentioned above, and the mass loss was calculated by:

$$(m_0 - m_d) / m_0 \times 100\% \quad (\text{Eq. 3.6})$$

Chapter 4

In vitro Rapamycin Release Study

4.1 Introduction

This chapter focuses on the in-glassware (*in vitro*) rapamycin release studies. Rapamycin is a very potent drug in anti-proliferation and is used for prevention of intravascular hyperplasia. It has more recently been used within a polymeric coating onto a metallic stent, to achieve sustained and controlled local rapamycin release. Localized rapamycin delivery has been shown to be highly efficient and efficacious, with less side effects. The local release of rapamycin has led to a remarkable decrease of restenosis in metallic stents compared to bare metal stents. With more and more attention paid to rapamycin in anti-hyperplasia, the mechanisms of rapamycin in inhibition of cell growth and proliferation have been well investigated. However, in contrast, little or no work has been reported on the effects of controlled release of rapamycin on cell growth/proliferation; and consequently, no light has been shed on the desired release rate for this drug in the anti-hyperplasia application. This therefore forms the basis of the work to be reported below. The polymer samples used in this study are listed and have been labeled accordingly in **Table 3.3**.

4.2 Screening of materials

The screening of polymer materials for degradation and thermal properties is crucial to the selection of appropriate polymers for the stent application. The drug release kinetics will be influenced by many factors, including the sample shape, presence or absence of a plasticizer, the release environment and the presence of an “overcoat” layer etc., but among them the most important factor is the properties of the polymers themselves.

4.2.1 Degradation study

Drug release from amorphous polymers is mainly controlled by degradation and diffusion. For some polymers, such as the ones used in this study, the degradation rates are high enough to dominate drug release. So degradation of PLGA 53/47 and PLGA 50/50 was particularly important to drug release from these two polymers. The trends of their degradation were monitored during the immersion in buffer 7.4. Different degradation profiles were compared and mechanisms were explained.

GPC graphs of Sample A, C, E and F are shown in **Figure 4.1**. X-axis is the elution time in GPC, Y-axis stands for the nRID signal. As plotted in **Figure 4.1 (a)**, peaks of PLLA 6.63 and PLGA 53/47 were clearly separated. Obvious difference can be observed for these two polymers with immersion time from day 0 to day 20. The PLGA 53/47 peak was seen to decrease in size and finally became undetectable at day 20. Part of the hydrolyzed polymer chains is released into the medium and results in a loss of polymer matrix and subsequently a smaller peak in GPC graph. Meanwhile, the PLGA 53/47 peak shifted from 6.5 minutes to about 7 minutes, suggesting the molecular weight was

becoming lower gradually due to degradation. The PLGA 53/47 peak also broadened particularly after day 7.

Fig 4.1 (b) shows the elution time for the combination of blank PLLA 6.63 coated by PLGA 53/47 incorporated with 5% PEG 4000. The GPC graph showed a faster degradation than for the pure PLGA 53/47. At day 3, the peak had broadened considerably but thereafter, the shape and position of peaks did not change as much as that of pure PLGA 53/47. By day 20, there was no visible PLGA 53/47 peak. An interesting fact is that the results also showed that the distinct PEG peak did not change much in height and width throughout. This may be due to some partitioning of PEG into the PLLA layer, and that most of the initial release of PEG is from the surface of either polymer; the bulk PEG appears not to be released into the buffer as degradation proceeded.

Fig 4.1 (c) shows the elution time for blank PLLA 6.63 with PLGA 50/50. Unlike the results in (a) for pure PLGA 53/47, the peak of PLGA 50/50 remained unchanged from day 0 to day 13. After day 13, the peak broadened and elution time shifted to right. This implies the degradation of PLGA 50/50 was much slower than PLGA 53/47.

Fig 4.1 (d) shows the elution time of PLGA 50/50 coated PLGA 53/47. The degradation of PLGA 50/50 was still slower than PLGA 53/47 even PLGA 53/47 was in the middle layer between PLLA and PLGA 50/50. The trend of molecular weight change for PLGA 50/50 is shown in **Fig 4.1 (c)**. The effect of coating PLGA 50/50 will be discussed in Sec 4.2.2.

(a)

(b)

(c)

(d)

Figure 4.1 GPC graphs of degradation studies. (a): PLGA 53/47 (Sample A in Table 3.3); (b): PLGA 53/47 with 5% PEG 4000 (Sample C); (c): PLGA 50/50 (Sample E); (d): PLGA 53/47 coated with 50/50 (Sample F)

Integrated GPC results of molecular weight change with increasing immersion time were plotted in **Figure 4.2**. In **Figure 4.2**, X-axis is the immersion time, Y-axis is the polymer molecular weight (g/mol). The first is PLGA 53/47 (Sample A), the second is PLGA 53/47 with a slight addition of 5% PEG (Sample C) and the third is PLGA 50/50 (Sample E). As seen in the results in **Figure 4.2**, the two PLGA 53/47 combinations have a higher initial molecular weight (7.7×10^4 g/mol) than the initial molecular weight of PLGA 50/50 (1.1×10^4 g/mol).

Figure 4.2 Molecular weight of PLGA 53/47 with and without PEG 4000 and PLGA 50/50

Both PLGA 53/47 films with and without PEG showed faster degradation rates. However, it is clearly seen that the addition of 5 % PEG accelerated the degradation of PLGA 53/47 especially at the initial stage. This expedition in degradation can be more clearly seen from **Figure 4.3**. Within 3 days, the molecular weight of PEG plasticized PLGA 53/47 dropped 31% while at the same time, molecular weight of pure PLGA 53/47 only dropped 5.5%. The molecular weight loss of film C was quite higher than film A before day 13. After day 13, the molecular weight loss was almost the same. Generally PLGA by itself degraded slowly initially, because of the time needed for the water to penetrate into the polymer matrix initially. After day 3, after sufficient water has ingress into the matrix to enable more water to penetrate into the film easily, the degradation rate increased, as shown in **Figure 4.4**. By day 13, chain scissions of the PLGA 53/47 occurred to form polymer chains of lower molecular weight. More cracks and pores could be observed during this time, suggesting more water penetration, which was proved by an abrupt water uptake for film A and C after day 14 as shown in **Figure 4.4**. Polymer mass loss was detectable after day 3 for both films, and increased considerably after 2 weeks (**Figure 4.5**). This was proven by FESEM results as shown in **Figure 4.6** and **Figure 4.7**. Only cracks were seen at day 7 for film A and C. At day 14, pores were observed for both films, but there were more pores on surface of film C than film A, which suggests an accelerating effect of PEG on PLGA degradation. There is also a self-catalytic effect due to the accumulation of the acidic end groups, resulting in an acceleration of the overall degradation rate. During this period, the average molecular weight decreased quickly. By day 20, the molecular weight of PLGA 53/47 dropped from 77,000 to only 3,300 g/mol.

The incorporation of 5 wt% PEG ($M_w = 4000$ g/mol) was seen to significantly increase the initial degradation of PLGA 53/47. From day 0 to day 3, the molecular weight

dropped from 77,000 to 52,000 g/mol, whereas the sample without PEG dropped marginally only to 72,000 g/mol. PEG is a hydrophilic polymer with a high moisture absorption. The addition of PEG effectively plasticized the polymer. On immersion into the release medium, the PEG on the surface was quickly dissolved, leaving space into the bulk polymer, allowing water to penetrate more easily, which was demonstrated by the water uptake study (Figure 4.4) and FESEM results in Figure 4.6 and 4.7. So during this time the amount of absorbed water by polymer was higher than by pure PLGA 53/47, thus the hydrolysis extent was higher. After that, the degradation was slowed down till day 13. By day 20, the molecular weight of PLGA 53/47 was too low to be detected by GPC, indicating that there was a remarkable decrease of molecular weight in PLGA 53/47. The incorporation of PEG expedited the degradation of PLGA 53/47 compared to the pure polymer without plasticization.

Figure 4.3 Molecular weight losses of PLGA 53/47 and PLGA 50/50

Figure 4.4 Water uptake of PLGA 53/47 and plasticized PLGA 53/47 with PEG

Figure 4.5 Mass losses of PLGA 53/47 and plasticized PLGA 53/47 with PEG

Figure 4.6 Surfaces morphologies of PLGA 53/47 and plasticized PLGA 53/47 with PEG without immersion and after immersion for 7 days. (a): Sample A at day 0; (b): Sample A at day7; (c): Sample C at day 0; (d): Sample C at day 7; (e): Sample D at day 0; (f): Sample D at day 7

Figure 4.7 Surface morphologies of PLGA 53/47 and plasticized PLGA 53/47 with PEG with immersion of 14 and 21 days. (a): Sample A at day 14; (b): Sample A at day 21; (c): Sample C at day 14; (d): Sample C at day 21; (e): Sample D at day 14; (f): Sample D at day 21

From **Figure 4.6** and **4.7**, we can observe the main difference in polymer surface morphology between pure and plasticized PLGA 53/47 appeared during day 0 to day 14. During this time, there were more cracks and pores on films plasticized with PEG. However, from microscope graphs of day 21, this accelerating effect of PEG on PLGA was attenuated. Addition of a high concentration of PEG of 10 percent, resulted a faster degradation than low concentration of 5 percent. However, this accelerating effect on degradation also disappeared at day 21. **Figure 4.8** shows the SEM pictures of PLGA 53/47 cross sections at day 0 and day 14. Pores created by water penetration can be clearly seen at day 14 compared to graphs of day 0. The addition of PEG increased water uptake and subsequently resulted in more pores and water channels in PLGA 53/47, as shown in **Figure 4.8**.

Figure 4.8 Cross sections of PLGA 53/47 and plasticized PLGA 53/47 with PEG without immersion and after immersion for 14 days. (a): Sample A at day 0; (b): Sample A at day14; (c): Sample C at day 0; (d): Sample C at day 14; (e): Sample D at day 0; (f): Sample D at day 14

Over the duration of the test, the PLGA 50/50 system showed very slow degradation (**Figure 4.2** and **Figure 4.3**). PLGA 50/50 is an amorphous polymer with lauryl ester end groups as illustrated in **Figure 4.9**. The PLGA 50/50 degraded very slowly because its structure was different from that of PLGA 53/47; the end-capping by the methoxy groups appeared to also render the polymer less hydrophilic. There was only slight decrease in molecular weight till day 13, after day 13, the degradation was faster than before, though still quite slow. At day 20, there was a 33% loss of initial molecular weight (**Figure 4.3**).

Figure 4.9 Chemical structure of PLGA 50/50 with lauryl ester end group

4.2.2 Thermal properties

(a) T_g change of different polymers

Figure 4.10 shows the T_g change of PLGA 53/47 and PLGA 50/50 during immersion. From this picture, from day 0 to day 13, T_g of both polymers showed a slight decrease. However, after day 13, there was a significant T_g decrease. The T_g decrease of PLGA 53/47 was much faster than PLGA 50/50, which is in agreement to the results in degradation studies that PLGA 50/50 is structurally more stable than PLGA 53/47.

The initial molecular weights for PLGA 53/47 and PLGA 50/50 were 7.69×10^4 g/mol and 1.12×10^4 g/mol, respectively. For amorphous polymers, a lower molecular weight would indicate more free volume due to the shorter polymer chains. The results showed that as the time of immersion increased, both polymers showed lower T_g values, due to lowered molecular weights. In general, these data concur with the Mw-degradation results, with substantial drop occurring after about day 12.

The initial T_g for PLGA 53/47 was around 37 °C (the normal body temperature for humans). Thus, a lowering of the T_g of this material inside the human body would change the material into a rubbery phase, with a correspondingly faster degradation and thus increasing the release rate of rapamycin. The initial T_g of PLGA 50/50 was less than 30 °C, which may lead to some processing problems such as morphology change in polymer drying. Great care should be taken during casting, drying and some other treatments for PLGA 50/50.

Figure 4.10 Glass transition temperatures of PLGA 53/47 and PLGA 50/50 during immersion

(b) T_g change of plasticized PLGA 53/47

The overall T_g results for varying PLGA 53/47 samples with varying PEG addition, obtained using similar MDSC curves, have been plotted for comparison in **Figure 4.11**. For comparison, result for the PLGA 53/47 without PEG was also plotted in.

It can be seen that the addition of 5 and 10 % PEG had an immediate plasticizing effect. The T_g was reduced from 35.8°C to 31.1 °C and 23.7 °C, respectively for 5 wt% and 10 wt% addition of PEG. This was due to the fact that the addition of PEG 4000 interrupted the polymer chain packing and made it more randomly. The interruption enabled polymer chains to have more free volumes for rotation and movement as the temperature rises.

Figure 4.11 Glass transition temperature of plasticized PLGA 53/47

During immersion, plasticized PLGA 53/47 showed different trend of T_g change. For pure PLGA 53/47, there was only a slightly decrease of T_g within the first two weeks. In contrast, the plasticization effect of PEG not only reduced the initial T_g of polymer, but also accelerated the drop of T_g during immersion time. The faster drop within first 2 weeks was due to the degradation of polymer generating shorter chains. After 2 weeks, the decreasing trend of T_g for pure PLGA 53/47 was faster than that of plasticized PLGA 53/47. This can be explained by mass loss as shown in **Figure 4.5**. In **Figure 4.5**, a significant decrease of mass loss can be observed in pure PLGA 53/47 after day 14, while the same decreasing trend happened after day 21 for both of 5% and 10% PEG plasticized PLGA 53/47. A higher loading of PEG resulted in an even lower initial T_g , but the T_g trends subsequently were similar to each other, probably because the free volume

generated by 10 wt% PEG was not significantly higher than that of 5 wt% during the immersion.

(c) T_g change of PLGA 53/47 coated with PLGA 50/50

The effect of PLGA 50/50 coating to T_g of PLGA 53/47 is shown in **Figure 4.12**. PLGA 53/47 was between layers of PLLA 6.63 and PLGA 50/50, so the water penetration into the middle PLGA 53/47 layer was difficult. The ‘sandwich’ structure offered barriers at both sides of PLGA 53/47 and prevented the plasticization of water molecules. PLGA 53/47 with the PLGA 50/50 coating did not show any decrease in T_g until after 12 days. After 12 days, PLGA 50/50 began to degrade and the resulting T_g drop is due to degradation of both PLGA 50/50 and PLGA 53/47, followed by a delayed decreasing slope. PLGA 50/50 coating effectively stabilized T_g of PLGA 53/47 and delayed the drop.

Figure 4.12 Glass transition temperature of PLGA 53/47 coated with PLGA 50/50

4.3 Drug release study

Drug loading and control of its release are the significant advantages for using a fully polymeric coronary stent. More drug can be incorporated and released in a controlled manner for a fully polymeric stent. Therefore the aggregation of blood cells and proliferation of smooth muscle and fibroblast cells can be inhibited effectively so as to eliminate restenosis. In vitro release profile is crucial in selecting the stent material, stent design and the amount of drug to be loaded. A sustained release kinetics close to zero-order will be more acceptable compared with release profile of other types. The following section discusses the release profiles of rapamycin from various combinations of polymers and different modifications.

4.3.1 Different drug loadings

Figure 4.13 Rapamycin of different drug loadings released from PLGA 53/47

The rapamycin release profiles for 1 % and 2 % drug loadings are shown in **Figure 4.13**. At these loadings, the rates of release were seen to be quite similar. The usual burst effect is not observed, presumably because rapamycin is hydrophobic, it can be mixed homogeneously with polymer solution and there is not much surface segregation of rapamycin. Release of rapamycin was slow until day 11 until the polymer degraded rapidly. This initial slow release is probably the stage where diffusion-control was dominant. After day 11, PLGA 53/47 degraded faster and rapamycin release increased accordingly. Both concentrations exhibited similar release profiles.

For polymer samples with a slab shape, it is a zero-order release in degradation-controlled stage, the following equation can be used to calculate the release constant:

$$Mt/M = k t \quad (\text{Eq. 4.1})$$

Where the Mt/M is the fraction of released rapamycin, k is constant, t is release time.

The release rate constant in degradation controlled stage was calculated and plotted in **Figure 4.14**. Data points after day 11 was chosen to calculate the constant. The k of 2 wt% of rapamycin is 2.8759 %/day, while the k of 1 wt% of rapamycin is 2.4235 %/day. The rates are comparable. So low drug concentrations at 1 % and 2% loading resulted similar release rates in PLGA 53/47.

Figure 4.14 The release rate constant of PLGA 53/47 with 1% and 2 % of rapamycin

4.3.2 Plasticization effect

The plasticization effect of polyethylene glycol (PEG 4000) was studied. Polyethylene glycol is a hydrophilic polymer, it dispersed into polymer matrices as a plasticizer. Two loadings of PEG, 5 wt% and 10 wt%, were added into PLGA 53/47 to modify the rapamycin release profile.

Figure 4.15 Rapamycin release from PLGA 53/47 with and without addition of 5 wt% PEG

The release profile of rapamycin from PLGA 53/47 was shown to be significantly modified with the addition of PEG in **Figure 4.15**. With 5 wt % addition of PEG, the release profile became more linear and faster. The incorporation of PEG probably resulted in a degradation dominant release (instead of the slower diffusion-controlled release), approximating to an almost zero order release kinetic. Evidently, a faster degradation caused by the presence of the hydrophilic PEG resulted in a corresponding increase in the drug release.

Figure 4.16 Rapamycin release profiles from PLGA 53/47 plasticized by 5 wt% and 10 wt% PEG

Figure 4.16 compares the results of the drug release for 5 and 10 wt % addition of PEG. The addition of more PEG (10%) did not have any further effect on rapamycin release than 5% PEG. Although rapamycin release was slightly higher in 10 wt% PEG plasticized films from day 11 to day 23, over the entire period of study, release rates appeared to be similar for 5% and 10% PEG addition. Water uptake was accelerated by hydrophilicity of PEG (**Figure 4.4**), which affects the hydrolysis and drug release rates, as observed from the GPC and MDSC results in **Figure 4.2** and **4.11**.

Figure 4.17 Rapamycin release constant of PLGA 53/47 with 5 wt% and 10 wt% PEG

Both of these PEG plasticized films show a linear profile in rapamycin release. Trendline were added in Figure 4.17. Unlike the trendline in Figure 4.14, the trendlines in Figure 4.17 were plotted through the whole data range of the rates of release are comparable, and appear to be linear (zero-order) in nature, attesting to the lack of diffusion-control.

4.3.3 Different polymers

PLGA 53/47 and PLGA 50/50 were chosen as basic polymers to study the rapamycin release from different matrices considering they were both amorphous polymers and of

low molecular weight. A main difference between these two polymers is the structure of the end group. The end group of PLGA 50/50 is lauryl ester, as demonstrated in section 4.2, whereas PLGA 53/47 has -COOH and -OH end groups.

Figure 4.18 shows the rapamycin release profiles of PLGA 53/47 and 50/50. PLGA 50/50 had a lower rapamycin release compared to PLGA 53/47. The Rapamycin released up to day 11 was lower than 1% of drug loading in PLGA 53/47, with the release from day 5 to day 11 showing a diffusion-controlled release. After day 11, rapamycin in PLGA 50/50 was released faster due to the start of polymer degradation. As discussed previously, the degradation of PLGA 50/50 was considerably slower than the degradation of PLGA 53/47 (**Figure 4.2** and **4.3**). The difference in end groups resulted in different release profiles. The lauryl ester end group in PLGA 50/50 renders the polymer less hydrophilic, and this delayed the onset and rate of polymer degradation. As the degradation of PLGA 50/50 was very slow, the release curve exhibited smoother and no abrupt increase was observed as compared to PLGA 53/47 from day 16 to 23.

The T_g of PLGA 50/50 matrix is 28.43 °C (**Figure 4.10**), such a low T_g made the polymer rubbery at 37 °C, the drug release temperature. T_g for PLGA 53/47 is around 37 °C, about 10 degree higher than PLGA 50/50. However, the lower T_g did not play a key role in rapamycin release. Lower T_g usually results in faster diffusion kinetics, but this was not observed here. We believe this may be due to the fact that PLGA 53/47 absorbs more water, hence its T_g decreased below that of PLGA 50/50 (plasticization effect), thus making the T_g 's closer after immersion in buffer at 37 °C.

Figure 4.18 Rapamycin release profiles of PLGA 53/47 and PLGA 50/50

Figure 4.19 Release constant of PLGA 53/47 and PLGA 50/50

Figure 4.19 shows the release constant of PLGA 53/47 and PLGA 50/50. From the k value, we can see that the rapamycin release rates during degradation-controlled stage were quite similar. Since it is a plot of the accumulated release, the difference in rapamycin release profile was due to the delay in PLGA 50/50 degradation.

4.3.4 Polymer coating effect

Figure 4.20 shows the rapamycin release profile of PLGA 53/47 coated with PLGA 50/50. As a comparison, release profiles of PLGA 53/47 and PLGA 50/50 were added in. As shown in **Figure 3.2 (b)**, the thickness of PLGA 50/50 layer is 60 μm , the total thickness of this triple-layer film is 220 μm . The bottom layer is PLLA 6.63, a crystalline polymer and the diffusion of rapamycin into PLLA layer may be negligible. From day 0 to day 11, the release of rapamycin of Sample E exhibited a very similar profile to the drug release from PLGA 50/50. This is possibly because the partitioning of rapamycin from PLGA 53/47 layer into PLGA 50/50 layer, and released out from the PLGA 50/50 side. In the diffusion-controlled stage, the coating does not appear to influence drug release much; however, the onset of degradation-controlled release and the subsequent rate appears to be affected by the coating, although not substantially. Release of rapamycin from PLGA 53/47 was deferred by PLGA 50/50 layer since the water penetration through PLGA 50/50 was slow. The release rate of rapamycin in the triple-layer was between the release from PLGA 53/47 and PLGA 50/50 alone.

Figure 4.20 Rapamycin release profiles of PLGA 53/47 with blank PLGA 50/50 coating

4.4 Summary

The polymer materials were screened for their studies of degradation and thermal properties. Drug release was monitored and profiles were figured out. The release of rapamycin can be ranged from faster to slower as: PLGA 53/47 with 5% (10%) PEG 4000 > PLGA 53/47 > PLGA 53/47 coated with PLGA 50/50 > PLGA 50/50. Thus release profiles of rapamycin were successfully modified. Results were discussed in this chapter. Three films among the above were chosen for cell culture studies.

Chapter 5

Drug Release Study in Cell Culture

5.1 Introduction

This chapter reports on the results obtained for tests on the release kinetics for rapamycin in cell culture. The tests investigate into the effects of changes in the release environment from buffer pH 7.4 to the cell culture medium with cells, on the release profiles for rapamycin. Understanding the difference in release kinetics between in-glassware study and under cell culture conditions would elucidate knowledge into the real release situation when the release profile is to be predicted in culture medium, according to the results in buffer system.

In this chapter, the amount of rapamycin released into the cell culture medium from different film samples would be shown. Based on results of Chapter 4, PLGA 53/47 with 1% and 2% rapamycin as well as PLGA with 1% rapamycin but plasticized with 5% PEG would be studied, as different release rates were observed for these films in buffer system. Here, we will also deduce the relationship between the amount of released rapamycin and its inhibition effect on human smooth muscle cell growth, which would be further discussed in greater detail in the next chapter.

5.2 Degradation study

Figure 5.1 shows the plot of the decrease in molecular weight for various polymer samples over time, as the rapamycin was released in either the buffer or cell culture

solutions. The tested samples include samples combining blank PLLA with PLGA 53/47 and samples combining blank PLLA with PLGA 53/47 plasticized by 5% PEG.

The results show that no difference in molecular weight loss for PLGA 53/47 in cell culture and in buffer. As for the samples containing additional 5 % PEG, it was observed that they degraded faster, due to the plasticizing effect of PEG, in both the buffer and cell culture medium. The samples with 5 % PEG have shown a faster initial degradation in the buffer medium than in the cell culture medium. But by day 7, the sample in cell culture has increased in rate to catch up and even exceed the former. The change of release medium from buffer to cell culture systems generally increased the degradation of plasticized PLGA 53/47.

Figure 5.1 Degradation study of PLGA 53/47 in buffer and in cell culture medium

5.3 Drug release study

Results for the drug release studies in this section have been divided into two parts: for rapamycin release by weight percentage, and for the amount of rapamycin released in cell culture, in order to obtain more instructive insight into the cell culture studies.

5.3.1 Rapamycin release by percentage

Figure 5.2 compares the results of the drug release for identical 1 % drug loaded PLGA 53/47 samples, for both the cell culture and the buffer medium. The results clearly show that the drug release was significantly faster in the cell culture medium. **Figure 5.3** similarly compares the results for the drug release, but with a higher 2 % drug loading. A similar faster rate of release was observed for the cell culture medium compared to the buffer medium. In fact, the difference seemed greater for this higher drug loading. **Figure 5.4** shows similar results comparing the PLGA 53/47 with 5 % PEG samples. The results show that while there was still a greater release rate in the cell culture medium compared to the buffer medium, the difference in this case was a lot less.

Figure 5.2 Compare rapamycin release from PLGA 53/47 (1% rapa) in cell culture medium with in buffer 7.4

Figure 5.3 Compare rapamycin release from PLGA 53/47 (2% rapa) in cell culture medium with in buffer 7.4

Figure 5.4 Compare rapamycin release from PLGA 53/47 (1% rapa, 5% PEG) in cell culture medium with in buffer 7.4

As listed in **Table 5.1**, for 1% of rapamycin loading, there was accumulated 31.7% and 37% released for the first and second week respectively. In first 2 days there was about 15.9% rapamycin released, as a contrast, there was only about 3% rapamycin released in buffer at day 3. For 2 % of rapamycin loading, the difference was even more distinct. There was 22.4% of rapamycin release at day 2 and a totally 53% release at day 17. For PLGA 53/47 with 5% PEG, the increase in rapamycin release was not as remarkable as the other two films compared to the result in buffer, but still more than the released in buffer. From Figure 5.5, the rapamycin release at day 7 was 32.7%, while at day 17, there was 68.2% rapamycin released out.

Table 5.1 Rapamycin release by percentage

Release Time	Accumulated rapamycin release (%)		
	PLGA53/47 (1% rapamycin)	PLGA53/47 (2% rapamycin)	PLGA53/47 (1% rapa,5% PEG)
Two days	15.91	22.4	8.33
The 1 st week	31.71	29.65	32.71
The 2 nd week	37.05	47.90	48.03
Total in 17 days	44.51	53.85	68.15

Figure 5.5 Rapamycin release in cell culture medium

Figure 5.5 plotted together the results for different drug loadings of 1 and 2 %, as well as for the same loading of 1 % but in samples containing 5 % PEG. The results show that the release profiles of these three films were very similar to each other up to 7 days. Thereafter, the higher loaded sample and the sample containing 5 % PEG degraded visibly faster than for the 1 % drug loaded PLGA 53/47 sample. Compared to the drug release profile in buffer, the change in release system from buffer to cell culture medium resulted in a faster release in the sample of 2 % drug loading after day 7, however, this change could not be explained solely by polymer degradation, but more significantly, it is caused by a higher partition coefficient in cell culture conditions, the external driven force to rapamycin release.

The difference in rapamycin release between in buffer and in cell culture systems could not only be attributed to a significant difference in polymer degradation, as shown from the results in **Figure 5.1**. Though this explanation would be insufficient, the difference in release rates in both medium must be explained by other factors instead.

Rapamycin specifically binds to FKBP-12, which forms the rapamycin-FKBP12 complex, which then interacts with the mTOR to inhibit the cell proliferation. The study by *Levin et al.*¹⁰⁸ has shown that the FKBP12 was most abundant in vascular smooth muscle cells at a concentration of about 10^{-5} M. However, the Rapamycin is not stable in cells. It has a half life of about 135 hr in the human blood system. When bound to plasma, its half life is even lower at 7.2 hr¹⁰⁹. *Hosoi et al.* has also shown that in rhabdomyosarcoma cell lines, the biological half-time of rapamycin is only about 9.9 hours¹⁰¹. The low stability of rapamycin in blood and cytosol is due to the hydrolytic degradation. Another reason contributing to its instability is the dissociation of rapamycin-FKBP12 complex. As mentioned in Chapter 2, the formation of rapamycin-FKBP-12 complex stabilizes

rapamycin in cellular system, and elongates its half life. However, the dissociation of this complex results in a continuous demand for rapamycin. This ability of the drug to bind itself to the cells is a significant force for rapamycin release in cell culture system.

From results in Sec 5.3.2, it was shown that about 5.76 μg of rapamycin was released into cell culture medium during the first week, for PLGA 53/47 with 1% rapamycin. The culture medium was changed thrice within the first week, so the concentration of rapamycin in cell culture medium was about 1.05×10^{-5} M, which is very close to the concentration of FKBP-12 in vascular smooth muscle cells of about 10^{-5} M. That may suggest, for PLGA with 1% rapamycin, rapamycin release in the first week was enough for the saturation of all FKBP-12 in human vascular smooth muscle cells. If the degradation of rapamycin is considered in cell culture conditions, the binding to FKBP-12 is a significant driven force to rapamycin release.

Another possible force driving the higher drug release rate is the cell culture medium itself. The medium contained fetal bovine serum added for smooth muscle cell growth. There are over 1,000 different components found in serum, including proteins, electrolytes, lipids, carbohydrates, hormones, enzymes, and other miscellaneous undefined constituents. In human blood, many drugs bind extensively to serum albumin (97%), α 1-acid glycoprotein and lipoproteins. So the albumin, lipoprotein and other unknown components in cell culture medium probably also contribute to the faster rapamycin release.

Other than polymer degradation, these two factors combined to accelerate the rapamycin release profile in the cell culture medium. They changed the Partition Coefficient (K) mainly, and enhanced the release of rapamycin in medium and cells by changing the

apparent solubility of rapamycin in the release medium. In contrast, in the buffer medium, the drug release was influenced by the limited solubility of the drug within the medium and was controlled almost exclusively by polymer degradation, considering the fact that the buffer was replenished frequently.

5.3.2 Rapamycin release by amount

Although the results of rapamycin release plotted in percentage give us the information from a view of released rapamycin relative to the initial loading, they are unable to let us know exactly the amount released out during the study. However in a matter of fact, the amount of rapamycin released into cell culture medium is more directly related to the inhibition effects which will be discussed in Chapter 6. Thus it is necessary to discuss the release of rapamycin by amount following the Sec 5.3.1.

Figure 5.6 Rapamycin release profile by amount

The actual amount of rapamycin released was thus measured for some films, using the HPLC and through the calibration curve in **Figure 3.8**. The amount of Rapamycin released over time has been plotted in **Figure 5.6**. For PLGA 53/47 with 5 % PEG loaded with 1% rapamycin, 3.4µg was released into the culture medium within one week, and 8.0µg within 17 days. For PLGA 53/47 only with 1% rapamycin, the amounts released were 5.8 and 8.5 µg for 7 days and 17 days, respectively. When the drug loading was increased to 2 % for the PLGA 53/47 only film, the amount released was seen to more than double for the sample with 1 % loading. If we assume there was no rapamycin degradation in culture medium, the rapamycin daily released in medium after day 2 can be calculated as shown in **Table 5.2**. Daily released rapamycin was all above 309.5 ng per well, which was enough to inhibit the cell proliferation in the 96-well plate (see Chapter 6). Released rapamycin from 2% loading dose was three times of 1% drug loading. The accumulated release of rapamycin for PLGA 53/47 with PEG was lower than without the PEG, because the initial release in 2 days was a lot lower probably due to the loss in sterilization process. However, rapamycin in PLGA 53/47 with PEG released slightly faster than PLGA 53/47 without PEG. If we consider that 100ng/ml of the drug can effectively inhibit the proliferation of PDGF derived human vascular smooth muscle cell proliferation¹⁰⁴, the amounts released from the PLGA 53/47 samples are seen to be more than enough to inhibit proliferation.

Table 5.2 Rapamycin released by amount

Polymer	PLGA53/47 (1% rapamycin)	PLGA53/47 (2% rapamycin)	PLGA53/47 (1% rapa,5% PEG)
Rapamycin release ($\mu\text{g}/\text{well}\cdot\text{day}$)	0.3095	0.9359	0.4106
Concentration ($\mu\text{g}/\text{ml}\cdot\text{day}$)	1.5475	4.6795	2.053
Released Rapamycin in the 1st week	5.76 $\pm$ 0.04	15.46 $\pm$ 0.57	3.40 $\pm$ 0.47
Released Rapamycin in 2 weeks	6.71 $\pm$ 0.28	19.97 $\pm$ 1.59	5.34 $\pm$ 0.31

5.4 Summary

Rapamycin release properties in cell culture were studied in this chapter. Three different rapamycin loaded films were chosen in this study. Generally, rapamycin release rates were considerably enhanced in cell culture conditions compared to the results in buffer. Since there was no significant change in polymer degradation, the increased release of rapamycin were due to the change of drug partition coefficient in cell culture, as the binding of rapamycin to FKBP12 in hcaSMC and to serum molecules as well.

Chapter 6

Effect of Rapamycin on Cell Growth and Proliferation

6.1 Introduction

The growth of SMCs within the coronary artery results in the occurrence of stenosis with fatal results. These quiescent SMCs are stimulated by the presence of both endothelial cells and white blood cells, activating a change from a contractile to a proliferating state¹⁰⁷. The activated SMCs migrate from the media layer through to the intima layer and proliferate continuously there on, over-growing and constricting the artery.

Rapamycin have been shown to effectively inhibit the proliferation and migration of these coronary artery SMCs, in particular those that are largely induced by Platelet-derived growth factor (PDGF)¹⁰⁴. In clinical trials, metallic stents coated with rapamycin have shown promising results in significantly reducing stenosis. Recent studies by *Hafizi et al.* revealed the inhibition effect of smooth muscle proliferation in cell culture conditions and proposed some possible mechanisms¹⁰⁴. However, there has been very few studies reported on the effect of controlled delivery of rapamycin from fully biodegradable polymer film to date.

In this study, the effect of a controlled release of rapamycin from biodegradable polymers on the inhibition of smooth muscle proliferation was investigated. The release kinetics of rapamycin from PLGA, with and without PEG plasticizer, has been reported in Chapter 4. The release profiles of the same polymers with different rapamycin loading in a smooth

muscle cell culture medium have also been reported in Chapter 5. In this chapter, the effects of control-released rapamycin on the proliferation of human coronary artery SMCs' growth were studied. Cell viability, cell proliferation and recovery study were done in triplicates. Data after subtracting background absorbance (by incubating complete culture medium with WST-8 or BrdU kit) was averaged and the standard deviation was calculated.

6.2 Control-released rapamycin inhibits DNA synthesis in SMCs

Rapamycin works by arresting SMC proliferation in the late G1 phase, before entering into S phase by inhibiting mTOR activity. This in turn influences the p70S6K, eukaryote initiation factor 4E, thus reducing the translation activity and protein synthesis. Rapamycin also regulates the mRNA transcription by inhibiting the phosphorylation of retinoblastoma binding protein (pRb) by mTOR, thus prevents the starting of mRNA transcription. Mechanisms of mTOR regulation and rapamycin inhibition were reviewed in Chapter 2. In this study, the DNA synthesis during S phase in proliferating SMCs was studied by BrdU assay.

BrdU (5-bromo-2'-deoxyuridine) is an analogue to thymidine and has been used widely in cell cycle studies. When quiescent SMCs are stimulated, they enter the cell cycle from G0 phase to G1 phase, and prepare for DNA synthesis which happens in S phase (Figure 2.1). When cells finish the preparation and pass through the 'checkpoints' in late G1 phase, cells proceed into S phase. During the S phase in cell cycle, BrdU is to be incorporated into newly synthesized DNA chromatids, the concentration of incorporated BrdU can be measured by the intensity of absorbance at certain wavelength. With the

detection of BrdU, the proportion of cells enter S phase and lead to proliferation can be obtained. Rapamycin inhibits SMCs at G1 phase, inhibited cells are static without BrdU labeling, thus in BrdU assay, these cells are undetected. This chapter therefore also reports on the BrdU activity relating to the DNA synthesis under effect of rapamycin.

Figure 6.1 BrdU study for DNA synthesis in SMCs

Figure 6.1 shows the BrdU incorporation into newly synthesized DNA in proliferating SMCs. Labeling of polymer samples in cell culture study can be referred to **Table 3.4**. Labeling is listed again as: A: PLGA 5347 with 1 % rapamycin; B: PLGA 5347 with 2 % rapamycin; C: PLGA 5347 with 1 % rapamycin and 5% PEG 4000. AB0: Control for A and B, PLGA 5347 without rapamycin; C0: Control for C, PLGA 5347 with 5% PEG 4000 but without rapamycin; Cell: blank cells without polymer samples. X-axis is time (day), Y-axis is integrated BrdU absorbance value subtracted by system error.

From the results, we can see the quiescent cells were activated with the stimulation of growth factors including hEGF, hFGF-B, insulin and increased FBS. Cells started to grow slowly from G0 to G1 phase, making preparation for S phase, the DNA synthesis. During G1 phase, cells grow to increase the cellular size, synthesize protein needed for mRNA transcription and DNA synthesis. In S phase, cells incorporated BrdU into duplicated DNA, continue proceeding to G2 and M phase thus complete the cell cycle to split into two daughter cells.

From **Figure 6.1**, most of the cells stimulated by growth factors finished G1 phase within 2 days, then proceeded to S phase, which occurred from days 2 to 7. During this period, the BrdU incorporation was high. The DNA synthesis reached a peak at day 4. After day 4, it slowed down and most of the cells finished the S phase. Only a small number of cells showed DNA synthesis activity between days 7 to day 10. During this time, the BrdU incorporation was remarkably lower than S phase. So the time from day 4 to day 10 was deduced to be the G2 and M phase for the first cell cycle after cell activation, and possibly the G1 phase for the 2nd cell cycle. It is a complete cell mitosis process from cell activation till day 10. After day 10, some cells started a new cycle which can be seen from

the increase of BrdU incorporation again. In some wells, cell density nearly reached confluence and the DNA synthesis was stopped due to cell contact inhibition effect.

The results show that rapamycin reduced the DNA synthesis activity in human coronary artery SMCs. The data for day 2 and day 4 in Figure 1 shows that the BrdU incorporation was lower in cells treated by rapamycin, though more than 50 % of the cell population was still proceeding into S phase successfully. Interestingly, rapamycin treated cells incorporated more BrdU than cells with control samples in the 2nd cell cycle. This may suggest a delay of cell proliferation by rapamycin rather than total inhibition.

Cells growth for the control samples showed similarity with growth without the polymer films. Cells without rapamycin peaked at day 4 and proceeded into G2 and M phase. Thus the DNA synthesis was followed by a remarkable decrease from day 4 to day 10. After day 10, the DNA synthesis of cells with control samples was much less than cells treated by rapamycin. This may be caused by the faster growth previously, so cells reached a confluence after the 1st cell cycle.

Figure 6.2 DNA synthesis by percentage of control samples

The proportion of proliferating cell number to total cell population against time of rapamycin treatment was plotted in **Figure 6.2**. This figure shows about 40 % of DNA synthesis was inhibited by rapamycin in the first 4 days. After that, most cells went into G2 phase, while there were still some cells remaining in the S phase, which can be seen from the data of day 7 and day 10. During this time, the DNA synthesis of rapamycin treated cells increased to almost the same level as cells with control samples. These cells may be those initially inhibited by rapamycin and slowly proceeding to the S phase following the cell cycle. After day 10, some cells entered into the 2nd cell cycle. Even with the existence of rapamycin released from polymer films, about 150% DNA synthesis (compared to the control cells) can be observed. Although it was not possible to deduce accurately the delay time from this BrdU study, we can still conclude that rapamycin

hindered the DNA synthesis, thus delaying the cell division but not stopping the cell cycle completely. The significant increase in BrdU incorporation after day 10 was partly due to the proliferation of cells with rapamycin, but more important is the confluence of control cells. However, if we assume the cells with control samples could proliferate into more cell cycles, the proliferation of cells with rapamycin should be still under inhibition and DNA synthesis were less than control cells.

From **Figure 6.1**, the BrdU synthesis in cells with control films is similar to that in blank cells, the influence of PLGA and PLLA films on cell proliferation was not observed. Compared to cells without any polymer samples, the control polymer films with plasticizer PEG (Mw = 4000) released into cell culture medium showed no negative effect to DNA synthesis of human SMCs. During the whole experiment, the DNA synthesis still remained in the same level as that of control samples and blank cells, that means any released low molecular weight PEG could not interfere the processes with DNA, probably because the PEG could not be transported across the cell membrane.

From the comparison of DNA synthesis with blank cells, the degradation of PLLA and PLGA over 2 weeks had no effect on SMCs' DNA synthesis. Long term degradation effect of PLLA and PLGA on the smooth muscle proliferation can not be deduced from this study.

6.3 Effect of rapamycin on cell growth

WST-8, [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] was used in the study of smooth muscle cell growth. WST-

8 is a modified tetrazolium salt similar to WST-1, but has better sensitivity and stability. WST-8 with 1-Methoxy PMS measures the cell growth via electron transporter NADH/NADPH which located in cytosol. 1-Methoxy PMS transports the electron from NADH/NADPH to WST-8 at the membrane area which makes the tetrazolium salt to be reduced to formazan in orange color. The color intensity can be measured using microplate reader at 450 nm. For normal coronary artery SMCs, the absorbance at 450 nm is in proportion to the cell number. If the cell growth is to be inhibited, the absorbance of inhibited cells will be lower than control samples without drug treatment. In this study, WST-8 assay was not used directly to count the cell number, but as an indicator of cell growth or cell viability. For WST-8, it measures the metabolic activity of cells.

Figure 6.3 Cell growth study by WST-8 assay.

Figure 6.3 shows the effect of rapamycin and control samples on the growth of SMCs by WST-8 assay. X-axis is time (day), Y-axis is WST-8 absorbance value representing cell growth. As for the case for BrdU assay, the WST-8 study also covered a complete cell cycle of human coronary artery SMC.

The 1st cell cycle, in which cell numbers of all samples remained constant, stretched up to day 10. After day 10, cells finished the mitosis and proceeded into the 2nd cell cycle, which was similarly observed for the BrdU study. The results shown for the control samples at days 14 and 17 showed a significant increase of cell growth, evidently due to the proliferation of the cells.

Figure 6.3 shows that sustained rapamycin treatment effectively reduced the human coronary artery SMC growth. Cells treated with rapamycin-releasing samples showed a remarkable decrease of cell growth in all three films, reducing growth to only about 50-60% of control cells. Considering that there was almost no growth up to day 10 and the cell viability was stable, it was evident that rapamycin exerted no cytotoxic effect on proliferating SMCs, but cytostatic effect. A higher concentration of rapamycin did not seem to reduce growth significantly, also suggesting that the effect of rapamycin was cytostatic rather than cytotoxic. The growth of some cells was inhibited and cell growth was arrested or became very slow. This suppression of cell growth lasted throughout this study. After day 10, although cells were able to grow with the existence of rapamycin, it still maintained the same inhibition level, limiting growth to no more than 60-70% of the control cells. This is more evident in **Figure 6.4**.

Chapter 6 *Effect of Rapamycin on Cell Growth and Proliferation*

The similar results obtained for both the control cells and the growth without addition of polymer films would suggest that the control polymer samples had no negative effect on the cell growth. Inhibition came mainly by the presence of rapamycin only. The addition of PEG also did not show any inhibiting effect on SMCs. Long term effect of polymer on cell growth is unknown in this study.

The results also showed that an increase in rapamycin dosages did not significantly vary the effects of the rapamycin’s inhibiting behavior. Evidently, 2 % loading was well above the required minimum for inhibition.

Figure 6.4 Cell growth by percentage of control samples

Figure 6.4 shows the rapamycin-treated cell growth compared to control samples. The results indicate that up throughout the duration of study from days 2 to 17, cell growth fell to 50-70% of control cells. For all cases, a slight increase in growth was observed after day 10. The sample/control ratio of rapamycin-treated cells increased very slightly, showing the continuing inhibition effect of rapamycin.

The inhibition by rapamycin of smooth muscle cell growth is believed to be via the regulation of mRNA transcription, translation and protein synthesis. The target of rapamycin in SMCs is mTOR, which regulates many mechanisms in eukaryotic cells. In response to amino acids and growth factors, mTOR controls the phosphorylation of p70 ribosomal S6 kinase (p70S6K) and inhibition of eukaryotic initiation factor 4E binding protein (4E-BP1) ⁹⁹.

The mTOR kinase activates the p70 ribosomal S6 kinase by phosphorylation or possibly inhibition of phosphatases such as protein phosphatase 2A (PP2A). The activation of S6K1 phosphorylates the S6 protein of the 40S ribosomal subunit, increases the translation of many mRNA transcripts including ribosomal proteins and elongation factor (eEF-2) for protein synthesis ⁹⁹. Rapamycin inhibits the activation of p70S6K via the inhibition of mTOR kinase ⁹⁸.

Another factor mTOR regulates in translation and protein synthesis is 4E-BP1. 4E-BP1 is the binding protein of eukaryote IF-4E, which inhibits the initiation factor of translation. The mTOR phosphorylates the 4E-BP1, thus activating and initiating the translation process. Rapamycin inhibits the mTOR and also prevents the dissociation of 4E-BP1 from eIF-4E, thus inhibits the translation ⁹⁸. Protein synthesis is very important to cell

growth. Cell growth mainly refers to the synthesis of proteins and the subsequent increase in cellular size. Cell growth mostly happens in G1 and G2 phase during the cell cycle.

WST-8 assay results reflect the fact that the sustained-release of rapamycin inhibited smooth muscle cell viability in vitro. Results suggest that rapamycin is cytostatic to proliferating SMCs. Combined with the results of BrdU study in Section 6.2, control-released rapamycin not only inhibited the DNA synthesis of SMCs, but also reduced the cell growth by inhibition of translation and protein synthesis.

6.4 Cell growth recovery study

With rapamycin, proliferation and growth of human coronary artery SMCs were inhibited successfully. From BrdU assay and cell growth study above, the influence of rapamycin to SMC growth after removal cannot be understood clearly. The cell recovery study after removal of rapamycin gave us the information of post-rapamycin cell growth by WST-8 assay.

Two kinds of treatment with different rapamycin releasing time were used in this study. One set of cells were cultured with rapamycin released from polymer films for 7 days while the other set of cells were cultured with rapamycin from identical films but for 14 days. After culture with rapamycin released from polymer film for 7 or 14 days, the polymer samples were removed from the 96-well plates and the cells were allowed to grow in a fresh medium, with no drug present at all. The cell metabolic activities of both these two sets were monitored by WST-8 assay at regular time intervals, after removal of rapamycin.

Figure 6.5 Cell recovery after rapamycin treatment for 7 days

Figure 6.5 shows the cell recovery after rapamycin treatment for the first group of cells cultured for 7 days. The zero point in X-axis represents the day rapamycin was removed (or polymer film control was removed), when the cells began to recover. The results shows that control sample cells grew faster than cells inhibited by rapamycin up to day 7. Between day 7 and day 10, cells with control samples peaked. Thereafter, growth decreased, indicating that contact inhibition effect had occurred. However, for cells cultured with 1 or 2 % rapamycin, growth was immediately inhibited. Cells reached confluence about 1 week after that of cells with control samples. The samples with the higher 2% rapamycin showed a higher inhibition as well as a longer effective inhibition effect on cell growth, compared to the samples with 1% rapamycin. The effective inhibition time was also prolonged up to day 7, after which the rate was similar to the control samples. From this aspect, a higher rapamycin loading dose of 2 wt% appeared to have an advantageous effect against proliferating SMCs.

Rapamycin-FKBP12 complex enables the drug to prolong its presence within the cells¹⁰¹. However, the dissociation of rapamycin-FKBP12 requires a longer time with rapamycin to saturate the FKBP12 in cells, which will inhibit the activity of mTOR. Thus a long rapamycin release time is preferred for better inhibition result. However, if the rapamycin remains within the cells for a relatively short period, such as only 1 week, a higher loading would be needed to inhibit cell growth effectively. In other words, if rapamycin release cannot be sustained, then a higher dose is required to maintain suppression.

Figure 6.6 shows the results for the recovery of cells treated with rapamycin for up to 14 days. The base zero-point represents the start of cell recovery. The results show that control cell growth peaked on day 0 and started to decrease afterwards in this study. Contrarily, cells treated with rapamycin for 14 days continued to grow to confluence till day 11 after rapamycin removal.

Figure 6.6 shows no significant difference in cell growth with increase in drug loading from 1 to 2 %. This phenomenon may be caused by the accumulated amount of rapamycin in cells. Rapamycin accumulated in long time release as 14 days eliminate the difference between 1% and 2% drug as seen in **Figure 6.5**. This is because the accumulated rapamycin in cells are more than the available FKBP12. Here the availability of FKBP12 becomes the limiting factor. In this case, small concentration of rapamycin with a relatively long-term release will probably show the same inhibition effect as a high dosage with faster release; while in **Figure 6.5** the inhibition of 2% rapamycin was higher than 1% since the rapamycin inhibition lasted for only 1 week.

Figure 6.6 Cell recovery after rapamycin treatment for 14 days

Figure 6.7 compares the growth trends after removal of rapamycin. The time axis is the total time for both when rapamycin was present and the time of cell recovery. **Figure 6.7 (a)** and **(b)** shows the results for 1 and 2 % drug loading respectively, indicating a significant difference between the cells treated with rapamycin for 7 days and 14 days. The reason for these different growth curves lay in the different starting points of these two recovery studies. Cells containing rapamycin up to 7 days have their RNA translation and protein synthesis inhibited, so the cells grew slowly. By this time, the cells are in the G2/M phase, and some cells were unable to pass into the S phase. As such, their preparation for mitosis was not completed. Once the rapamycin was removed after 7 days, the cells were then able to synthesize DNA and proteins for their separation. The cells grew slowly, then proliferated finally to the confluence.

For cells inhibited by the presence of rapamycin for 14 days, on removal of the drug, the cells would have been in their G1/S phase, as shown in **Figure 6.1**. We can deduce that rapamycin still inhibited the cell growth beyond day 14 to day 22, after which cells began proliferating and finally towards a confluence. Evidently the effect of rapamycin well into the G1 phase resulted in a sustained inhibition as shown in **Figure 6.7**. Beyond day 22, the growth showed a high increase, presumably because the majority of cells were proliferating concurrently.

Generally, for cells started to grow without rapamycin from day 7, these cells were in the late stage of cell cycle and cells were beginning to separate, so it showed an increasing recovery growth rate. But cells which started recovery from day 14 would have completed the first cell cycle, and even going in the 2nd cycle. For these cells, the prolonged presence of rapamycin would cause a strong inhibiting delay in cell growth. Thus, from the two sets of data (7- and 14-day treatment) it is clear that the inhibition effect of rapamycin depends considerably on the duration of its release relative to the overall cell cycle time: in other words, if the release is sustained through one complete cycle, the inhibition effects are stronger and last longer.

Figure 6.8 shows the cell growth curves for varying periods of 1% drug treatment on the cells. Cells treated for only 7 days grew faster than cells treated with rapamycin for 14 and 17 days. However, interestingly cells treated for 14 days seemed to show a different growth curve to all the others, including the one that was exposed to rapamycin for 17 days. The removal of rapamycin could have led some changes to cells and resulted in a different growth curve.

Figure 6.7 Cell recovery comparison between rapamycin treatment of day 7 and day 14

Figure 6.8 Different growth curves due to different treatments

6.5 Cell attachment to drug-eluting polymer film

The ability of the SMCs to attach themselves onto the polymer substrate affects its proliferation and growth rate. Reducing SMCs attachment onto stent surface, while promoting the growth of endothelial cells on polymer is crucial for the early wound healing and prevention of restenosis.

Figure 6.9 - 6.11 show the SEM pictures of smooth muscle cell attachment at day 1, 3 and 7, respectively. **Figure 6.9 (a)** shows that very little cells were able to attach themselves to the rapamycin-eluting film. The very low cell density on the film was likely to be due to the inhibiting presence of the rapamycin to cytoskeleton or microtubules, which are required for cells to be attached and to grow. Cells which could not attach to the substrate subsequently died. **Figure 6.9 (b)** shows the results for the control film, without Rapamycin, after 1 day. It is evident that a lot more cell attachment is now evident. The SMCs seeded onto control films showed rapid attachment and were able to fully spread themselves even from day 1. The morphology exhibited in **Figure 6.9 (b)** was different to the behavior on cell culture plastics like flask and plate, as many long filopodia extended in radial fashion and these filopodia were still observed at day 3. SMCs could even form a net shape in high cell density area.

(a)

(b)

Figure 6.9 Cell attachment to PLGA 5347 film at day1. (a): Cell attachment to film with 1 wt% rapamycin; (b): Cell attachment to control film without rapamycin

Figure 6.10 shows similar results for the cell attachment to PLGA films, after 3 days of seeding. For the film containing rapamycin, it can be seen that very little cells were able to attach themselves to the film or to spread over the surface. Cells were like rod shape and shrunken instead of spreading out and contacting with other cells. The direct contact with rapamycin greatly inhibited the cell attachment and growth. SEM pictures indicated the cell number attached to rapamycin-eluting films was only slightly more than that of day 1.

In contrast, the sample without drugs shown in **Figure 6.10 (b)** indicate that the cells were attaching themselves much better and had moved outward randomly and also shrank their cellular size. Cells were smaller than those in samples for day 1. As we know from

mentioned above that the cells require more than 7 days to double in number, these smaller cells are not new-born cells.

(a)

(b)

Figure 6.10 Cell attachment to PLGA 5347 film at day3. (a): Cell attachment to film with 1 wt% rapamycin; (b): Cell attachment to control film without rapamycin

Figure 6.11 shows the results after 7 days of cell growth. Again, **Figure 6.11 (a)** shows very little cell growth on the rapamycin containing sample. The cell morphology was seen to be similar to those found for **Figure 6.10 (a)**, after day 3. Cells on the control film were much smaller and moved out more than day 3. The change in morphology and the movement to other areas were the normal response of SMCs to undesirable circumstances.

From the day of cell seeding to day 7, rapamycin highly inhibited the coronary artery smooth muscle cell attachment to PLGA films. Very few cells could attach to the polymer surface with the effect of rapamycin. For those cells that could attach to the film after a relatively longer incubation time, their morphology was changed compared to the cells on control films. Considering the suppression by rapamycin of smooth muscle cell proliferation and growth which were mentioned above, the cells would find it difficult to live normally with rapamycin released from substrate. The growth of cells was decreased, the proliferation was inhibited and the low cell density even could not allow cells to form a colony. Cells could attach to control films much easier than those with rapamycin, they could grow or even possibly proliferate on PLGA films. The morphology for cells on PLGA surface was almost the same as SMCs cultured in plastic flask, they spread and moved out as well as contacting with other cells for communication. However, the PLGA is not an appropriate substrate for SMCs although they could attach to it. Cells changed their morphology to smaller cellular size and moved to other regions. Since this experiment lasted only for 7 days, more time should be expected for the cell proliferation and confluence on control PLGA films, which is the basis of tissue engineering such as for artificial blood vessel.

(a)

(b)

Figure 6.11 Cell attachment to PLGA 5347 film at day7. (a): Cell attachment to film with 1 wt% rapamycin; (b): Cell attachment to control film without rapamycin

(a)

(b)

Figure 6.12 (a) hCASM on cell culture plastic by inverted microscope x 400 (Passage 4); (b) hCASM on control PLGA 5347 film by SEM (x 500) 5 days after cell seeding

Figure 6.12 indicated the difference of hcaSMC morphology between cells cultured on plastic flask and control PLGA 53/47 film. Cells on plastic flask were larger in size and few radial filopodia extended outside, while as a contrast, SMCs cultured on PLGA 53/47 film showed a smaller size, more spread out and many more filopodia extended radially. These mean the conditions for SMC growth on PLGA films were not as good as the surface of culture flask, although the PLGA surface was pre-coated with the deposit of proteins from culture medium before cell seeding. The treatment of PLGA surface is necessary for fast attachment and normal cell growth as well as proliferation.

6.6 Summary

Effects of control-released rapamycin to the inhibition of human coronary SMCs in proliferation, growth, recovery and attachment were investigated in this chapter. Sustained release of rapamycin to 17 days delayed the proliferation of SMCs, inhibited the cell growth and interrupted the signaling pathways. Cells showed different recovery profiles which were related to the inhibition time and the position of cell cycle when the rapamycin was removed from culture medium. If cell recovery occurred after S phase, the recovery profile was similar to cells not exposed to rapamycin, but the growth was much faster than cell recovery starting in G1/S phase. If cell recovery happened in G1/S, cells entered another cell cycle which needed more time for recovery. Rapamycin-eluting films inhibited the cell attachment. Even at day 7, still few cells could attach. The inhibition to cell attachment has special significance for polymer stent since it will be difficult for the cell deposition and the proliferation afterward. From cell metabolic assay, rapamycin exhibited a cytostatic effect, not cytotoxic, even for high rapamycin loading.

Chapter 7

In vivo Inhibition Effect on Neointimal Hyperplasia by Rapamycin

7.1 Introduction

Sirolimus-eluting coated metallic stents showed great success in inhibiting restenosis in humans^{6,113-115}. Long-term assessments of helical polylactide stents were conducted in human and in rabbits, which proved the reliability and feasibility of biodegradable stents^{9,116}. Paclitaxel-eluting double-helical PDLLA stents were also studied in a porcine model¹¹⁷. However, no animal trial with sirolimus-loaded biodegradable stents has so far been reported.

The effects of rapamycin on smooth muscle cell proliferation, growth, recovery and attachment (in vitro) were studied in Chapter 6. In this chapter, the *in vivo* effects of control released rapamycin from double-layered PLLA/PLGA stents are discussed in a rabbit femoral artery model. PLLA/PLGA 53/47 with 1% and 2% rapamycin respectively were chosen for *in vivo* study, because they gave different release rates in cell culture study and are expected to result in marked difference in inhibition of restenosis for animal study.

7.2 Stent expansion evaluation

Stent expansion plays a key role in stent performance. Successful stent expansion enables stent to fix inside the artery wall quickly and completely, to reduce the chance of thrombus formation and stent migration. In this study, the stent expanded through self-expansion at body temperature without the help of a balloon.

The initial stent outer diameter (OD) before self-expansion study is about 1-1.2 mm, which enables stent to be inserted into femoral artery via a 4F sheath easily. Stents were self-expanded to OD of 1.5 mm within 1 minute, to OD of 1.8 mm in the following 2 minutes, as shown in **Figure 7.1(a)**. The fast expansion fixes the stent into position quickly, and reduces the chance of stent migration in the femoral artery, thus avoiding thrombus formation and initial blockage by blood cells. From **Figure 7.1(b)**, the sterilization by ethanol slightly changed the expansion ratio because of the plasticization effect of ethanol on polymer, which made the stent more expandable.

Figure 7.2 shows the stent expansion after stenting in rabbit femoral artery. Expanded polymer stent maintained its shape in situ and supported the femoral artery at 40mm above the ligation site, as shown in **Figure 7.2(a)**. During stenting, the polymer stent was fully expanded to 4 rings with a final OD of 2mm and stent length of 9mm, as observed from **Figure 7.2 (b)** and **(c)**. The *in vivo* stent dimension is very similar to the dimension obtained in the *in vitro* test.

(a)

(b)

Figure 7.1 Stent self-expansion with and without sterilization

Figure 7.2 Stent dimension after insertion (a): Stent in rabbit femoral artery; (b): Stent taken out from artery. Outer diameter of fully expanded stent is around 2 mm, with marker at two ends; (c): length of fully expanded stent is about 9 mm

7.3 Histochemical and immunohistological studies

7.3.1 Morphology and inflammatory studies with hematoxylin & eosin staining

Results of Hematoxylin and eosin (H&E) staining of rabbit femoral arteries after stenting for 2 weeks were shown in **Figure 7.3**. The PLLA layer of polymer stent can be clearly observed in these pictures, which structurally maintained the shape and support the artery. However the PLGA layer is seen to be missing from **Figure 7.3** because of the *in vivo* degradation of this layer after two weeks. As discussed in Chapter 4, the PLGA 53/47 layer is seen to be completely degraded after 3 weeks in 7.4 buffer solution. The cell attachment and secretion of cellular components may significantly enhance polymer hydrolysis. Stenting destroyed the endothelium while structurally pressing on the arterial wall, which induced thrombosis and caused an inflammatory response to activate the proliferation of smooth muscle cells, thus leading to the neointimal hyperplasia. The neointima was significantly thickened in arteries with the control stents, but were seen to be less for the 1% rapamycin-loaded stents (**Figure 7.3 (a) and (c)**) and was greatly inhibited in those arteries inserted with 2% rapamycin-loaded stents (**Figure 7.3 (b)**, shown by arrow). Evidently, the higher released amount of rapamycin from 2% rapamycin-loaded stents during the first two weeks, which is 19.97 µg and 6.71 µg for 1% rapamycin-loaded stent in *in vitro* study (**Table 5.2**), greatly inhibited the cell proliferation in intima and cell attachment onto stent surface, confirming the results in Chapter 6. *Lincoff et al* reported that the inflammatory response to poly-L-lactic acid was associated with the polymer weight that low molecular weight polymer (80kDa) causes a significant inflammation while high molecular weight polymers (321kDa) do not induce a

significant reaction¹¹⁸. The PLLA layer in these bilayer stents was facing the lumen and in contact with blood, so it was the main target of inflammation responses. The molecular weight of PLLA used in this study was 1.28×10^3 kDa, which is much higher than 321 kDa, and is not expected to induce significant inflammation reaction according to *Lincoff's* theory. In this study, there was only mild inflammatory reaction after two weeks with leukocytes and multi-lobed neutrophils around control stents and 1% rapamycin-loaded stents, especially in layer between stent and lumen, as shown in **Figure 7.4**. The concurrent presence of neutrophils and leukocytes, as well as a small number of multi-nuclear macrophages in the control and 1% rapamycin-loaded stents, suggests it is in transition from acute to chronic inflammation at 2 weeks. The neovascularization with many new capillaries, arterioles and venules buried in fibrin are apparently observed in **Figure 7.5**, which was another proof of chronic inflammation. Almost no inflammatory reaction was observed in the 2% rapamycin stent. The reduction in inflammation was attributed to the inhibition effect of released rapamycin on the leukocytes, macrophages and chronic inflammatory cells. No thinning of the media layer was detected, suggesting no medial necrosis was induced by control and rapamycin-eluting stents, as was observed in paclitaxel-eluting coated stents¹¹⁹⁻¹²¹.

Figure 7.3 Morphology of femoral artery after stenting for 2 weeks with H&E staining. (a): artery with 1% rapamycin-loaded stent (Magnification $\times 50$), (b): artery with 2% rapamycin-loaded stent (Magnification $\times 50$), (c): artery with control stent (Magnification $\times 50$). Abbreviations: I: intima, L: lumen, M: media layer, S: stent, T: thrombus

Figure 7.4 Inflammatory reactions after 2 weeks (stained with H&E). (a): tissue with control stent. L: location of the lumen, F: fibrin, M: macrophage. Arrow shows the monocytes while the arrowhead points to the lymphocyte. The polymorphonuclear cells are marked in a circle. (b): tissue with 1% stent. L: location of the lumen, S: stent material. Arrow shows polymorphonuclear cells, while the arrowhead shows the lymphocyte. Monocytes are marked in a circle. (Magnification $\times 400$)

Figure 7.5 Angiogenesis in neointima. The arrows point to capillaries. Abbreviations: A: arteriole, V: venule, F: fibrin. (Magnification $\times 200$)

7.3.2 Fibrin deposition study with Masson trichrome and anti α -actin staining

The results of Masson trichrome staining after stenting for 2 weeks were shown in **Figure 7.6**. The panoramic view of the artery shows extensive neointimal hyperplasia in control stent, and the lumen size was significantly decreased (**Figure 7.6 (a)**). There were considerable numbers of RBCs in the media and adventitia (**Figure 7.6 (b)**). This phenomenon could suggest that hemorrhage might have occurred, with the infiltration of

RBCs in tunica media and adventitia of femoral artery caused by stretching of artery due to the stenting procedure, and this was also observed in paclitaxel-eluting stents¹¹⁹. **Figure 7.6 (d)** is a magnified picture, from which the biconcave disks' shape of RBCs could be clearly observed. A thick layer of fibrocellular hyperplasia can be seen in the intima close to the stent, identified as the pink color area labeled by double asterisks (**Figure 7.6 (c)**). The area near the lumen was heavily stained with blue color, suggesting this part was a collagen-rich area. Smooth muscle cells were in the matrix of fibrin and collagen, especially toward lumen, but many were in stellate instead of spindle shape as in the media layer, which was demonstrated by anti α -actin staining (**Figure 7.7 (a)** and **(b)**). Indications of angiogenesis could be seen in the intima and the neocapillaries were buried in fibrin and collagen fibers, as shown in **Figure 7.6 (c)**. The fibrin deposition and erythrocyte remnants are present near the 2% rapamycin-loaded stent (**Figure 7.6 (e)**), but to a lesser degree compared to the control stent.

Figure 7.6 Masson trichrome staining of femoral artery with stenting for 2 weeks. From (a) to (d): femoral artery with control stent (Magnification: $\times 50$, $\times 100$, $\times 200$ respectively), (e): femoral artery with 2% rapamycin-loaded stent (Magnification: $\times 200$). *Abbreviations:* A: adventitia, I: intima, M: media, S: stent. *Symbols:* * collagen, ** fibrin. Arrow in (b) shows the internal elastic lamina; arrow in (c) points to new arteriole in the intima; arrowhead in (e) shows the fibrin deposition around stent

Figure 7.7 Anti α -actin staining after stenting for 2 weeks (Magnification: $\times 400$). (a): the media layer; (b): the intima layer.
Abbreviation: A: Adventitia, M: media

7.3.3 Cell proliferation study with Anti Ki-67 staining

The expression of Ki-67 is strictly associated with cell proliferation. The Ki-67 antigen is expressed in G1, S, G2 and mitosis stages of cell cycle, but not in resting cells ^{118, 122}.

Figure 7.8 shows the anti Ki-67 staining results after stenting for 2 weeks. The nuclei of proliferating cells, most of which are smooth muscle cells, were stained in brown or black, whereas nuclei of resting cells were stained blue. Staining intensity was increasing from S phase to M phase ¹²². From **Figure 7.8**, the proliferating cells were mainly in the neointima layer and was seldom found in the media layer. The amount of proliferating cells showed a decreasing trend for arteries with control stents, 1% and 2% rapamycin-loaded stents. Rapamycin released from these polymer stents penetrated into the cytoplasm and inhibited mTOR by forming rapamycin-FKBP12. This stopped cell cycle at the G1 phase, therefore preventing the cell proliferation and entering into the intima. The faster and higher release of rapamycin from 2% rapamycin-loaded stents showed a more effective inhibition of cell proliferation than 1% loading.

Figure 7.8 Anti Ki-67 staining of femoral artery with stenting for 2 weeks. (a): artery with the control stent (Magnification $\times 200$), (b): artery with 1% rapamycin-loaded stent (Magnification $\times 400$), (c): artery with 2% rapamycin-loaded stent (Magnification $\times 400$). *Abbreviations:* I: intima, M: media, S: stent

7.4 Summary

The double-layered PLLA/PLGA stents were successfully inserted and anchored into the rabbits' femoral arteries. Hematoxylin and eosin staining, Masson trichrome, anti α -actin and anti Ki-67 stainings were performed in arteries stented for 2 weeks. The results obtained from the two-week cell study were compared to the *in vivo* study, which lasted for 2 weeks. The effects of rapamycin sustained release on inflammation and neointimal hyperplasia was discussed. There was evidence of a mild inflammation along with a significant neointimal thickening for the control stents. Stents with controlled release of rapamycin was seen to have inhibited any significant inflammatory response, reduced the fibrin deposition and also decreased the amount of proliferating cells, thus effectively reducing the restenosis caused by the stent. Based on *in vitro* results discussed in Chapter 6, the 2% rapamycin-loaded stents have higher release rate than 1% rapamycin-loaded stents. In this rabbit study, a higher rapamycin loading and a faster release rate appeared to result in more inhibition effects.

Chapter 8

Conclusions and Recommendations

8.1 Conclusions

As a proven anti-proliferation drug that could be used in a fully polymeric coronary artery stent, rapamycin was chosen in this study to examine the drug release kinetics, inhibition of proliferation, growth, recovery and attachment of hcaSMCs. Rapamycin, anti-proliferation drug, has been shown to be released at different rates from a fully polymeric coronary artery stent.

Rapamycin release from amorphous polymer PLGA 53/47 and PLGA 50/50 showed both diffusion- and degradation-controlled kinetics. Because it is a hydrophobic drug, there was no burst effect observed during the initial release stage. The diffusion stage was short, followed by a degradation-controlled phase. The chemical structure of PLGA 50/50 retarded the release of rapamycin by inhibiting water penetration. Different polymer modifications were used and their respective release profiles were studied. Polyethylene glycol 4000 was added as a plasticizer at 5% and 10% by weight. It has been shown that the addition of PEG accelerated the degradation of PLGA 53/47 thus increasing the release of rapamycin. A linear release profile was obtained for PEG plasticized PLGA 53/47. PLGA 53/47 coated with PLGA 50/50 exhibited a release profile between that of films with only PLGA 53/47 and films with only PLGA 50/50. Most of the drug was released from the PLGA 50/50 side, which needed a period for the diffusion of rapamycin

into PLGA 50/50, therefore delayed the release from PLGA 53/47 layer. This ‘sandwich’ structure can be used to shape a profile between two polymers.

Rapamycin released in cell culture conditions was significantly faster than in the buffer system. The FKBP-12 in proliferating SMCs binds a considerable amount of rapamycin. Lipoproteins and albumins in serum also play an important role in enhancing the solubility of rapamycin. All these factors resulted in a faster release of rapamycin in cell culture conditions. Results of this study are useful in the prediction of release profiles of drugs in cell culture.

Rapamycin not only inhibited the DNA synthesis in hcaSMCs, but also inhibited the cell growth. Cells inhibited by rapamycin might become resting cells, and the growth was very slow. But interestingly, even under the high concentration of rapamycin, some DNA synthesis and mitosis continued. The cells continued to proliferate although under some inhibition. Theoretically all cells should be ‘static’ if the mTOR pathways were entirely ‘cut off’ by excessive rapamycin in medium. It was probably that cells still maintained a set of mechanisms even when these pathways were inhibited and thus continued to proliferate. Actually this was also observed in some cancer cells possessing the ability of resistance to rapamycin¹⁰¹.

Recovery from rapamycin inhibition depends on the position of cell cycle that cells were in when rapamycin was removed. Cells in G2/M phase recovered faster than cells in G1/S phase, as the latter entered a new cell cycle and needed a longer time to be recovered. Longer incubation with rapamycin resulted in a longer inhibition effect, the recovery also

lasted longer. Results also showed that, the effect of rapamycin to cells were cytostatic rather than cytotoxic, even with a high concentration of rapamycin.

Although several formulations were studied, with varying degrees of control over release of rapamycin, no effect of the details of controlled release was seen in the inhibition results. This may be primarily due to the fact that under the conditions of the study, the amount of rapamycin released per day was well above the minimum inhibitory concentration, hence small deviations in this amount were not reflected in the inhibition results. However, the data clearly point the need to sustain the release of rapamycin over at least one or two cell cycles to prevent proliferation.

Rapamycin inhibited cell attachment to polymer film. Only few cells could attach to PLGA 53/47 with effect of rapamycin. Even at day 7, attached cell number was still low. The inhibition of SMC attachment is related to anti-proliferation. The elimination of SMC attachment enables unhindered rapamycin release and avoids the hyperplasia on stents. However, whether the normal attachment of endothelial cells occurs under rapamycin inhibition is still to be studied.

Controlled release of rapamycin successfully inhibited neointimal hyperplasia in rabbit study up to 2 weeks. Inflammatory reaction, fibrosis and cell proliferation were significantly inhibited by rapamycin compared to control polymer stent.

A correlation exists between in buffer, in cell culture and in vivo studies. The drug release profiles were significantly changed in cell culture conditions compared to in buffer system. In cell culture, samples with 2% rapamycin loading showed a faster release rate

than 1% loading (47.9% vs. 37.05% after 2 weeks). Although lacking the drug release data in animal study, based on the correlation of drug release in buffer and in cell culture, the drug release rate in animal study is expected to be even faster than in cell culture and the drug release rate of 2% rapamycin-loaded stents is expected higher than 1% drug-loaded stents. Subsequently, the stent with 2% rapamycin greatly reduced in-stent stenosis compared to 1% rapamycin-loaded stent. In this study, a faster rapamycin release rate leads to a greater inhibition to stenosis. In cell culture study, control-released rapamycin effectively inhibited the SMC proliferation, growth and attachment to polymer films. These effects were further proved by the inhibition on stenosis and the lack of SMC overgrowth in vivo. However, in cell culture, the low cell seeding density leads to insignificant dose-dependent effects compared to results in vivo. Therefore, the correlation among in buffer, in cell culture and in vivo drug release as well as cells/tissue responses was partially established and will guide the future design of drug-eluting cardiovascular stents.

8.2 Recommendations

The inhibition effect of control-released rapamycin on SMCs' proliferation and growth was studied. However, the influence of rapamycin to the function of cardiovascular cells still remains to be extensively investigated for better understanding and to optimize the design of coronary artery polymer stent. Following studies are recommended:

1. Study of controlled release of rapamycin on SMC migration

The study to date reported mostly the effects of rapamycin release on SMC proliferation in a static sense. However, migration of SMCs must also be inhibited to prevent overall proliferation, as the migration of SMC from media into intima and secretion of growth factors stimulates SMC to change from contractile into synthetic phenotype and results in the subsequent proliferation. The inhibition of SMC migration will greatly reduce the proliferation and thus prevent the neointimal formation. Rapamycin was demonstrated to be capable of inhibition of rat and human vascular cell migration. The study of a local controlled delivery of rapamycin will enable us to learn more of the inhibition of hcaSMC migration and will be useful to the adjustment of rapamycin loading and release profiles for biodegradable stent. In the migration study, a modified Boyden Chamber with a pore size 5 or 8 μm filter may be used. The platelet-derived growth factor (PDGF) is recommended for use.

2. Study the inhibition of proliferation and attachment of endothelial cells

The endothelial proliferation plays a key role in angiogenesis, which is important in wound healing as it will close the contact between injured artery and blood elements such as platelets and white blood cells. However, rapamycin is able to inhibit the proliferation of endothelial cells too¹¹¹. A study of control-released rapamycin on the inhibition of endothelial cells will be necessary to examine if and how this restriction is dose-dependent. Thus the rapamycin release profile can be optimized and applied to biodegradable stent.

The study of the attachment of endothelial cells to rapamycin-eluting polymer films is also recommended to know its biocompatibility. Fast and thorough attachment of endothelial cells to polymer film will greatly reduce neointimal thrombosis.

3. Effects of long-term release of rapamycin, the inhibition and cytotoxicity

Although the inhibition effect of rapamycin on hcaSMC was studied, the experiment was designed only in a 96-well plate, which was a very small scale and the inhibition time is only 2 weeks. Such a short time was enough for 96-well plate study, but will be not suitable for a longer inhibition, especially considering the SMC proliferation was controlled at a relatively low speed and without extra growth factors such as PDGF in this study. So a further study on inhibition is recommended, in which PDGF is suggested to be used. A wider range of release rates are also to be studied.

However, with the inhibition by rapamycin for a long time, the cytotoxicity or hypersensitivity issue must be taken account¹¹². Local high concentration of rapamycin may possibly bring cell apoptosis or change cells to some degree, so a monitoring of cell cytotoxicity is suggested.

4. Study the effects of long-term controlled release of rapamycin in vivo

The long-term study of sustained rapamycin release in rabbit will give more information on inhibition of restenosis by rapamycin-eluting stent. Chronic inflammatory responses to rapamycin-loaded and unloaded stents, endothelialization and wound healing will be

investigated. The pharmacokinetics of release in vivo will also be available by studying the release rate and distribution of rapamycin in the rabbit model.

Stent samples with markedly different release rates of rapamycin, e.g., stents incorporated with 5% (10%) PEG, will be applied in animal study. The drug release kinetics in vivo and stenosis degree will be compared to the results obtained in this study. Thus the correlation of drug release kinetics between in vitro and in vivo will be thoroughly established.

5. Further study of in vitro- in vivo correlations

A higher cell seeding density should be applied to observe the effect of different rapamycin release rates on cell growth and proliferation. The amount of rapamycin released in vivo will be monitored and the release rate will be calculated to compare with in vitro data. Stent samples with markedly different release profiles in vitro, e.g., stents incorporated with 5% (10%) PEG, will be applied in animal study. And a higher rapamycin loading (e.g., 5% rapamycin) will also be used. The drug release kinetics in vivo, inflammatory reaction and stenosis degree will be compared to the results with 1% and 2% rapamycin-loaded stents in this study. Thus the correlation of drug release kinetics and their effects between in vitro and in vivo will be thoroughly established.

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