Drug delivery in the anterior chamber of the eye for islet transplantation as a diabetic treatment

Fan, Yanliang

2018

Fan, Y. (2018). Drug delivery in the anterior chamber of the eye for islet transplantation as a diabetic treatment. Doctoral thesis, Nanyang Technological University, Singapore.

<http://hdl.handle.net/10356/75793>

<https://doi.org/10.32657/10356/75793>

NANYANG
TECHNOLOGICAL
UNIVERSITY

**DRUG DELIVERY IN THE
ANTERIOR CHAMBER OF THE EYE
FOR ISLET TRANSPLANTATION AS
A DIABETIC TREATMENT**

FAN YANLIANG

Interdisciplinary Graduate School
NTU Institute for Health Technologies

2018

**DRUG DELIVERY IN THE ANTERIOR CHAMBER OF
THE EYE FOR ISLET TRANSPLANTATION AS A
DIABETIC TREATMENT**

FAN YANLIANG

**INTERDISCIPLINARY GRADUATE SCHOOL
NTU INSTITUTE FOR HEALTH TECHNOLOGIES**

A thesis submitted to the Nanyang Technological University
in partial fulfillment of the requirement for the degree of
Doctor of Philosophy

2018

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research and has not been submitted for a higher degree to any other University or Institution.

02 June 2018

.....
Date

.....
Fan Yanliang

Acknowledgements

The successful completion of my thesis was made possible because of the invaluable contribution of a number of people. Just saying “thank you” is not enough to express my gratitude. They are all angels sent to me by Our Heavenly Father.

First, I would like to express my deep appreciation to all my supervisors, Associate Professor Joachim Loo, Professor Per-Olof Berggren, Assistant Professor Yusuf Ali and Associate Professor Sierin Lim for their intellectual support, guidance and constant encouragement throughout the course of doctorate. The professional knowledge, technical skills and research experience learnt from them have been one of the greatest treasures to me, which will surely benefit me in my future research work and life. Without their inspiration, guidance and advice, I would not be able to accomplish this PhD project within this limited time frame.

Special thanks are extended to Dr. Lee Wei Li, Dr Lui Yuan Siang Dr Baek Jongsuep, and Dr Tan Chuan Hao (Grant) for their valuable suggestions, inspiring discussion and kind support throughout my graduate studies. They have, imparted to me with valuable research knowledge and skills, as well as necessary information for me to conduct and execute research experiments smoothly. I also want to thank Dr. Rafael Arrojo e Drigo for the challenges he imposed upon the project, which grew my endurance and built my character. I am also grateful to Dr. Hou Han Wei, Tay Hui Ming and Dr. Mustafa Hussain Kathawala, for their collaboration in performing all the complex cell work and help in my first publication. I would like to extend my appreciation to all members from Berggren’s group, especially to Dr Zheng Xiao Feng, for their kind support in animal work, knowledge sharing and in-depth discussion. Furthermore, I want to extend my thanks to fellow colleagues from Loo’s group,

FACTs Lab and Biomaterial Lab for their selfless assistances in instrument operation, knowledge sharing and support over these years. I would like to thank the students (Jessica Kim Tendi, Lam Ngo Cheung (Gabriel), Wee Zhi Qi (Allicia), Dionaldo Zudhistira) for their assistance provided in this research work. I would like to thank my friends, especially Lim Ming Pin (Alan), Ng Feng Lin, Chen Hui Zhi, Tan Wei Ni (Dulcia), Chan Jingni, and Pua Jie Liang for their supportive spirit in this journey full of ups and downs.

Last but not least, I would also like to express my utmost gratitude to my lifetime friend Aravind Muthiah, and my parents. Their continuous support and motivation gave me the courage and a determined mind to achieve this milestone in my life.

Above all, I thank my Lord Jesus Christ, for giving me the intellect to understand the complexity of material science, and giving me strength to sustain me through the hardship and complete this doctorate. Most specially, thank you for sending your angels when I needed them the most.

Publications

1. Fan, Y. L., Hou, H. W., Tay, H. M., Guo, W. M., Berggren, P. O., and Loo, S. C. J.*, Preservation of Anticancer and Immunosuppressive Properties of Rapamycin Achieved Through Controlled Releasing Particles. AAPS PharmSciTech, 2017: p. 1-10

2. Fan, Y. L.[†], Tan, C. H.[†], Lui, Y. S., Zudhistira, D., and Loo, S. C. J.* Mechanistic Formation of Drug-encapsulated Janus Particles through Emulsion Solvent Evaporation. RSC Adv, 2018 (Manuscript accepted)

[†] Equal contribution

3. Fan, Y. L.[†], Zheng, X. F.[†], Ali, Y., Berggren, P. O., and Loo, S. C. J.* Local Release of Rapamycin by Microparticles Delays Islet Rejection within the Rodent Anterior Chamber of the Eye, (Manuscript submitted)

[†] Equal contribution

Conference Presentations

1. Fan, Y. L., Hou, H. W., Tay, H. M., and Loo, S. C. J.*, Biodegradable biopolymer based microparticle formulations effect on hydrophobic drug release and preservation *in vitro*, in *Society of Plastics Engineers (SPE) Asean Annual Seminar 2016*: NTU, Singapore. 06 May 2016 (**Poster**)

2. Fan, Y. L., Hou, H. W., Tay, H. M., and Loo, S. C. J.*, Novel microparticle delivery system for preservation and long-term release of rapamycin, in *The 11th International Particle Toxicology Conference*: NTU, Singapore. 26-30 Sep 2016 (**Poster**)

3. Fan, Y. L., Hou, H. W., Tay, H. M., and Loo, S. C. J.*, Long-Acting Sirolimus Microparticle Formulation and Its Antiproliferative Effect in Jurkat Cell, in *American Association of Pharmaceutical Scientists (AAPS) Annual Meeting and Exposition 2016*: Denvor, USA. 13-17 Nov 2016 (**Poster**)

4. Fan, Y. L., Lui, Y. S., Zudhistira, D., and Loo, S. C. J.*, One-Step Synthesis of Drug-Loaded Biodegradable Janus Particle for Control Release, in *Fall Meeting of the Materials Research Society 2016*: Boston, USA. 27 Nov-02 Dec 2016 (**Poster**)

5. Fan, Y. L., Lui, Y. S., and Loo, S. C. J.*, Mapping the Drug Distribution Preference in Heterogenic Microparticles Using Confocal Raman, in *The International Symposium on Polymer Analysis and Characterization (ISPAC) 2017*: Linz, Austria. 11-14 Jun 2017 (**Oral Presentation, NTU CoE ISPAC Travel Award Winner**)

Table of Contents

Acknowledgements	i
Publications	iii
Table of Contents	v
List of Abbreviations	xv
Abstract.....	1
1 Chapter 1 Introduction	3
1.1 Background and Significance	3
1.2 Motivation.....	4
1.3 Problem Statement and Aim	6
1.4 Objectives	8
1.5 Organization of the Dissertation	11
2 Chapter 2 Literature Review	17
2.1 Diabetes and its Unmet Needs	17
2.1.1 Diabetes Definition and Classification	18
2.1.2 Pancreas Anatomy	19
2.1.3 Pancreatic β -cell and Mechanism of Insulin Release	20
2.1.4 Brief Diabetes Pathophysiology and Pathogenesis.....	21
2.1.5 Diabetes Treatment	23
2.1.6 The Unmet Need of Diabetes.....	25
2.2 Islet Transplantation – Old Idea with New Approach	26
2.2.1 Emerging Treatment of Diabetes	26
2.2.2 The Approach of Islet Transplantation	27
2.2.3 Limitations of Islet Transplantation.....	28
2.3 Novel Site for Islet Transplantation – Anterior Chamber of the Eye (ACE).....	29
2.3.1 ACE as New Emerging Site of Islet Treatment	29
2.3.2 ACE Internal Environment	31
2.3.3 Islets Transplanted into ACE are Subject to Immune Attack..	35

2.4	Immunosuppression Strategies Associated with Islet Transplantation	36
2.4.1	Common Immunosuppressive Therapy and Its drawbacks	36
2.4.1.1	Rapamycin – A Distinctive Immunosuppressive Drug.....	37
2.4.2	Right Delivery System Aid the Therapeutic Effects	39
2.4.3	Characteristics of Each Drug Delivery Systems.....	39
2.5	Current Drug Delivery Systems for Islet Transplantation	40
2.5.1	Microparticle System for Allotransplant Rejection	40
2.5.2	Bioengineered System for Islet Transplantation.....	42
2.5.3	Current and Future Research Direction for Islet Transplantation	
	43	
3	Research Methodology	57
3.1	Engineer a Controlled Releasing Microparticle Formulation for Long-term Release of Immunosuppressive Agent Rapamycin	57
3.1.1	Materials	57
3.1.2	Microparticle Fabrication	58
3.1.3	Particle Characterization.....	59
3.1.3.1	Particle Morphology and Average Size (Scanning Electron Microscopy).....	59
3.1.3.2	Drug Encapsulation Efficacy	59
3.1.4	In vitro Drug Release Study.....	60
3.1.4.1	Rapamycin Degradation Study	60
3.1.4.2	Rapamycin Liquid Chromatography–Mass Spectrometry	60
3.1.4.3	Long Term Release Study In vitro	61
3.2	In vitro Assessment of Rapamycin Microparticle Formulations	61
3.2.1	Materials	61
3.2.2	Effect of Rapamycin Microparticles on MCF-7 Cell Proliferation	62
3.2.3	Long Term Effect of Rapamycin Microparticles on Jurkat Cell Proliferation	63
3.2.4	Ex vivo Assessment of Rapamycin Microparticle Impact on Rodent Islets	64
3.2.4.1	Islets Isolation from Rodent	64

3.2.4.2	Islet Glucose Stimulated Insulin Secretion (GSIS) Assay	65
3.3	In vivo Transplantation of Rapamycin Microparticle and Islets	65
3.3.1	Material	65
3.3.2	Particle Preparation Prior to Surgery	66
3.3.3	Transplantation of Pancreatic Islets and Microparticles to the ACE	66
3.3.4	In vivo Imaging of Islets Transplanted to the ACE	67
3.3.4.1	Islet Rejection and Survival Quantification	67
3.4	Drug-loaded Janus Particle Formulation Development	68
3.4.1	Materials	68
3.4.2	Drug-loaded Janus Particle Fabrication	68
3.4.3	Interfacial Tension Measurement	69
3.4.4	Particle Characterization	69
3.4.4.1	Confocal Raman Microscope	70
3.4.4.2	Laser Scanning Confocal Microscope	70
3.4.5	In vitro Drug Release Study	71
4	Development of Controlled Releasing Microparticle Formulation for Preserving the Anti-cancer and Immunosuppressive Properties of Rapamycin¹	73
4.1	Introduction	73
4.2	Results	75
4.2.1	Rapamycin Released from Microparticle Formulations	75
4.2.2	MP Formulations Preserve Bioactivity of Rapamycin	78
4.2.3	Inhibition Efficacy of Released Rapamycin on Cell Lines	82
4.3	Discussion	84
4.4	Summary	88
5	Local Release of Rapamycin by Microparticles Delays Islet Rejection within the Rodent Anterior Chamber of the Eye – An In Vitro and In Vivo Study	93
5.1	Introduction	93
5.2	Results	95
5.2.1	Effective Dosing of Rapamycin Released from Biodegradable Microparticles in vitro	96

5.2.2	Rapamycin Microparticles Delay Islet Graft Immune Rejection in vivo	99
5.3	Discussion	103
5.4	Summary	106
6	One-step Synthesis of Novel Drug Carrier, Janus Particle, Encapsulating Diabetes Drug for Control Release²	111
6.1	Introduction	111
6.2	Results	113
6.2.1	Glibenclamide Drives Janus Particle Formation at a Specific Polymer Weight Ratio	113
6.2.2	Negative Charge of Glibenclamide Determines JP Formation at 20:10 Polymer Weight Ratio	117
6.2.3	Glibenclamide Alters the Interfacial Tensions in the Emulsion System	120
6.2.4	Modulation of Interfacial Tensions by Polymer Weight Ratio Allows the Formation of Lidocaine- and Rapamycin-loaded Janus Particles	124
6.2.5	Janus Particles Demonstrate Greater Control over Burst Release of Glibenclamide Compared to the Core-shell Particles	126
6.3	Discussion	127
6.3.1	Janus Particle Hemisphere Forms When Polymer Phase Shows Equivalent Interfacial Tension	128
6.3.2	Interfacial Tension is Susceptible to Change Induced by Drug	129
6.3.3	Janus Particle Inhibits Burst Release Better Than Core-Shell Microparticle	133
6.4	Summary	135
7	General Conclusion and Future Work	141
7.1	Perspective	141
7.2	Summary of Results	141
7.2.1	Rapamycin Microparticle Formulation Development	142
7.2.2	Co-transplant Rapamycin Microparticle with Islets to ACE in Rodent Model	142

7.2.3	One-step Synthesis of Drug-loaded Janus Particle, a Novel Microparticle System for ACE	143
7.3	Novel Contribution	144
7.4	Recommendations for Future Directions	145

List of Tables

Table 2.1 Estimated prevalence and number of people with diabetes (adults 18+ Years).....	17
Table 2.2 Asia diabetes market size distribution in 2009	18
Table 2.3 Information of ACE in human vs mouse	34
Table 3.1 Weight of PLGA and PCL for each mass ratio used for fabrication	69
Table 4.1 Microparticle formulations were listed with its diameter, drug loading and encapsulation efficiency	76
Table 5.1 Mass of rapamycin calculated from mice ACE volume & target concentration of 20 nM.....	97
Table 6.1 Interfacial tensions and spreading coefficients at different PLGA/PCL weight ratios	123
Table 6.2 Interfacial tensions and spreading coefficients at PLGA/PCL weight ratio 19:11	123
Table 6.3 Calculation of interfacial tension between PLGA and PCL in DCM system	124
Table 6.4 Alteration of interfacial tensions by drugs at different polymer weights.....	133

List of Figures

Figure 2.1	Schematic diagram of pancreas and Islet of Langerhans. .	19
Figure 2.2	Insulin release of β -cell after glucose uptake.	20
Figure 2.3	The complex pathogenesis of β -cell destruction in T1D...	22
Figure 2.4	Schematic illustration of the <i>in vivo</i> imaging platform to study pancreatic islet/ β -cell physiology and pathology...	30
Figure 2.5	Illustration of islet transplantation into the anterior chamber of the eye	31
Figure 2.6	The location and structure of anterior chamber of eye. The flow of aqueous humor was illustrated by blue arrow.....	33
Figure 2.7	Schematic illustration of the trabecular meshwork conventional outflow pathway.....	33
Figure 2.8	Concentration of various solutes in aqueous humor of rabbit as compared with the values of dialysate of plasma.....	34
Figure 4.1	SEM images of microparticle cross section and surface morphology.	77
Figure 4.2	Cumulative release of rapamycin from different microparticle.	78
Figure 4.3	Degradation of rapamycin in aqueous condition and MP.....	79
Figure 4.4	Chromatography and mass spectrometry profiles of Rapamycin.	80
Figure 4.5	Degradation rate of rapamycin in aqueous condition.....	81
Figure 4.6	Inhibition efficacy of rapamycin microparticle on breast cancer cell (MCF-7) proliferation over 30 days.	83
Figure 4.7	Proliferation of Jurkat cell with microparticle formulation PLGA (■), PLGA-Acid (◆), free drug rapamycin (●), blank microparticle (●) and Healthy (●, dash line) as the control.	84
Figure 5.1	Rapamycin microparticles and its release <i>in vitro</i>	97
Figure 5.2:	Effect of rapamycin microparticles and blank microparticles in glucose-stimulated insulin secretion (GSIS) <i>in vitro</i> of islets..	99

Figure 5.3	Effect of blank and rapamycin microparticles on transplanted islet grafts <i>in vivo</i> .	101
Figure 5.4	Effect of microparticle number on islets rejection.	102
Figure 6.1	Microparticles fabricated at different PLGA/PCL weight ratios in the presence or absence of glibenclamide.	115
Figure 6.2	Distribution of glibenclamide in a Janus particle.	116
Figure 6.3	Size distribution of Janus particle.	116
Figure 6.4	Microparticles fabricated with different concentrations of glibenclamide.	118
Figure 6.5	Microparticles fabricated at the PLGA/PCL weight ratio 20:10 in the presence of drugs with different charges (A) or charge densities (B).	119

List of Abbreviations

ACE	Anterior chamber of the eye
ACN	Acetonitrile
AqH	Aqueous Humor
BSA	Bovine Serum Albumin
CLSM	Confocal Laser Scanning Microscopy
DCM	Dichloromethane
DDS	Drug delivery system
DI	Deionised
EDX	Energy dispersive X-ray Spectroscopy
EE	Encapsulation Efficiency
FDA	Food and Drug Administration
FT-IR	Fourier Transform Infra-red
GA	Glycolic acid
hr	Hour, hours
HPLC	High performance liquid chromatography
JP	Janus Particle
IV	Intrinsic viscosity
LA	Lactic acid
Log P	Partition Coefficient
O/W	Oil in water (emulsion)
PBS	Phosphate buffer saline
DPBS	Dulbecco's Phosphate Buffer Saline
PCL	Polycaprolactone
PLGA	Poly (lactide-co-glycolide)
PLLA	Poly (L-lactide)
PVA	Poly-vinyl alcohol
MeOH	Methanol
min	Minut, minuts
MP, MPs	Micropartiles
MW	Molecular weight
SEM	Scanning Electron Microscopy
UV/Vis	Ultraviolet/Visible
µm	Micrometer
w/v	Weight by volume
w/w	Weight by weight

Abstract

Transplantation of pancreatic islets restores β -cell mass, reduces exogenous insulin dependence and provides tight glycemic control for diabetic patients. However, the challenge of maintaining the functionality of grafted islets and preventing their mass loss in the long-term is yet to be addressed. A novel site Anterior Chamber of the Eye (ACE) is employed as potential clinical site of transplantation because islets transplanted easily engraft and re-vascularize well on the vessel-rich iris. In addition, it acts as a natural body window for non-invasive longitudinal studies of islets condition during the post-transplantation period. Grafted allogeneic islets, however, are subject to immune rejections. In mouse models, T-cells attack allograft islets and their mass gradually decreases to zero over 21 days post transplantation. To address the immune rejection locally, a microparticle drug delivery system that ensures sustained release of the immunosuppressive drug rapamycin inside ACE was developed. Rapamycin was encapsulated by microparticle formulations developed using biodegradable polymers of poly(lactic-co-glycolic acid) (PLGA), poly-L-lactide (PLLA) and polycaprolactone (PCL). These microparticles protected the encapsulated rapamycin from degradation. The investigation of *in vitro* release showed a mixture of two selected formulations at 1:1 ratio released rapamycin higher than the target rate for 30 days. The subsequent co-transplantation of rapamycin microparticles and islets to ACE was successfully performed on mice. These transplanted microparticles effectively delayed the rejection onset of islets grafts and prolong the grafted islets' survival period significantly in comparison to controls. This project is the first few to transplant drug carriers into ACE and monitor islet interaction with microparticles *in vivo*. Building on the success

of this model, a novel microparticle, Janus particle (JP), was proposed for encapsulating dual drugs, rapamycin and glibenclamide, to preserve islets grafts and stimulate insulin secretion, simultaneously. The challenge of drug-loaded JP fabrication was overcome after series investigations. The formation of drug-loaded JP can be defined thermodynamically that is strongly governed by the emulsion interfacial tensions in accordance with the classic equilibrium spreading coefficient. A mechanistic insight into the fabrication of drug-loaded JP was provided and laid the necessary foundation for dual drug loaded JP fabrication. In summary, the novel microparticles and novel approach of islet transplantation developed in this thesis would pave a path for an improved clinical treatment method for diabetes.

1 Chapter 1 Introduction

1.1 Background and Significance

Diabetes mellitus is one of the top ten causes of death, killing 1.6 million people worldwide in 2015 [1]. Its typical symptom, hyperglycemia or high blood glucose level, can be caused by defects of insulin secretion and/or insulin action [2]. When autoimmune destruction of insulin producing β -cells in the pancreas causes the secreted insulin fall below the sufficient level, the condition is termed type 1 diabetes (T1D). Alternately, some pathogenic process lead to a progressive condition of insulin resistance and/or inadequate insulin secretion, a condition termed as type 2 diabetes (T2D). The underlying cause of deficient or inadequate insulin secretion is dysfunction of the islet of Langerhans, the endocrine cell clusters embedded in the pancreas [3]. Among the endocrine cells, β cells are responsible for generating insulin and secret insulin in response to blood glucose change [3, 4].

The fluctuation in glucose levels can be rapid and unpredictable. Therefore, the long-term complications of diabetes can be potentially fatal, ranging from blood vessel damage, nerve damage, kidney failure to cardiovascular disease [5]. To minimize such complications, exogenous insulin injections and medicines promoting insulin release are commonly prescribed to manage the blood glucose level within the normal range. However, such approaches have challenges of imprecise dosage, acute hypoglycemia risk, depletion of the remaining β cells and high fluctuation of the blood glucose level. In addition, they burden patients with life-long medication and complex self-care routines. The development of diabetes management has been extensive, but is yet to achieve full control to prevent the complications of the disease from progressing.

The most natural way to manage diabetes is through boosting endogenous insulin production. Islet is a smart organ as it first senses blood sugar levels and secretes an appropriate amounts of insulin. Such a mechanism makes it intrinsically better than exogenous insulin administration in providing a tight control of blood glucose level [6], therefore more suited for avoiding the long-term complications effect of hyperglycemia [7, 8].

A current long-term effective treatment option for T1D is to infuse (transplant) healthy pancreatic islets into the hepatic portal vein; a procedure known as the Edmonton protocol. A transfer of 500,000 to 1,000,000 islets into a severely diabetic person resolves hyperglycemia, ‘rescinds’ insulin dependence and reduces progression of diabetic complications [9]. A major downside to islet transplantation is the need for lifelong immunosuppressive drugs to prevent host-mediated destruction of the infused islets. Endocrinologists are faced with a delicate decision of determining patient suitability and the patient’s ability to sustain lifelong immunosuppressive drugs is a key factor in this decision. In most instances, the upside of being independent from insulin still outweighs the undesirable side-effects of immunosuppressive agents. Till now, there have been many such transplants around the world with millions of diabetics still on the wait-list registry for islet-allotransplantation [10].

1.2 Motivation

Built upon the success of the Edmond protocol, most clinical islet transplantations are performed on the liver site through hepatic portal vein [11]. Islet transplantation is now routinely carried out in many diabetic centers around the world [12]. The benefits of islet transplantations includes improvement in blood glucose control, partial to complete reduction of insulin injections needed to control diabetes, and prevention of hypoglycemia. However, follow-up studies have reported the recurrence of insulin dependence possibly due to the

impairment of the function of the transplanted islets [13] and gradual mass loss of the grafted islets in the post-transplantation period. Many factors contribute to the loss of functional grafted islets including damage during isolation and transplantation procedure, nonspecific inflammation, recurrence of autoimmunity and toxicity of immunosuppressive agent used [14]. Real-time assessment of the graft islet condition is not readily available but is required to investigate the reason for failure. Although the liver has been the traditional site for infusing the donor islets, the site-specific properties of liver markedly impair the metabolic function of intraportally transplanted islets [13]. In this regard researchers are investigating alternative sites.

The Anterior Chamber of Eye (ACE) as a novel site for islet transplantation to study islet cell physiology and pathology *in vivo* has been developed and explored by Berggren and coworkers [15-18]. The transparency of the cornea makes the ACE a natural body window allowing easy access for non-invasive longitudinal studies at single-cell resolution [15]. In comparison to other invasive techniques of either creating a body-window [19] or by using an imaging agent for magnetic resonance imaging (MRI) [20], imaging of grafts in the ACE is a non-invasive and readily repeatable method. Furthermore, the iris has a high concentration of blood vessels that enables fast vascularization of the grafted islets. The multiple advantages of ACE being a transplantation and imaging site have been shown in islet allografts in diabetic mice and baboons [21]. Islets that are transplanted into the ACE have been reported to be well-vascularized, functional and capable of contributing to glucose homeostasis. With a sufficient number of islets allografts in diabetic baboons, insulin-dependence was shown to be significantly reduced over time [21].

With such promising results, it is imperative to address the key prerequisite of islet survival in ACE: the long-term immunosuppression in post transplantation period. In mice, immune response toward allogenic islets transplanted to ACE occurred after 7 days of post-transplantation.

Over the subsequent 7-14 days, T-cells infiltrate around and within islet grafts and eventually islet grafts are fully rejected by post-transplantation day 21 [15]. It is very clear that a post-transplant treatment to suppress immune rejection in ACE is required to preserve these insulin-secreting islets grafts. The conventional method here is to systemically administer patients with immunosuppressive agents at full-body dose, which is probably much higher than the dose needed at transplantation site. Hence, multiple drawbacks such as off-target side effects, poor patient tolerance and cytotoxicity severely limit the adaption of islet transplantation as the first choice of T1D therapy.

Another area for improvement is the efficacy of transplanted islets in maintaining blood glucose homeostasis. The amount of islets required to ensure sufficient insulin-producing grafted islets is high due to significant islet dysfunction or even loss [22]. The scarcity of islet donor would require a better solution. One solution is to improve the efficacy of islet transplantation instead of simply scaling up number of islets injected. In a transplantation site like the ACE, which has limited volume, the space needs to be used efficiently. Instead of overfilling it with a large number of islets, improvement of insulin-secretion ability per islet would be a better approach to increase the independence for exogenous insulin injection. It is, therefore, of interest to engineer clinically practical solutions to address these limitations for treatment of diabetes.

1.3 Problem Statement and Aim

With the advancement in materials engineering, a biodegradable microparticle particulate drug delivery system can be fabricated to encapsulate hydrophobic and/or hydrophilic drugs, and designed to elicit controlled release capabilities [23-25]. In comparison to conventional drug delivery, controlled release is better in terms of customizable duration, tunable dosage, improved efficacy and reduced drug dose fluctuation [26, 27]. Applying engineered particulate drug delivery systems in the ACE islet transplantation model has not yet been

tested but it could potentially solve the issues of long-term immunosuppression and low functional efficacy. Thus, the engineered microparticle based drug delivery system is tailorable, easy-to-inject and biodegradable, and easy to adapt into a clinical setting.

The aim of this thesis is to first develop functional novel microparticles which encapsulate and release immunosuppressive agent and/or insulin-releasing agent to protect, preserve, and promote islet function during post-transplantation in ACE. It is also our aim to implant the developed microparticles and to evaluate their effect on immune rejection to islets transplanted into the ACE *in vivo*.

1.4 Objectives

We hypothesize that a novel microparticle system delivering functional drugs such as immunosuppressive agents and insulin secretion stimulating agents can be implanted together with transplanted islets in the ACE to overcome the drawbacks of immune rejection and islet dysfunction. The overall targeted outcome is that grafted islets will receive prolonged immunosuppression with a minimized dosage to enhance their survival and insulin secreting function while minimizing side effects. Implanting such drug delivery microparticle into ACE allows the delivery of immunosuppressing agents to be local, bypassing the negative systemic effects of immunosuppression. In comparison to systemic delivery, it is anticipated that the local delivery will require a lower dosage to achieve the same therapeutic effect in terms of immune rejection prevention. Consequently, the deleterious effect of chronic immunosuppression and cytotoxicity to grafted islets can be mitigated through such local delivery. It is predicted that local delivery of the immunosuppressive agents in ACE will protect islets from immune rejection in an allogeneic transplantation setting and perhaps lengthen the survival time of islets when compared to systemic delivery.

To the best of my knowledge, this project is the first attempt at implanting a drug delivery system together with islet in rodent ACE. Once this proof-of-concept model is successful in achieving the desired outcome, more delivery candidates (e.g. modulators of islet function) can be encapsulated and delivered using a similar concept in ACE for enhanced islet functionality and a better therapeutic outcome.

The specific goals are

(a) Engineer a Controlled Releasing Microparticle Formulation for Long-term Release of Immunosuppressive Agent Rapamycin.

The first challenge is to develop a microparticle formulation that sustains release of rapamycin over 30 days to meet the minimum doseage requirement for local immunosuppression in rodent ACE. We

anticipate a significant effect in preserving transplanted islets if rapamycin is delivered for 30 days as it is twice the duration of natural islets rejection in mice ACE. The ability of different biodegradable polymers to preserve and release rapamycin will be determined. Strategies to enhance rapamycin release will be explored and implemented to extend its release duration. The formulation selected from the best candidates will be evaluated for its long-term effect *in vitro* by exposing them to cells of immune system. In addition, the effect of the delivered rapamycin on islet function *in vitro* will be studied to determine whether any toxicity effect will be exerted on transplanted islets and whether insulin release is being impacted by the presence of rapamycin microparticle.

(b) Preserve the Allogeneic Transplanted Islets by Implantation of Rapamycin Microparticle into ACE.

The procedure of islet and particles implantation will be established to transplant a sufficient number of particles to achieve the required dosage within the confines of the Aqueous Humor (AqH). The post-transplantation condition of the islets and particles will be assessed by confocal microscopy and quantitative changes of islet size over time. The rejection onset of islets with rapamycin microparticles implanted in ACE will be compared with the baseline rejection onset of allogeneic transplanted islets without any rapamycin exposure (blank microparticle). The change of islet volume over time, as a quantitative measurement of islet rejection, will be tracked every 7 days. With a blank microparticle implanted in the ACE as the control, any difference in rejection onset with islets surrounded by rapamycin microparticles will be attributed by rapamycin released from the microparticle in the ACE.

(c) Design a Multi-drug Delivery System – Janus Particle – for Both Immunosuppression and Enhanced Insulin Secretion.

Multiple drug loading into a single particle can extend the function of microparticles beyond immunosuppression alone without complicating

the surgical procedure. A novel delivery system – the Janus particle – is proposed for incorporation of two drugs: rapamycin and glibenclamide (a drug that stimulates insulin release from islets). The benefit of this novel system is the potential of allowing a simultaneous release of two drugs to holistically address current challenges in islet transplantation. The Janus particle design fits into our vision as it makes the formulation design stage challenging but simplifies the transplantation step, by removing the mixing step, shortening the surgery time and remove the requirement of controlling the particle mixing ratio. When establishing the delivery system, the challenge of loading the drug into Janus particle without changing its anisotropic structure will be addressed. This synthesis principle will be established based on interfacial tension and the spreading coefficient theory, showing feasibility in the loading of two different drugs into one microparticle. The investigation of Janus particle formation mechanism in the presence of different drugs will help to provide empirical guidance for future modification when new drug is required for diabetes treatment. The *in vitro* release profile of drug-loaded Janus particle will be assessed for future transplantation with islets.

1.5 Organization of the Dissertation

This dissertation is organized as follows:

Chapter 1 introduces the background of diabetes and its treatment. The emerging treatment trends, particularly islet transplantation, are briefly explained and their advantages and disadvantages are compared. The limitations are highlighted, providing the motivation and objective of this research work.

Chapter 2 introduces the working principle, procedure and benefits of ACE islet transplantation. It explains, in detail, the common reasons limiting the long-term success of insulin-independence, the different types of engineered drug delivery systems for sustained drug release and the suitable drug candidates and engineering strategies which can address those limitations.

Chapter 3 describes the experimental procedures for the fabrication of the microparticles, the investigation of drug release from microparticles and evaluation of its effect on cell culture. It also briefly introduces the principles and equipment used in the characterization of the microparticles *in vitro* and *in vivo* as well as the transplantation procedure.

Chapter 4 investigates the degradation rate of rapamycin in solution and the ability of different microparticles to protect it from degradation. It also reports on the formulation development and optimization process to prepare the long-acting rapamycin microparticles and further investigates its effect in two cell lines: the breast cancer cell (MCF-7) and the human lymphocyte cell (Jurkat).

Chapter 5 reports the toxic effect of rapamycin from microparticles on isolated islets in *in vitro*. It also briefly explains the protocol for implanting particles along with islets into mice ACE. The delayed immunorejection caused by rapamycin microparticle in an *in vivo* environment is also reported.

Chapter 6 demonstrates the one-step method of synthesizing the biodegradable, multi-drug loaded Janus particle which encapsulates both rapamycin and glibenclamide. The underlying mechanism and

principles of the process are discussed. Furthermore, it reports the effect of the sustained release of the drug from the Janus particles for future application in particle implantation in mice ACE.

Chapter 7 provides a general conclusion of the findings of this dissertation and future recommendations to push this pilot study forward towards practical implementation.

1.6 References

- [1] WHO. (2017, 06 Feb). *The top 10 causes of death-The Fact Sheet*. Available: <http://www.who.int/mediacentre/factsheets/fs310/en/>
- [2] "Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus," *Diabetes Care*, vol. 20, no. 7, pp. 1183-1197, 1997.
- [3] M. S. Islam, *The Islets of Langerhans [electronic resource] / edited by Md. Shahidul Islam* (Advances in Experimental Medicine and Biology: 654). Dordrecht : Springer Science+Business Media B.V., 2010., 2010.
- [4] S. A. Paschou, N. Papadopoulou-Marketou, G. P. Chrousos, and C. Kanaka-Gantenbein, "On type 1 diabetes mellitus pathogenesis," *Endocrine Connections*, vol. 7, no. 1, pp. R38-R46, 11/30
- [5] J. E. Fradkin, "Confronting the urgent challenge of diabetes: an overview," *Health Aff (Millwood)*, vol. 31, no. 1, pp. 12-9, Jan 2012.
- [6] E. Mannucci, M. Monami, I. Dicembrini, A. Piselli, and M. Porta, "Achieving HbA1c targets in clinical trials and in the real world: a systematic review and meta-analysis," *Journal of Endocrinological Investigation*, journal article vol. 37, no. 5, pp. 477-495, May 01 2014.
- [7] S. J. Livingstone *et al.*, "Estimated life expectancy in a Scottish cohort with type 1 diabetes, 2008-2010," (in eng), *Jama*, vol. 313, no. 1, pp. 37-44, Jan 06 2015.
- [8] M. Lind *et al.*, "Glycemic Control and Excess Mortality in Type 1 Diabetes," *New England Journal of Medicine*, vol. 371, no. 21, pp. 1972-1982, 2014.
- [9] A. M. Shapiro *et al.*, "International trial of the Edmonton protocol for islet transplantation," (in eng), *N Engl J Med*, vol. 355, no. 13, pp. 1318-30, Sep 28 2006.

- [10] M. Qi *et al.*, "Five-year follow-up of patients with type 1 diabetes transplanted with allogeneic islets: the UIC experience," (in eng), *Acta Diabetol*, vol. 51, no. 5, pp. 833-43, Oct 2014.
- [11] L. Jansson and P. O. Carlsson, "Graft vascular function after transplantation of pancreatic islets," (in English), *Diabetologia*, Review vol. 45, no. 6, pp. 749-763, Jun 2002.
- [12] A. M. J. Shapiro, M. Pokrywczynska, and C. Ricordi, "Clinical pancreatic islet transplantation," *Nat Rev Endocrinol*, Review vol. advance online publication, 11/11/online 2016.
- [13] J. Lau *et al.*, "Implantation site-dependent dysfunction of transplanted pancreatic islets," *Diabetes*, vol. 56, no. 6, pp. 1544-50, Jun 2007.
- [14] P. Wang, Z. Medarova, and A. Moore, "Molecular Imaging: A Promising Tool to Monitor Islet Transplantation," *Journal of Transplantation*, vol. 2011, p. 202915, 10/15
- [15] M. H. Abdulreda *et al.*, "High-resolution, noninvasive longitudinal live imaging of immune responses," *Proc Natl Acad Sci U S A*, vol. 108, no. 31, pp. 12863-8, Aug 2 2011.
- [16] E. Ilegems *et al.*, "Reporter islets in the eye reveal the plasticity of the endocrine pancreas," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 51, pp. 20581-20586, Dec 2013.
- [17] S. Speier *et al.*, "Noninvasive in vivo imaging of pancreatic islet cell biology," (in English), *Nature Medicine*, Article vol. 14, no. 5, pp. 574-578, May 2008.
- [18] I. B. Leibiger and P.-O. Berggren, "Intraocular in vivo imaging of pancreatic islet cell physiology/pathology," *Molecular Metabolism*, vol. 6, no. 9, pp. 1002-1009, 2017/09/01/ 2017.
- [19] S. Bertera *et al.*, "Body window-enabled in vivo multicolor imaging of transplanted mouse islets expressing an insulin-Timer fusion protein," (in eng), *Biotechniques*, vol. 35, no. 4, pp. 718-22, Oct 2003.

- [20] K. Oishi *et al.*, "In Vivo Imaging of Transplanted Islets Labeled with a Novel Cationic Nanoparticle," *PLOS ONE*, vol. 8, no. 2, p. e57046, 2013.
- [21] V. L. Perez *et al.*, "The anterior chamber of the eye as a clinical transplantation site for the treatment of diabetes: a study in a baboon model of diabetes," *Diabetologia*, vol. 54, no. 5, pp. 1121-6, May 2011.
- [22] A. Agarwal and K. L. Brayman, "Update on Islet Cell Transplantation for Type 1 Diabetes," *Seminars in Interventional Radiology*, vol. 29, no. 2, pp. 90-98, 2012.
- [23] W. L. Lee, E. Widjaja, and S. C. Loo, "One-step fabrication of triple-layered polymeric microparticles with layer localization of drugs as a novel drug-delivery system," *Small*, vol. 6, no. 9, pp. 1003-11, May 7 2010.
- [24] W. L. Lee and S. C. Loo, "Revolutionizing drug delivery through biodegradable multilayered particles," *J Drug Target*, vol. 20, no. 8, pp. 633-47, Sep 2012.
- [25] W. L. Lee, Y. C. Seh, E. Widjaja, H. C. Chong, N. S. Tan, and S. C. Loo, "Fabrication and drug release study of double-layered microparticles of various sizes," (in eng), *J Pharm Sci*, vol. 101, no. 8, pp. 2787-97, Aug 2012.
- [26] Z. Abouelfadel and E. D. Crawford, "Leuprorelin depot injection: patient considerations in the management of prostatic cancer," *Therapeutics and Clinical Risk Management*, vol. 4, no. 2, pp. 513-526, 04/ 2008.
- [27] J. R. Baker, Jr., "Dendrimer-based nanoparticles for cancer therapy," (in eng), *Hematology Am Soc Hematol Educ Program*, pp. 708-19, 2009.

2 Chapter 2 Literature Review

2.1 Diabetes and its Unmet Needs

Diabetes is regarded as one of the leading causes of death [28]. Since 1980, the prevalence of diabetes has almost doubled (Table 2.1) [29]. WHO 2014 data showed 422 million people living with diabetes worldwide [29]. Amongst all the regions, the South-East Asia Region has more number of diabetic patients. The global burden of diabetes has been increasing and the alarming number of diabetic patients in the South-East Asia region calls for attention in early preparation of the huge demand of diabetes management in the future.

In the healthcare industry, the constant increase in number of diabetic patients creates a huge market demand for diabetes treatment. Based on FiercePharma data, the annual market will hit \$58 billion by 2018 [28]. The growing diabetic patient numbers in Asia region generates a large market size in the regional leading countries that is hard to be neglected (Table 2.2). The incidence of diabetes in Singapore is fast rising and it is estimated that one million Singaporeans may suffer from diabetes by 2050. To meet the demand from a growing diabetic patient population, an increasing number of scientists worldwide have been looking for effective therapies for diabetes treatment.

Table 2.1 Estimated prevalence and number of people with diabetes (adults 18+ Years) [30]

WHO Region	Prevalence (%)		Number (millions)	
	1980	2014	1980	2014
African Region	3.1%	7.1%	4	25
Region of the Americas	5.0%	8.3%	18	62
Eastern Mediterranean Region	5.9%	13.7%	6	43
European Region	5.3%	7.3%	33	64
South-East Asia Region	4.1%	8.6%	17	96
Western Pacific Region	4.4%	8.4%	29	131
Total ^a	4.7%	8.5%	108	422

^aTotal include non-Member States

Table 2.2 Asia diabetes market size distribution in 2009 [31]

2009	China	India	South Korea	Taiwan	TOTAL
Total Diabetes Therapeutics Market ('000)	1,576.6	404.0	361.3	196.6	2,538.5
Insulin Market ('000)	800.0	113.0	68.5	40.2	1,021.7
Non-insulin Market ('000)	776.6	291.0	292.8	156.4	1,516.8

2.1.1 Diabetes Definition and Classification

Diabetes, or diabetes mellitus, is a class of metabolic diseases characterized by high levels of blood glucose resulting from defects in insulin secretion, insulin action, or both. Insulin is a 51-amino acid polypeptide that regulates tissue glucose uptake [32]. It promotes glucose absorption from blood to skeletal muscles and other tissues. Therefore it regulates and removes excess blood glucose. Without sufficient amount of insulin, chronic hyperglycemia (i.e. high blood glucose) leads to various microvascular complications (diabetic nephropathy, neuropathy and retinopathy) and macrovascular complications (coronary artery disease, peripheral arterial disease, and stroke) [33]. The long-term complications of diabetes have severe impact on patient's morbidity and mortality, resulting in a drastic deterioration in quality-of-life.

There are two main types of diabetes: T1D and T2D. When pancreatic β -cells are undergoing autoimmune destruction, their mass is significantly reduced. Therefore, the insulin secretion is decreased to an insufficient level. This is characterized as the phenotype of T1D. On the other hand, insulin resistance and impaired β -cell function result in T2D. About 90-95% of diabetic patients have T2D, while 10% have T1D. Other types of diabetes are gestational diabetes, maturity-onset diabetes of the young (MODY) and secondary diabetes caused by multiple reasons. Their percentages are much smaller compared to T1D and T2D.

2.1.2 Pancreas Anatomy

The pancreas mainly consists of four interrelated components (Figure 2.1): the exocrine tissue, the endocrine cells, the ducts and the connective tissue [3]. The endocrine cells form a cluster, termed islets of Langerhans, and secrete hormones into the hepatic portal vein. The Islets contain five types of endocrine cells: α -cell, β -cell, δ -cell, PP cell (pancreatic polypeptide cell) and Ghrelin cells [34]. Each type of cell secretes different endocrine hormones and they together form a balanced mixture to maintain glucose homeostasis. The β -cell is responsible for the secretion of insulin to increase glucose uptake (Figure 2.2), while the α -cell secretes the hormone glucagon, which promotes glucose release [35]. Both α -cell and β -cell function can be inhibited by somatostatin, the hormone produced by δ -cell. Hence, these hormones tightly modulate the blood glucose levels. The communication between these endocrine cells is crucial in maintaining glucose homeostasis [36]. Besides these cells, other cells such as immune cells, and autonomic nerve system endings [37] are also housed in the islet. An abundant vascular network serves the connection between all these different parts in islets.

Figure 2.1 Schematic diagram of pancreas and Islet of Langerhans [38].

2.1.3 Pancreatic β -cell and Mechanism of Insulin Release

Insulin acts as a critical regulator of metabolism. Its secretion is primarily based on glucose stimulation. It is synthesized inside β -cell as preproinsulin and processed to proinsulin [39]. Afterwards, the proinsulin is converted to insulin by cleavage of C-peptide, a by-product of insulin secretion. β -cell stores both insulin and C-peptide inside a storage granule awaiting release on demand (Figure 2.2). In human, Type 1 glucose transporters (GLUT1) predominantly mediate glucose uptake, when blood glucose level increases. But in rodent, additional Type 2 glucose transporters (GLUT2) is there for the same function [40]. Increased amount of ATP is generated by glycolysis. As a response, the ATP sensitive potassium channel closes, leading to cell depolarization. Subsequently, calcium channel opens up. Large amount of insulin is released by exocytosis [41]. Hence, the primary insulin secretion signal is an increase in intracellular Ca^{2+} concentration [42]. The glucose-induced insulin secretion can be elevated by other nutrients such as free fatty acids and amino acids. In addition, various hormones, such as melatonin, estrogen, leptin, growth hormone, and glucagon like peptide-1 (GLP-1) regulate insulin secretion.

Figure 2.2 Insulin release of β -cell after glucose uptake [42].

2.1.4 Brief Diabetes Pathophysiology and Pathogenesis

A decrease in both mass and insulin secretory function of β -cell is the common characteristic observed in both T1D and T2D. T1D is a chronic autoimmune disorder, in which immune system is triggered to respond against altered pancreatic β -cell antigens, or molecules in β -cells that resemble a viral protein [43]. Presence of immune activity was found in histological analysis of the pancreas from T1D patients [44]. Hence, autoimmunity plays a crucial role in the development of T1D. Several potential factors, such as genetic susceptibility, environment and dietary factor, are identified to be associated with the autoimmune disorder. To further the understanding, innate immune response linked to T1D development is widely studied as it is involved in the initiation of the autoimmune process. However, the associated molecular pathways are yet to be elucidated [45]. The pathogenesis of selective β -cell destruction within the islet in T1D is difficult to follow due to marked heterogeneity of the pancreatic lesions (Figure 2.3). However, it is known that β -cell destruction is mainly caused by adaptive immunity during T1D development [46]. Recent studies suggest that T1D impairment of β -cell function is an early feature of disease pathogenesis. Hence, β -cell mass replacement in an early stage of diabetes may be a viable treatment option [35].

Figure 2.3 The complex pathogenesis of β -cell destruction in T1D. This figure maps the different immunological phases and represents the β -cell mass or function change at each corresponding phase. Please refer to ‘How T1D might arise’ for a detailed pathogenesis explanation [43].

T2D is a result of chronic insulin resistance and loss of β -cell mass and function [47]. Obesity is a one of the leading pathogenic factors for insulin resistance development. Obesity is associated with impairment in energy metabolism, leading to increased intracellular fat content in the pancreatic islets, skeletal muscle and liver [48]. Chronic insulin resistance progresses to T2D when β -cells are unable to secrete adequate amounts of insulin to compensate for decreased insulin sensitivity. Two main factors, insulin secretory dysfunction and loss of functional β -cells, contribute to decreased insulin sensitivity [47, 49, 50]. Indeed, patients with T2D always manifest increased β -cell apoptosis and reduced β -cell mass [51]. When insulin resistance lead to β -cell dysfunction, T2D progresses to a full-blown status [52].

2.1.5 Diabetes Treatment

As multiple factors are associated with diabetes development, maintaining a healthy diet and an active exercise program are the most recommended method in the prevention and management of diabetes. However, majority of patients need pharmacological intervention to regain glycemic homeostasis. Current strategies to treat diabetes include supplementing insulin with exogenous insulin, increasing endogenous production with sulfonylurea and meglitinides, reducing insulin resistance using glitazones reducing hepatic glucose production through biguanides, and limiting postprandial glucose absorption with alpha-glucosidase inhibitors.

The discovery and extraction of insulin in the early 19th century marked the beginning of the mainstream diabetes treatment through exogenous insulin administration. The challenge of this treatment method is to introduce accurate amounts of insulin into patients. In order to do that, instant blood glucose level analysis is usually carried out right before the insulin injection. Hypoglycemia (low blood sugar) is one of the most common problems associated with insulin injection caused by insulin overdose [27]. It is dangerous as it could lead to headache, passing out when patient has an inadequate supply of glucose and even coma. Current therapies such as artificial pancreas allow patients to keep blood glucose level in control in order to avoid detrimental consequences caused by hyperglycemia. Artificial pancreas is able to provide a closed-loop control of blood glucose, including a glucose sensor, a control algorithm, and an insulin infusion device. The insulin infusion device has been constantly upgraded with new mechanism, injection model, and blood sugar measurement to provide instant and long-acting blood glucose control. However, it has limitation such as time delay between detection and injection; delivery of insulin based on interstitial glucose level instead of blood glucose level; and constant need of wearing a device [53]. Furthermore, the issue of blood glucose fluctuation associated with any external insulin injection has not yet been resolved.

Many insulin injection types and blood sugar measurement devices are developed to address such problems. Current therapies such as insulin pumps allow patients to keep blood glucose level in control in order to avoid detrimental consequences caused by hyperglycemia. With the advanced device and well-established insulin self-injection procedures, diabetes is now a disease of reduced morbidity with careful management.

The understanding of insulin release mechanism facilitates drug development to improve endogenous insulin production. For example, a class of sulfonylureas drug was discovered to block the potassium ion channel, causing the β -cell to depolarize and therefore enhance insulin secretion [54]. New generation of sulfonylureas was pushed to market to minimize the side effect and toxicity. Similarly, when incretin hormones were found to encourage insulin production, a new class of diabetes drug was developed based on the Glucagon-like peptide-1 (GLP-1) [55]. Various drugs aim to boost insulin secretion or reduce insulin rejection for T2D patients.

Hepatic Glucose Production (HGP) describes the net release of glucose from the liver, the main contributor to diabetic hyperglycemia [56]. Insulin functions as inhibitor of gluconeogenesis, hence controls HGP. In T2D, HGP is higher in the post-absorptive state, and fails to be properly suppressed by insulin [57]. Biguanides, such as metformin [58], decreases HGP and intestinal absorption of glucose and improves insulin sensitivity by increasing peripheral glucose uptake and utilization. They are often combined with other diabetic drugs to form a cocktail treatment for better management of blood glucose level. Delaying the breakdown of complex polysaccharides in upper gastrointestinal (GI) tract is an alternative route for modulating the surge of blood glucose after a meal [59]. A class of α -glycosidase inhibitor, such as acarbose [59], can delay this breakdown from the GI tract until the small intestine so that postprandial glucose peak is reduced.

2.1.6 The Unmet Need of Diabetes

The recent development in tools and technologies treating diabetes has helped patients to control cost and improve their health. However, the nature of diabetes is progressive due to its complex pathogenesis. For example, T2D patients who start with a single drug therapy could progress to multiple drugs (i.e. cocktail) therapy. When β -cell mass decreases further and insufficient insulin is secreted, exogenous insulin injection will be introduced at the latter stage of treatment to achieve glucose homeostasis. In view of this, the expenses on medication for one's entire life can be a huge financial burden, not only to the patient but also to the economy. Furthermore, the long-term complication developed from hyperglycemia may cause far more severe consequences such as blood vessel damage, nerve damage, kidney failure, and cardiovascular problem. Therefore, diabetes is often termed as a silent killer. Diabetes care has been constantly seeking effective ways of slowing down this progression.

Both environmental and genetic factors are potential triggers of the autoimmune attack on β -cells in T1D. The development process of T1D stretches for years. Hence, it is difficult to identify diabetes until the patient shows significant symptoms, by when it is too late and the majority of the β -cells are destroyed. Attempts are being made to interfere with the diabetes development. Recently, one immunotherapy of T1D focused on the β -cell antigen, aiming to protect the β -cell during the T1D development [60].

There is still room for improving the current treatment methods. Many current medicines possess dose-limiting side effects. More importantly, the glycemic control in both T1D and T2D remains suboptimal. In many cases, it is still difficult to inject the correct amount of insulin into individual patients. Moreover, as the mode of application is by injection, patients are constantly exposed to pain and risk of infections. As such,

the comprehensive and effective management and treatment of diabetes have been and remain active areas of study [61].

2.2 Islet Transplantation – Old Idea with New Approach

2.2.1 Emerging Treatment of Diabetes

With the progress in diabetes research and a deeper understanding of its physiology and pathology, a series of prominent and promising preclinical strategies has arisen for treating diabetes. Next-generation insulin analogs aim to provide more aggressive treatment of hyperglycemia with lower risk of life-threatening hypoglycemia. For example, a combination of basal insulin with GLP-1 agonist has clinically shown to improve glycemic control with less hypoglycemia and weight gain [62, 63]. Furthermore, more effort is being directed towards the development of novel hormone drugs, such as leptin and FGF21. Multi-hormone combination therapies adjust enteroendocrine response and contributes to massive and rapid metabolic improvements [64].

Scientists are looking for alternative ways to cure diabetes by aiming for the root cause: the β -cell/islets/pancreas. Pancreas and islet transplantations are considered effective β -cell replacement methods for diabetes treatment. It is a straightforward concept but difficult to execute due to biological complications [65]. Pancreas transplantation has been studied and experimented earlier than islet transplantation [55]. It requires major organ donation and systemic immunosuppression. It has been found that pancreas transplantation done simultaneously with kidney transplantation have better success rates [65]. The high risk of major surgery and scarcity of donors limit the number of patients that can undergo this treatment.

Transplanted islets can maintain glucose homeostasis in the recipient, reducing both glucose excursion and plasma glycated haemoglobin (HbA1c). Although neither pancreatic transplantation nor islet transplantation is an entirely new procedure, recent progress has made these invasive therapies appealing. Transplantation of isolated pancreatic islets from cadaveric donors has emerged as the most promising therapy for T1D and for c-peptide-/ insulin-deficient T2D patients [66].

On the another hand, β -cell regeneration is a new research area that has recently emerged, due to advancements in tissue engineering [67]. However, diabetes pathogenesis has not been completely understood. Therefore, protecting these regenerated β -cell from autoimmunity remains a challenge [68]. Furthermore, the ability of transferring alpha, delta and other endocrine cells to β -cell is limited and difficult to control. Therefore, further studies are required to improve feasibility of β -cell regeneration [67, 68].

2.2.2 The Approach of Islet Transplantation

Replacement of β -cell through pancreas or islet transplantation is currently the only curative treatment for T1D. Pancreas transplantation is a major surgery that is associated with high morbidity and mortality. In contrast, pancreatic islet transplantation is less invasive, and therefore, an attractive alternative therapy. There has been significant progress in the field of β -cell replacement therapy through islet transplantation [9, 69, 70]. The intrahepatic portal system is currently the site of choice for clinical islet transplantation. Islet transplantation becomes feasible after the islet-isolating method was developed [71]. A group of investigators in Edmonton, Alberta, Canada, developed a new approach of islet transplantation on T1D patients in 1999 [72]. Unlike other mediocre results of earlier trials, this study reported seven patients were rendered insulin-free [73]. The protocol used in that study was

termed Edmonton Protocol and became the foundation of subsequent islet transplantations. This study showed that successful islet transplantation could reverse patient diabetes and restore endocrine hormone balance, highlighting the possibility of diabetes therapy.

Islet transplantation has its unique advantages. Islet transplantation replaces only the dysfunctional region of the pancreas, leaving the healthy part to function as it is. The islets are only 2% of the pancreas. Thus, islet transplantation is on a smaller scale and therefore easier to handle. Human body regulates blood glucose in a smart way that is hardly matched by current external insulin injection. Islet transplantation restores the β -cell mass and leaves the self-regulating job to the human body [65]. Diabetic patients could be free of medication once successfully transplanted with islets. Compared to life-long medication bills, islet transplantation is rather attractive despite its high initial cost. Nowadays, medical facilities and conditions are much better than at the time of the Edmonton case. We are in a better position to re-examine the potential of β -cell replacement method as a possible therapy. Source of islet could be addressed by tissue culture or isolation of islet from other species to resolve the issue of scarcity.

Islet transplantation is not merely for treating T1D patients, but also could be beneficial to T2D patients. The latter have a certain degree of insulin resistance, which leads to hyperglycemia. With islet transplantation, the insulin resistance could be compensated by increased insulin release, resulting in possibly resolving the issue of hyperglycemia. Therefore there is a potential benefit of islet transplantation for both types of diabetes.

2.2.3 Limitations of Islet Transplantation

Massive cell death during isolation, culture, and post-transplantation periods limits the widespread implementation of islet transplantation

[74]. A typical transplantation requires islets from more than two donors, and many patients need more than one transplant to become insulin independent, whereas the final mass of islet tissue engrafted may correspond to only 20% of the mass of a healthy individual [75]. Investigation showed low oxygen tension and the induction of an inflammatory response have been considered as the major contributors to β -cell death and dysfunction. The oxygen tension of liver (5mmHg) is much lower compared to that of the pancreas (40mmHg). About 70% of the islets are hypoxic 1 day after intraportal transplantation, which impairs β -cell function and triggers endoplasmic reticulum (ER) stress mediated cell death [76-79]. Furthermore, there is massive loss of islets in the immediate post-transplantation period due to inflammation after blood contact and activation of the hepatic microenvironment [80, 81]. Difficulty in monitoring the islet grafts and the relatively high intrahepatic levels of immunosuppressive drugs that could be toxic for islets results in an additional concern. Therefore, improving methods of islet transplantation remains an active area of research and a priority for funding agencies [82, 83].

2.3 Novel Site for Islet Transplantation – Anterior Chamber of the Eye (ACE)

2.3.1 ACE as New Emerging Site of Islet Treatment

Building upon the success of Edmond protocol, islet transplantation is becoming a promising therapy for T1D treatment [12]. In recent years, research has been focused on experimenting different transplantation sites to address the decrease in mass of grafted islets over time. Various signs suggest that the transplantation site has a major role in graft survival and functionality post-transplantation [84]. In most clinical transplantations, islets are transplanted into an environment with close proximity to pancreas, such as liver [13]. The procedure of transplantation to liver via the portal vein has been extensively

optimized and showed positive results. Despite the careful approach, the grafted islets after transplantation have substantial cell death and impaired functionality [13]. Alternative sites have been investigated for improved efficacy, however, none were found to provide significantly better engraftment of islets.

In addition, it is difficult to access and evaluate the condition of islet grafts after transplantation without invasive approach. It remains questionable whether grafted islets diminished due to immune attack from autoimmune/alloimmune responses after transplantation, or due to challenges in re-vascularization [12]. Although indirect methods could be used to measure the functionality of grafted islets, a direct observation will be necessary to obtain data for the above questions. A novel site – Anterior Chamber of the Eye (ACE) – for islet transplantation was recently reported [17, 85, 86]. Cornea is transparent so the biology of the grafted islets in the ACE can be easily accessed. It is a non-invasive site of longitudinal observation to monitor the condition of engrafted islets up to single cell resolution by confocal microscopy [87]. Therefore, long-term observation *in vivo* for mice and non-human primate subjects was possible at this transplantation site.

Figure 2.4 Schematic illustration of the *in vivo* imaging platform to study pancreatic islet/ β -cell physiology and pathology. Following their isolation, pancreatic islets of Langerhans are transplanted into ACE, where they engraft on the iris, become vascularized and innervated. As the cornea represents a natural body-window, this transplantation site allows non-invasive, repetitive *in vivo* imaging of the islet with high resolution [17].

Besides that, several other factors make ACE to be an ideal site for islets to engraft. The iris of the eye is rich in blood vessels that facilitates the revascularization of transplanted islets. Islet vasculature has been suggested to play a critical role in maintaining islet function in ageing [37]. The islet vasculature is responsible for supplying both oxygen and nutrients, including key metabolic signal (glucose) and incretin hormones (such as GLP-1) [29, 30]. The islets transplanted inside ACE functions in the same way as the pancreatic islets [16]. Therefore, ACE is not only a suitable site for islet transplantation, but also enables real time imaging the behavior of grafted islets [15, 21, 86, 87].

Figure 2.5 Illustration of islet transplantation into the anterior chamber of the eye (a) and noninvasive *in vivo* imaging (b). (c) Photograph of islets engrafted on the iris. Scale bar - 2 mm. [15]

2.3.2 ACE Internal Environment

Anterior chamber refers to the small space between the iris and cornea in the eye (Figure 2.5). This space is transparent, allowing light to transmit through the cornea pupil for vision. But the chamber is filled with a fluid called aqueous humor (AqH), which creates a dynamic reservoir. The fluid dynamics are determined by a ciliary body, located at Posterior Chamber of Eye and constantly secretes fresh AqH into ACE (Figure 2.6). AqH flows into ACE via pupil and drains off by

trabecular pathway (major pathway) or uveoscleral pathway (minor pathway) (Figure 2.6).

AqH is a transparent, gelatinous fluid, serving as a blood surrogate in nutrition supply, metabolic waste removal, neurotransmitter transportation and ocular structure stabilization [88]. It shares similar composition as plasma with less protein content. The concentration of protein in the AqH of the human eye is approximately 0.02%, whereas protein concentration in plasma is 7% [89]. The typical composition of AqH includes water, electrolytes, ascorbic acid and glutathione. Figure 2.7 shows a detailed breakdown and comparison between AqH and plasma in rabbits [89]. The composition of AqH remains constant but the proportion of each component varies from species to species. Nevertheless, the simple composition of AqH allows the widely used medium phosphate buffered saline (PBS) to be a suitable release medium. Another buffer, balanced salt solution (BSS) [90] mimics AqH composition closely, therefore it is commonly used in intraocular surgery [91]. Therefore both buffer were used in this project to mimic the ACE condition.

Figure 2.6 The location and structure of anterior chamber of eye. The flow of aqueous humor is illustrated by blue arrow.

Figure 2 7 Schematic illustration of the trabecular meshwork conventional outflow pathway. Aqueous humor is produced by the ciliary body and it flows (dashed line shown with arrowheads) from the posterior chamber through the pupil into the anterior chamber. From there it flows out

through the trabecular meshwork into the Schlemm's canal and is subsequently absorbed into the episcleral veins via the collector channels [88].

Substance	Aqueous humour concentration (plasma concentration)	Concentration in dialysate (plasma concentration)
Na ⁺	0.96	0.945
K ⁺	0.955	0.96
Mg ⁺⁺	0.78	0.80
Ca ⁺⁺	0.58	0.65
Cl ²	1.015	1.04
HCO ₃ ²	1.26	1.04
Glucose	0.86	0.97
Modified from Davson H: Physiology of the ocular and cerebrospinal fluids, London, J & A Churchill, 1956.		

Figure 2.8 Concentration of various solutes in aqueous humor of rabbit as compared with the values of dialysate of plasma [88].

The rate of AqH secretion is a key factor for this project as it determines the required release rate and the amount of drug required inside the ACE. As AqH is responsible to maintain the inner pressure of the eye, its rate of formation has to be equivalent to the drainage rate. Table 2-3 summarizes differences in human and mouse AqH volume, drainage rate (i.e. secretion rates), and the turnover rate [92]. Hence, the AqH in mice would be completely replenished after 30-40 minutes.

Table 2.3 Information of ACE in human vs mouse [92]

	ACE Volume	Drainage Rate	Turnover (%)
Human	143.98 µL(n=10)	2.4±0.6 µL/min	1.0% - 1.5% /min
Mouse	5.9 ±0.5 µL(n=8)	0.18±0.05 µl/min (n=8)	2.5% /min

2.3.3 Islets Transplanted into ACE are Subject to Immune Attack

The anterior chamber of the eye (ACE) is often considered an immune-privilege site [93]. However, this privilege is broken for islets transplanted in ACE as immune cells were observed to surround grafted islets after 5-7 days after transplantation. With its easy access, longitudinal imaging of transplanted islets in ACE have been performed to assess the *in vivo* condition of graft islets post-transplantation. Abdulreda and colleagues transplanted both allogeneic and syngeneic islets into mice ACE for immune response observation up to 21 days [94]. Microscope observation showed allogeneic islets disappeared gradually (Figure 2.8A). The mass loss caused by allorejection started from day 3 (Figure 2.8B). On day 21, all allogeneic islets disappeared. Without administering any immunosuppression drug, the transplanted islets were subjected to attack by immune cells immediately. In the reported study, half of the grafted islets were lost within 14 days. Clearly, the duration of immunosuppression needed has to be longer than 14 days to preserve the islets grafts.

Figure 2.8 The longitudinal, noninvasive imaging and tracking of islets grafts transplanted into ACE. A) Images of mouse eye transplanted with allogeneic or syngeneic islets that have engrafted on the iris. B) Change in the average islet volume (circles) vs. the number (squares) of graft-infiltrating T-cells (blue, allogeneic; red, syngeneic).

2.4 Immunosuppression Strategies Associated with Islet Transplantation

Since the early days of pancreas transplantation, endless effort has been put in to identify the right type and amount of immunosuppressive agent to preserve the transplanted organs. The failure of early islet transplantation attempts to report positive results in restoring insulin independence is mainly attributed to poorly selected immunosuppression therapy. The situation was not changed until the Edmonton protocol (section 2.2.2.), which used a cocktail of rapamycin, tacrolimus and dacluzimab (Anti-CD 25, monoclonal anitbody), and showed greatly improved survival rate of grafted islets [95]. Hence, the right type, amount and combination of immunosuppressive agent is needed for an effective islet transplantation

2.4.1 Common Immunosuppressive Therapy and Its drawbacks

The post-transplant treatment includes both induction immunosuppressive therapy and maintenance immunosuppressive therapy [12]. The first therapy prevents acute rejection at the early post-transplantation period with a high degree of immunosuppression, while the second therapy target to maintain the allograft as well as managing the risk of infection and malignancy. Majority of induction immunosuppressive agents are biological antibody agents. Monoclonal antibodies like daclizumab and basiliximab, target to inhibit the T-cell signal activation [96]. These drugs have good tolerance and minor side effects, but they lack potency [72]. Polyclonal antibody, thymoglobulin, was used in combination with etanercept to deplete T-cell as an alternative approach of induction immunosuppressive therapy [97]. In addition, T-cell depletion therapy can be achieved by alemtuzumab (Anti-CD52 antibody) used in Edmonton Protocol. The emerging data

suggests alemtuzumab induction is better as it requires less intensive maintenance protocols [98].

The challenge remains in finding the ideal choice for maintenance of immunotherapy. Ciclosporin and tacrolimus are two of the most widely used drugs in this therapy [99]. They are calcineurin inhibitors, stopping the calcium and calcium dependent serine/threonine protein phosphatase from activating T-cells. However the dosage applied is limited due to its high potency and strong adverse effects [100]. They are used together with rapamycin in Edmonton protocol, which is identified as equipotent immunosuppressive agent [9] with fewer adverse effects.

2.4.1.1 Rapamycin – A Distinctive Immunosuppressive Drug

Rapamycin emerged as a foundation for long-term immunosuppressive therapy, after its extensive usage in Edmonton protocol [101]. It spurred researches' interest due to its unusual mechanism of action, different from the mainstream calcineurin inhibitors. Rapamycin (also known as sirolimus) is a macrolide antibiotic isolated from *Streptomyces hygroscopicus*. It binds to the FK binding protein 12 (FKBP12) and forms a complex that binds to multiple proteins, called the mammalian targets of Rapamycin (mTOR) [102]. The mTOR signaling pathway is involved in many disease, including cancer, cardiovascular disease and diabetes [102, 103]. Subsequently, DNA and protein synthesis of T-cells are inhibited, resulting in the arrest of the cell cycle in late G1 phase as it progresses to the S phase, therefore blocking T-cell proliferation, alloantigens, and mitogens. Rapamycin has demonstrated its immunosuppressive effect in various organs, such as skin, renal, small bowel, pancreatic, and pancreaticoduodenal allografts in a number of animal species, including mice, rats, rabbits, pigs, dogs, and primates [104]. Together with tacrolimus and mycophenolate mofetil, they are newer immunosuppressing agents in organ transplantation than ciclosporin [105].

Rapamycin can create synergetic effects if used in combination with tacrolimus due to their different sites of action. Tacrolimus inhibits calcineurin phosphatase so that T-cell cytokine transcription is inhibited and T-cell does not progress from G0 to G1 in cell cycle [104]. On the contrary, rapamycin inhibits the cell cycle stage after G1. As a result, the dual drug therapy inhibits the cell cycle more effectively, enhancing the immunosuppression. Similarly, rapamycin has the potential to synergize with other calcineurin inhibitors. The unique mechanism of rapamycin provides a possibility for dual or multiple drug delivery for a highly efficient immunosuppressive therapy.

However, there is a unique challenge associated with rapamycin structure and chemical property in terms of delivery. Rapamycin easily undergoes ring opening at its ester group and degrades over time (Figure 2.9). Both hydrolysis and autoxidation cause it to degrade rapidly [106]. There is no clear condition under which this degradation mechanism can be suppressed. The *in vitro* investigation showed that the rapamycin stabilities in plasma and whole blood are different among humans, rabbits and rats. The half-life of rapamycin in plasma is 2.2 hours in rats, whereas in the whole blood, it is 15 hours [107]. Its fast degradation imposes unique challenges in quantification analysis and drug delivery formulation.

Figure 2.9: Structure of rapamycin (1) and its degraded products (2: ring open after hydrolysis; 3: dehydration of 2) [106]

2.4.2 Right Delivery System Aid the Therapeutic Effects

Delivery of immunosuppressive agent is necessary for islets transplantation, however it can be a double-edged sword as it places patients at increased risk of infections due to the suppressed immune system. Higher dosage of immunosuppressing agents has also been reported to stress out the grafted islets and may contribute to islets mass loss in the long term [108]. Smart engineering solutions to overcome these undesirable effects of immunosuppressive agent have been proposed in this thesis. These drawbacks can be addressed by the sustained delivery of immunosuppressive agents locally inside ACE.

Currently, delivery of immunosuppressive agents, such as rapamycin, tacrolimus, ciclosporin, is mainly through oral or intravenous modes. But these systemic delivery methods result in poor patient tolerance and strong side effects. Localized drug delivery of immunosuppressive agent effectively minimizes these disadvantages. For lung transplant patients, tacrolimus was delivered in the form of crystalline nanoparticles via nebulization, a pulmonary drug delivery strategy [109]. It is found that tacrolimus absorption rate is high in mice lungs. On the other hand, ciclosporin has a narrowed therapeutic range and the risk of chronic nephrotoxicity. Particulate delivery system of ciclosporin allows the drug to be released in a controlled manner without a noticeable burst release [110]. Therefore it reduces dosing frequency, avoids burst release, minimizes side effect and lowers loading dose [111]. The microparticle system is versatile to load and controlled release physicochemical challenging drugs. Thus it is a suitable delivery system used for localized delivery of immunosuppressive agents.

2.4.3 Characteristics of Each Drug Delivery Systems

A suitable delivery device is selected based on the desired delivery profile and specific application. A number of biodegradable delivery systems offer a controlled and sustained drug release such as liposome, dendrimer, micelle, and spherical particles [112]. Each delivery system

has its unique advantages and has shown excellent therapeutic effect in numerous studies. Liposome, micelle and dendrimer are known for excellent biocompatibility, easy absorbance and fast excretion. However, liposome and micelle have short half-life, poor stability and limited drug loading capacity [112]. Dendrimer has a relatively larger drug loading capacity and is more versatile in formulation, but delivery period is shorter compared to the microparticle system [113]. Microparticle systems made of FDA approved biodegradable polymers has good biocompatibility, better stability, and long delivery period up to months [24]. Besides, the adjustable size and easiness in injection and visualization are additional advantages that suit delivery to grafted islets in ACE. Thus, using PLGA or other biodegradable polymers to deliver Rapamycin via microparticles is a highly feasible and suitable option.

Lee et al. have reported a multi-layer particle system capable of loading both hydrophobic and hydrophilic drugs; and they have customized the delivery profile by varying the polymer, particle size, layered structure, and delivery rate [24]. Recently, Lim et al. have demonstrated a one-step fabrication method to load water soluble drugs [114]. With further investigation, it has the potential to encapsulate and deliver peptides and proteins as well. Thus, it is suitable for various drug candidates designed for immunosuppression. In addition, the chosen delivery system needs be injectable into ACE. Therefore, its practicality in terms of size, portability and injectability should be considered.

2.5 Current Drug Delivery Systems for Islet Transplantation

2.5.1 Microparticle System for Allotransplant Rejection

Recent advances in the field of biomaterials have provided a possibility of biocompatible and biodegradable delivery systems that can be customized into targeted size and release kinetics specific to the environment. Several microparticle systems have been developed for

calcineurin inhibitor due to its poor oral bioavailability and the toxic side effects associated with systemic administration. Three groups of PLGA microparticles (30 μm , 1 μm and 0.2 μm) were fabricated to encapsulate cyclosporine A (CsA). The *in vivo* murine studies have shown the large sized microparticle (30 μm) gave the best release with steady levels of drugs in the blood for over three weeks [115]. Another microparticle formulation based on poly (L-lactic acid) (PLA) released the drug up to four weeks [116]. Both formulations were effective in reducing burst release and the symptoms of arthritis in rat model. Apart from synthetic materials, natural materials such as chitosan and fibrin have been used to fabricate nanoconstructs for CsA delivery, however its effects in animals are not yet confirmed [117].

Drug delivery system for rapamycin is especially attractive because it is one drug with many effects [118]. The deregulation of the mTOR pathway has been implicated in multiple diseases besides diabetes. Several attempts to deliver rapamycin using particles have thus been reported. For example, solid-lipid microparticle, Ac-Dex microparticle and elastin-like polypeptides fused with FKBP12 were recently developed for encapsulating and delivering rapamycin [119-121]. These carriers have increased the solubility limit of rapamycin and have demonstrated high potential for use in autoimmune diseases. Jhunjhunwala et al. reported in 2009 on the sustained delivery of rapamycin via 3.4 μm PLGA MP to dendritic cells [122]. In 2012, the same group delivered rapamycin using a different PLGA MP formulation together with other proteins for induction of regulatory T-cells [123]. However, both formulations showed successful therapeutic effects in T-cells only for four days. Also, the stability of rapamycin delivered through these delivery systems was never explored nor discussed extensively. Despite the known intrinsic instability of rapamycin in water, the literature rarely reports on the functionality of rapamycin after its release.

Another approach of microparticle delivery is to release the antimetabolite mycophenolic acid (MMF). MMF is an anti-proliferative immunosuppression drug. It inhibits rapidly proliferating cells such as T-cells and B-cells via inosine monophosphate dehydrogenase-2 (IMPDH2). A PLGA-based MMF nanoparticle system has been proposed to prolong murine skin transplants better than systemic administration in terms of thickness [124]. The use of microparticle delivery has moved further from drug to genetic material. Specifically double stranded small interfering RNA (siRNA) can silence or deregulate expression of specific genes of interest in the inflammatory pathway [125]. A delivery system can protect the fragile siRNA and provide a better release kinetics over direct infusion. However, stability of the genetic materials and regulatory hurdles associated with cell therapy are the current challenges [126].

There are plenty of successful examples of microparticle formulations encapsulating and control releasing immunosuppressive agent for allogeneic rejection. These results not only lay a good foundation for new formula development but also demonstrate the benefits of a delivery system to biological applications.

2.5.2 Bioengineered System for Islet Transplantation

Compared to prevention in immune destruction of transplanted islets, improvement of transplanted islets is a challenging approach that few have tried. A recent article has reported the development of a microporous polymer scaffold system to act as a vehicle for islet transplantation [127]. The structure of the scaffold was designed to enhance cell infiltration and therefore the re-vascularization of islets after transplantation. The study demonstrated the possibility of using scaffold as a platform for extrahepatic islet transplantation. However, the efficiency of islet survival decreases when mass of engrafted islets increases. In contrast, ACE as a transplantation site can take a high amount of islets engrafts as a metabolic transplantation without

compromising the efficiency of islet survival. Despite the inconclusive result from the scaffold system, the new approach of improving islet transplantation from an engineering perspective is inspiring.

2.5.3 Current and Future Research Direction for Islet Transplantation

With the positive news of islet transplantation in ACE, a bold direction to take in future research is to co-transplant drug delivery carriers with islets to improve the islet survival and function over a long term. There is barely any research that has tried this approach so far. Relevant studies focused solely on novel drug carrier design to enhance function or a biological approach to enhance condition of islets in post-transplantation. This project is the first to combine an engineering design with an emerging biomedical solution for diabetes. It would provide a new approach to address the major limitation of islet transplantation and ease the burden of post-transplantation management. We foresee the success of this procedure will relieve diabetic transplant recipients from harsh systemic immune suppression, while achieving improved glycemic control and reduced insulin dependence. It would also help to develop a raw solution for prolonging the survival of islets in the host and reveal the interaction between biomaterials and living organs *in vivo*.

2.6 References

- [3] M. S. Islam, *The Islets of Langerhans [electronic resource] / edited by Md. Shahidul Islam* (Advances in Experimental Medicine and Biology: 654). Dordrecht : Springer Science+Business Media B.V., 2010., 2010.
- [9] A. M. Shapiro *et al.*, "International trial of the Edmonton protocol for islet transplantation," (in eng), *N Engl J Med*, vol. 355, no. 13, pp. 1318-30, Sep 28 2006.
- [12] A. M. J. Shapiro, M. Pokrywczynska, and C. Ricordi, "Clinical pancreatic islet transplantation," *Nat Rev Endocrinol*, Review vol. advance online publication, 11/11/online 2016.
- [13] J. Lau *et al.*, "Implantation site-dependent dysfunction of transplanted pancreatic islets," *Diabetes*, vol. 56, no. 6, pp. 1544-50, Jun 2007.
- [15] M. H. Abdulreda *et al.*, "High-resolution, noninvasive longitudinal live imaging of immune responses," *Proc Natl Acad Sci U S A*, vol. 108, no. 31, pp. 12863-8, Aug 2 2011.
- [16] E. Ilegems *et al.*, "Reporter islets in the eye reveal the plasticity of the endocrine pancreas," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 51, pp. 20581-20586, Dec 2013.
- [17] S. Speier *et al.*, "Noninvasive in vivo imaging of pancreatic islet cell biology," (in English), *Nature Medicine*, Article vol. 14, no. 5, pp. 574-578, May 2008.
- [21] V. L. Perez *et al.*, "The anterior chamber of the eye as a clinical transplantation site for the treatment of diabetes: a study in a baboon model of diabetes," *Diabetologia*, vol. 54, no. 5, pp. 1121-6, May 2011.
- [24] W. L. Lee and S. C. Loo, "Revolutionizing drug delivery through biodegradable multilayered particles," *J Drug Target*, vol. 20, no. 8, pp. 633-47, Sep 2012.
- [28] W. H. Organization. (2013, 25 Aug). *Diabetes Fact Sheet*. Available: <http://www.who.int/mediacentre/factsheets/fs312/en/>

- [29] W. H. Organization, "Global report on diabetes," 2016 2016.
- [30] "Worldwide trends in diabetes since 1980: a pooled analysis of 751 population-based studies with 4·4 million participants," *The Lancet*, vol. 387, no. 10027, pp. 1513-1530.
- [31] E. Palmer. (2013, 25 Aug). *10 Top-selling Diabetes Drugs - 2012*. Available: <http://www.fiercepharma.com/special-reports/10-top-selling-diabetes-drugs-2012>
- [32] J. P. Mayer, F. Zhang, and R. D. DiMarchi, "Insulin structure and function," *Peptide Science*, vol. 88, no. 5, pp. 687-713, 2007.
- [33] M. J. Sheetz and G. L. King, "Molecular understanding of hyperglycemia's adverse effects for diabetic complications," (in eng), *Jama*, vol. 288, no. 20, pp. 2579-88, Nov 27 2002.
- [34] P. In't Veld and M. Marichal, "Microscopic Anatomy of the Human Islet of Langerhans," in *Islets of Langerhans*, vol. 654, M. S. Islam, Ed. (Advances in Experimental Medicine and Biology, 2010, pp. 1-19.
- [35] C. Chen, C. M. Cohrs, J. Stertmann, R. Bozsak, and S. Speier, "Human beta cell mass and function in diabetes: Recent advances in knowledge and technologies to understand disease pathogenesis," *Molecular Metabolism*, vol. 6, no. 9, pp. 943-957, 2017/09/01/ 2017.
- [36] R. Arrojo e Drigo, Y. Ali, J. Diez, D. K. Srinivasan, P. O. Berggren, and B. O. Boehm, "New insights into the architecture of the islet of Langerhans: a focused cross-species assessment," (in eng), *Diabetologia*, vol. 58, no. 10, pp. 2218-28, Oct 2015.
- [37] R. Rodriguez-Diaz and A. Caicedo, "Neural control of the endocrine pancreas," *Best Practice & Research Clinical Endocrinology & Metabolism*, vol. 28, no. 5, pp. 745-756, 2014/10/01/ 2014.
- [38] ANATOMY. (August 06, 2017). *islets of Langerhans*. Available: <https://www.britannica.com/science/islets-of-Langerhans>

- [39] Z. Fu, E. R. Gilbert, and D. Liu, "Regulation of Insulin Synthesis and Secretion and Pancreatic Beta-Cell Dysfunction in Diabetes," *Current diabetes reviews*, vol. 9, no. 1, pp. 25-53, 2013.
- [40] A. De Vos *et al.*, "Human and rat beta cells differ in glucose transporter but not in glucokinase gene expression," (in eng), *J Clin Invest*, vol. 96, no. 5, pp. 2489-95, Nov 1995.
- [41] C. J.P. Insulin - from secretion to action [Online]. Available: http://www.betacell.org/content/articleview/article_id/1/page/2/glossary/0/
- [42] B. Draznin, "Intracellular calcium, insulin secretion, and action," (in eng), *Am J Med*, vol. 85, no. 5a, pp. 44-58, Nov 28 1988.
- [43] T. L. van Belle, K. T. Coppieters, and M. G. von Herrath, "Type 1 diabetes: etiology, immunology, and therapeutic strategies," (in eng), *Physiol Rev*, vol. 91, no. 1, pp. 79-118, Jan 2011.
- [44] A. K. Foulis, M. A. Farquharson, and R. Hardman, "Aberrant expression of class II major histocompatibility complex molecules by B cells and hyperexpression of class I major histocompatibility complex molecules by insulin containing islets in type 1 (insulin-dependent) diabetes mellitus," (in eng), *Diabetologia*, vol. 30, no. 5, pp. 333-43, May 1987.
- [45] S. C. Pino, A. J. Kruger, and R. Bortell, "The role of innate immune pathways in type 1 diabetes pathogenesis," (in eng), *Curr Opin Endocrinol Diabetes Obes*, vol. 17, no. 2, pp. 126-30, Apr 2010.
- [46] K. T. Tan and J. S. Cheah, "Pathogenesis of type 1 and type 2 diabetes mellitus," (in eng), *Ann Acad Med Singapore*, vol. 19, no. 4, pp. 506-11, Jul 1990.
- [47] D. A. Stoffers, "The development of beta-cell mass: recent progress and potential role of GLP-1," (in eng), *Horm Metab Res*, vol. 36, no. 11-12, pp. 811-21, Nov-Dec 2004.
- [48] C. Lara-Castro and W. T. Garvey, "Intracellular Lipid Accumulation in Liver and Muscle and the Insulin Resistance

- Syndrome," *Endocrinology and metabolism clinics of North America*, vol. 37, no. 4, pp. 841-856, 2008.
- [49] I. Cozar-Castellano *et al.*, "Molecular control of cell cycle progression in the pancreatic beta-cell," (in eng), *Endocr Rev*, vol. 27, no. 4, pp. 356-70, Jun 2006.
 - [50] P. Marchetti *et al.*, "Pancreatic islets from type 2 diabetic patients have functional defects and increased apoptosis that are ameliorated by metformin," (in eng), *J Clin Endocrinol Metab*, vol. 89, no. 11, pp. 5535-41, Nov 2004.
 - [51] A. E. Butler, J. Janson, S. Bonner-Weir, R. Ritzel, R. A. Rizza, and P. C. Butler, "Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes," (in eng), *Diabetes*, vol. 52, no. 1, pp. 102-10, Jan 2003.
 - [52] M. Pietropaolo and D. Le Roith, "Pathogenesis of diabetes: our current understanding," (in eng), *Clin Cornerstone*, vol. 4, no. 2, pp. 1-16, 2001.
 - [53] C. Cobelli, E. Renard, and B. Kovatchev, "Artificial Pancreas: Past, Present, Future," *Diabetes*, vol. 60, no. 11, pp. 2672-2682, 2011.
 - [54] L. C. Groop, "SULFONYLUREAS IN NIDDM," (in English), *Diabetes Care*, Review vol. 15, no. 6, pp. 737-754, Jun 1992.
 - [55] A. D. Association. (25 Aug). *History of Diabetes*. Available: <http://www.diabetes.org/research-and-practice/student-resources/history-of-diabetes.html>
 - [56] L. Monnier, C. Colette, G. J. Dunseath, and D. R. Owens, "The loss of postprandial glycemic control precedes stepwise deterioration of fasting with worsening diabetes," (in eng), *Diabetes Care*, vol. 30, no. 2, pp. 263-9, Feb 2007.
 - [57] R. A. Rizza, "Pathogenesis of fasting and postprandial hyperglycemia in type 2 diabetes: implications for therapy," (in eng), *Diabetes*, vol. 59, no. 11, pp. 2697-707, Nov 2010.
 - [58] M. Foretz *et al.*, "Metformin inhibits hepatic gluconeogenesis in mice independently of the LKB1/AMPK pathway via a decrease

- in hepatic energy state," (in eng), *J Clin Invest*, vol. 120, no. 7, pp. 2355-69, Jul 2010.
- [59] S. E. Inzucchi, "Oral antihyperglycemic therapy for type 2 diabetes: Scientific review," *JAMA*, vol. 287, no. 3, pp. 360-372, 2002.
 - [60] D. A. Schneider, A. M. Kretowicz, and M. G. von Herrath, "Emerging immune therapies in type 1 diabetes and pancreatic islet transplantation," *Diabetes Obesity & Metabolism*, vol. 15, no. 7, pp. 581-592, Jul 2013.
 - [61] M. A. Atkinson, M. von Herrath, A. C. Powers, and M. Clare-Salzler, "Current Concepts on the Pathogenesis of Type 1 Diabetes—Considerations for Attempts to Prevent and Reverse the Disease," *Diabetes Care*, vol. 38, no. 6, pp. 979-988, 05/12
 - [62] R. Balena, I. E. Hensley, S. Miller, and A. H. Barnett, "Combination therapy with GLP-1 receptor agonists and basal insulin: a systematic review of the literature," (in eng), *Diabetes Obes Metab*, vol. 15, no. 6, pp. 485-502, Jun 2013.
 - [63] S. K. Garg, "The role of basal insulin and glucagon-like peptide-1 agonists in the therapeutic management of type 2 diabetes--a comprehensive review," (in eng), *Diabetes Technol Ther*, vol. 12, no. 1, pp. 11-24, Jan 2010.
 - [64] C. Clemmensen, T. D. Müller, B. Finan, M. H. Tschöp, and R. DiMarchi, "Current and Emerging Treatment Options in Diabetes Care," in *Metabolic Control*, S. Herzig, Ed. Cham: Springer International Publishing, 2016, pp. 437-459.
 - [65] E. P. Joslin and C. R. Kahn, *Joslin's diabetes mellitus [electronic resource]*, 14th ed. Philadelphia, Pa. : Lippincott Williams & Wilkins, c2005, 2005.
 - [66] M. Vardanyan, E. Parkin, C. Gruessner, and H. L. Rodriguez Rilo, "Pancreas vs. islet transplantation: a call on the future," (in eng), *Curr Opin Organ Transplant*, vol. 15, no. 1, pp. 124-30, Feb 2010.
 - [67] N. Ben-Othman *et al.*, "From pancreatic islet formation to beta-cell regeneration," *Diabetes Res Clin Pract*, Feb 1 2013.

- [68] D. A. Schneider, A. M. Kretowicz, and H. M. G. von, "Emerging immune therapies in type 1 diabetes and pancreatic islet transplantation," (*Diabetes, Obesity and Metabolism* 15). 2013, p.^pp. Pages.
- [69] A. M. J. Shapiro *et al.*, "Islet Transplantation in Seven Patients with Type 1 Diabetes Mellitus Using a Glucocorticoid-Free Immunosuppressive Regimen," *New England Journal of Medicine*, vol. 343, no. 4, pp. 230-238, 2000.
- [70] S. Marzorati, A. Pileggi, and C. Ricordi, "Allogeneic islet transplantation," (in eng), *Expert Opin Biol Ther*, vol. 7, no. 11, pp. 1627-45, Nov 2007.
- [71] P. E. Lacy and M. Kostianovsky, "Method for the Isolation of Intact Islets of Langerhans from the Rat Pancreas," *Diabetes*, vol. vol. 16, no. 1, pp. 35-39, January 1967 1967.
- [72] A. M. Shapiro *et al.*, "Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen," (in eng), *N Engl J Med*, vol. 343, no. 4, pp. 230-8, Jul 27 2000.
- [73] R. F. Gibly, J. G. Graham, X. Luo, W. L. Lowe, Jr., B. J. Hering, and L. D. Shea, "Advancing islet transplantation: from engraftment to the immune response," *Diabetologia*, vol. 54, no. 10, pp. 2494-505, Oct 2011.
- [74] A. Plesner and C. B. Verchere, "Advances and Challenges in Islet Transplantation: Islet Procurement Rates and Lessons Learned from Suboptimal Islet Transplantation," *Journal of Transplantation*, vol. 2011, p. 6, 2011, Art. no. 979527.
- [75] E. A. Ryan *et al.*, "Five-year follow-up after clinical islet transplantation," (in eng), *Diabetes*, vol. 54, no. 7, pp. 2060-9, Jul 2005.
- [76] G. Miao *et al.*, "Dynamic production of hypoxia-inducible factor-1alpha in early transplanted islets," (in eng), *Am J Transplant*, vol. 6, no. 11, pp. 2636-43, Nov 2006.
- [77] R. Olsson, J. Olerud, U. Pettersson, and P.-O. Carlsson, "Increased Numbers of Low-Oxygenated Pancreatic Islets After

- Intraportal Islet Transplantation," *Diabetes*, vol. 60, no. 9, pp. 2350-2353, 2011.
- [78] W. Moritz *et al.*, "Apoptosis in hypoxic human pancreatic islets correlates with HIF-1alpha expression," (in eng), *Faseb j*, vol. 16, no. 7, pp. 745-7, May 2002.
 - [79] D. Espes, R. Selvaraju, I. Velikyan, M. Krajcovic, P.-O. Carlsson, and O. Eriksson, "Quantification of β -Cell Mass in Intramuscular Islet Grafts Using Radiolabeled Exendin-4," *Transplantation Direct*, vol. 2, no. 8, p. e93, 07/22 2016.
 - [80] O. Korsgren *et al.*, "Optimising islet engraftment is critical for successful clinical islet transplantation," (in eng), *Diabetologia*, vol. 51, no. 2, pp. 227-32, Feb 2008.
 - [81] F. Kenyon *et al.*, "The role of targeted selective treatments in the development of refugia-based approaches to the control of gastrointestinal nematodes of small ruminants," (in eng), *Vet Parasitol*, vol. 164, no. 1, pp. 3-11, Sep 16 2009.
 - [82] D. Mineo, A. Pileggi, R. Alejandro, and C. Ricordi, "Point: Steady Progress and Current Challenges in Clinical Islet Transplantation," *Diabetes Care*, vol. 32, no. 8, pp. 1563-1569, 2009.
 - [83] E. Cantarelli and L. Piemonti, "Alternative transplantation sites for pancreatic islet grafts," (in eng), *Curr Diab Rep*, vol. 11, no. 5, pp. 364-74, Oct 2011.
 - [84] A. Siedlecki, W. Irish, and D. C. Brennan, "Delayed Graft Function in the Kidney Transplant," *American Journal of Transplantation*, vol. 11, no. 11, pp. 2279-2296, 09/19 2011.
 - [85] M. H. Abdulreda, A. Caicedo, and P. O. Berggren, "Transplantation into the anterior chamber of the eye for longitudinal, non-invasive in vivo imaging with single-cell resolution in real-time," *J Vis Exp*, no. 73, p. e50466, 2013.
 - [86] S. Speier, D. Nyqvist, M. Kohler, A. Caicedo, I. B. Leibiger, and P. O. Berggren, "Noninvasive high-resolution in vivo imaging of cell biology in the anterior chamber of the mouse eye," *Nat Protoc*, vol. 3, no. 8, pp. 1278-86, 2008.

- [87] I. B. Leibiger, A. Caicedo, and P. O. Berggren, "Non-invasive in vivo imaging of pancreatic beta-cell function and survival - a perspective," (in eng), *Acta Physiol (Oxf)*, vol. 204, no. 2, pp. 178-85, Feb 2012.
- [88] M. Goel, R. G. Picciani, R. K. Lee, and S. K. Bhattacharya, "Aqueous Humor Dynamics: A Review," *The Open Ophthalmology Journal*, vol. 4, pp. 52-59, 09/03 2010.
- [89] R. L. Stamper, M. F. Lieberman, M. V. Drake, and B. Becker, *Becker-Shaffer's diagnosis and therapy of the glaucomas [electronic resource]*. [Edinburgh] : Mosby/Elsevier, c2009.
8th ed. / Robert L. Stamper, Marc F. Lieberman, Michael V. Drake., 2009, p. 20.
- [90] (2013, n.d.). *BSS PLUS® Irrigating Solution*. Available: <https://www.myalcon.com/products/surgical/bss-plus/bss-sterile-irrigating-solution.shtml>
- [91] V. Vasavada, V. Vasavada, N. V. Dixit, S. M. Raj, and A. R. Vasavada, "Comparison between Ringer's lactate and balanced salt solution on postoperative outcomes after phacoemulsification: A randomized clinical trial," *Indian Journal of Ophthalmology*, vol. 57, no. 3, pp. 191-195, 12/02 2009.
- [92] M. Aihara, "Aqueous Humor Dynamics in Mice," *Investigative Ophthalmology & Visual Science*, vol. 44, no. 12, pp. 5168-5173, 2003.
- [93] J. Y. Niederkorn, "Immune privilege in the anterior chamber of the eye," (in eng), *Crit Rev Immunol*, vol. 22, no. 1, pp. 13-46, 2002.
- [94] M. H. Abdulreda *et al.*, "High-resolution, noninvasive longitudinal live imaging of immune responses," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 31, pp. 12863-12868, Aug 2011.
- [95] S. Merani and A. M. Shapiro, "Current status of pancreatic islet transplantation," *Clin Sci (Lond)*, vol. 110, no. 6, pp. 611-25, Jun 2006.

- [96] S. Gabardi, S. T. Martin, K. L. Roberts, and M. Grafals, "Induction immunosuppressive therapies in renal transplantation," (in eng), *Am J Health Syst Pharm*, vol. 68, no. 3, pp. 211-8, Feb 01 2011.
- [97] G. Ciancio *et al.*, "A randomized trial of thymoglobulin vs. alemtuzumab (with lower dose maintenance immunosuppression) vs. daclizumab in renal transplantation at 24 months of follow-up," (in eng), *Clin Transplant*, vol. 22, no. 2, pp. 200-10, Mar-Apr 2008.
- [98] K. K. Chouhan and R. Zhang, "Antibody induction therapy in adult kidney transplantation: A controversy continues," *World Journal of Transplantation*, vol. 2, no. 2, pp. 19-26, 04/24 2012.
- [99] T. Van Belle and M. von Herrath, "Immunosuppression in islet transplantation," (in eng), *J Clin Invest*, vol. 118, no. 5, pp. 1625-8, May 2008.
- [100] S. Emre *et al.*, "Treatment of tacrolimus-related adverse effects by conversion to cyclosporine in liver transplant recipients," *Transplant International*, vol. 13, no. 1, pp. 73-78, 2000.
- [101] T. Berney and A. Secchi, "Rapamycin in islet transplantation: friend or foe?," (in eng), *Transpl Int*, vol. 22, no. 2, pp. 153-61, Feb 2009.
- [102] E. A. Dunlop and A. R. Tee, "Mammalian target of rapamycin complex 1: signalling inputs, substrates and feedback mechanisms," (in eng), *Cell Signal*, vol. 21, no. 6, pp. 827-35, Jun 2009.
- [103] R. Zoncu, A. Efeyan, and D. M. Sabatini, "mTOR: from growth signal integration to cancer, diabetes and ageing," (in eng), *Nat Rev Mol Cell Biol*, vol. 12, no. 1, pp. 21-35, Jan 2011.
- [104] R. N. Saunders, M. S. Metcalfe, and M. L. Nicholson, "Rapamycin in transplantation: A review of the evidence," (in English), *Kidney International*, Review vol. 59, no. 1, pp. 3-16, Jan 2001.
- [105] D. F. Mayer and S. S. Kushwaha, "Transplant immunosuppressant agents and their role in autoimmune

- rheumatic diseases," *Current Opinion in Rheumatology*, vol. 15, no. 3, pp. 219-225, 2003.
- [106] A. R. Oyler *et al.*, "Forced degradation studies of rapamycin: identification of autoxidation products," *J Pharm Biomed Anal*, vol. 59, pp. 194-200, Feb 5 2012.
- [107] G. M. Ferron and W. J. Jusko, "Species Difference in Sirolimus Stability in Humans, Rabbits, and Rats," *Drug Metabolism and Disposition*, vol. 26, pp. 83-84, 1998.
- [108] M. C. Fabian, J. R. Lakey, R. V. Rajotte, and N. M. Kneteman, "The efficacy and toxicity of rapamycin in murine islet transplantation. In vitro and in vivo studies," (in eng), *Transplantation*, vol. 56, no. 5, pp. 1137-42, Nov 1993.
- [109] P. Sinswat, K. A. Overhoff, J. T. McConville, K. P. Johnston, and R. O. Williams, 3rd, "Nebulization of nanoparticulate amorphous or crystalline tacrolimus--single-dose pharmacokinetics study in mice," (in eng), *Eur J Pharm Biopharm*, vol. 69, no. 3, pp. 1057-66, Aug 2008.
- [110] J. Azzi *et al.*, "Polylactide-cyclosporin A nanoparticles for targeted immunosuppression," (in eng), *FASEB J*, vol. 24, no. 10, pp. 3927-38, Oct 2010.
- [111] N. K. Varde and D. W. Pack, "Microspheres for controlled release drug delivery," (in English), *Expert Opinion on Biological Therapy*, Review vol. 4, no. 1, pp. 35-51, Jan 2004.
- [112] G. Orive, E. Anitua, J. L. Pedraz, and D. F. Emerich, "Biomaterials for promoting brain protection, repair and regeneration," *Nature Reviews Neuroscience*, vol. 10, no. 9, pp. 682-U47, Sep 2009.
- [113] M. M. Villiers, P. Aramwit, and G. S. Kwon, *Nanotechnology in Drug Delivery [electronic resource] / edited by Melgardt M. Villiers, Pornanong Aramwit, Glen S. Kwon* (Biotechnology: Pharmaceutical Aspects: X). New York, NY : Springer New York, 2009., 2009.
- [114] M. P. A. Lim, W. L. Lee, E. Widjaja, and S. C. J. Loo, "One-step fabrication of core-shell structured alginate-PLGA/PLLA

- microparticles as a novel drug delivery system for water soluble drugs," *Biomaterials Science*, vol. 1, no. 5, p. 486, 2013.
- [115] A. Sánchez, J. Vila-Jato, and M. J. Alonso, "Development of biodegradable microspheres and nanospheres for the controlled release of cyclosporin A," *International Journal of Pharmaceutics*, vol. 99, no. 2, pp. 263-273, 1993/10/15/ 1993.
- [116] T. Urata, K. Arimori, and H. Nakano, "Modification of release rates of cyclosporin A from poly(L-lactic acid) microspheres by fatty acid esters and in-vivo evaluation of the microspheres," (in eng), *J Control Release*, vol. 58, no. 2, pp. 133-41, Mar 29 1999.
- [117] G. Praveen, P. R. Sreerekha, D. Menon, S. V. Nair, and K. P. Chennazhi, "Fibrin nanoconstructs: a novel processing method and their use as controlled delivery agents," (in eng), *Nanotechnology*, vol. 23, no. 9, p. 095102, Mar 09 2012.
- [118] J. Li, S. G. Kim, and J. Blenis, "Rapamycin: one drug, many effects," *Cell Metab*, vol. 19, no. 3, pp. 373-9, Mar 4 2014.
- [119] B. K. Nanjwade, D. J. Patel, K. A. Parikh, V. K. Nanjwade, and F. V. Manvi, "Development and Characterization of Solid-Lipid Microparticles of Highly Insoluble Drug Sirolimus," *Journal of Bioequivalence & Bioavailability*, vol. 03, no. 01, 2011.
- [120] M. Shah *et al.*, "A rapamycin-binding protein polymer nanoparticle shows potent therapeutic activity in suppressing autoimmune dacryoadenitis in a mouse model of Sjogren's syndrome," (in English), *Journal of Controlled Release*, Article vol. 171, no. 3, pp. 269-279, Nov 2013.
- [121] K. J. Kauffman, N. Kanthamneni, S. A. Meenach, B. C. Pierson, E. M. Bachelder, and K. M. Ainslie, "Optimization of rapamycin-loaded acetalated dextran microparticles for immunosuppression," *Int J Pharm*, vol. 422, no. 1-2, pp. 356-63, Jan 17 2012.
- [122] S. Jhunjhunwala, G. Raimondi, A. W. Thomson, and S. R. Little, "Delivery of rapamycin to dendritic cells using degradable microparticles," *J Control Release*, vol. 133, no. 3, pp. 191-7, Feb 10 2009.

- [123] S. Jhunjhunwala *et al.*, "Controlled release formulations of IL-2, TGF-beta1 and rapamycin for the induction of regulatory T cells," *J Control Release*, vol. 159, no. 1, pp. 78-84, Apr 10 2012.
- [124] A. C. Shirali *et al.*, "Nanoparticle delivery of mycophenolic acid upregulates PD-L1 on dendritic cells to prolong murine allograft survival," (in eng), *Am J Transplant*, vol. 11, no. 12, pp. 2582-92, Dec 2011.
- [125] J. M. Williford, J. Wu, Y. Ren, M. M. Archang, K. W. Leong, and H. Q. Mao, "Recent advances in nanoparticle-mediated siRNA delivery," (in eng), *Annu Rev Biomed Eng*, vol. 16, pp. 347-70, Jul 11 2014.
- [126] V. Di Caro, N. Giannoukakis, and M. Trucco, "In vivo delivery of nucleic acid-formulated microparticles as a potential tolerogenic vaccine for type 1 diabetes," (in eng), *Rev Diabet Stud*, vol. 9, no. 4, pp. 348-56, Winter 2012.
- [127] R. F. Gibly *et al.*, "Extrahepatic islet transplantation with microporous polymer scaffolds in syngeneic mouse and allogeneic porcine models," (in eng), *Biomaterials*, vol. 32, no. 36, pp. 9677-84, Dec 2011.

3 Research Methodology

This chapter describes the materials, fabrication methods and characterization methods used in this dissertation and it is classified into four sections, including microparticle synthesis and characterization, *in vitro* control release assessment, *in vivo* transplantation and evaluation and novel microparticle synthesis investigation. It explains the synthesis method mechanism, details of experiments and analysis methods used to prove the hypothesis for this thesis. The first section explains the rapamycin microparticles formulation development and optimization, as well as the ability of microparticle preserving encapsulated rapamycin from degradation. The second section introduces the *in vitro* and *ex vivo* assessment of rapamycin microparticles performance in cell culture and with rodent islets, while the third section reveals the design of *in vivo* evaluation, transplantation procedure and long-term observation method for acquire rapamycin microparticle effect on islets in the post-transplantation. To investigate the mechanism of novel Janus particle formation, fabrication method and interfacial tension study are described in the fourth section.

3.1 Engineer a Controlled Releasing Microparticle Formulation for Long-term Release of Immunosuppressive Agent Rapamycin

3.1.1 Materials

Poly(lactic-co-glycolic acid) (PLGA) 50:50 (MW 11 kDa, MW 10 kDa, MW 7-17 kDa acid terminated), Polycaprolactone (PCL) (MW 10 kDa) and Poly(vinyl alcohol) (PVA) (MW 30–70 kDa) were obtained from Sigma-Aldrich (Singapore). PLGA 50:50 (MW 153 kDa, intrinsic viscosity (IV) 1.03) was obtained from Purac Biomaterials. Rapamycin from *Streptomyces hygroscopicus* was obtained from Apollo Scientific (UK). PLGA (PLGA 50:50, IV=0.2) polymers were obtained from

Corbion Purac. PCL 10K (MW 10 kDa), PVA (87-90% hydrolyzed, MW 30-70 kDa), High performance liquid chromatography (HPLC) column BC-Poroshell 120 (EC-C18, 4.6x100 mm, 2.7 μ m) was obtained from Agilent (Singapore). HPLC grade methanol (MeOH), dichloromethane (DCM) and acetonitrile were from Tedia (US). Dulbecco's Phosphate Buffer Saline (DPBS) of pH 7.4 was obtained from Life Technology (Singapore). Purified water was obtained from Milli-Q deionized H₂O (Biocel Ltd.). All items were used as received.

3.1.2 Microparticle Fabrication

Particle fabrication was based on oil-in-water emulsion solvent evaporation method [128, 129]. Four different MW of PLGA were used to fabricate PLGA monolayer particles, which were labeled as PLGA-HMW (MW 153 kDa), PLGA (MW 11 kDa), PLGA-LMW (MW 10 kDa) and PLGA-Acid (MW 7-17 kDa, acid terminated). 0.3 g of the PLGA polymer was dissolved with rapamycin (1.33% w/w) in DCM (3 ml for PLGA, PLGA-LMW, PLGA-Acid; 5ml for PLGA-HMW). Then the polymer solution was poured into a deionized (DI) water with PVA (0.5% w/v) and emulsified at 400 rpm (2000 rpm for PLGA-1) using an overhead stirrer (Calframo BDC1850-220) at ambient temperature for three hours. After DCM had completely evaporated, the particles formed were collected, washed with deionized water and lyophilized before being stored in a freezer. 0.3 g of PCL polymer was used in the preparation of particles through the same fabrication procedure as above with rapamycin (1.33% w/w). Mixtures of PCL and PLGA-HMW at 1:2 (w/w) and 1:1 (w/w) ratios were dissolved in DCM (3 ml) to fabricate PCL/PLGA21 and PCL/PLGA11 double-layered particles as per the procedure stated above. Drug loading percentage of all samples was kept the same.

3.1.3 Particle Characterization

3.1.3.1 Particle Morphology and Average Size (Scanning Electron Microscopy)

Particles were imaged under the JEOL JSM-6360A Scanning Electron Microscope (5 kV) for surface morphology and cross-section. The particles were first mounted onto a carbon tape, and then rapidly frozen in liquid nitrogen for 60s. Subsequently, particles were cut with a razor blade and air dried at room temperature. The dried particles were mounted onto metal stubs and coated with gold using a sputter coater (SPI-module model) for imaging. The particle size (diameter) was measured from SEM images using the ImageJ software. At least two independent batches for each type of particle were imaged and only representative images with consistent morphology were used. For each batch, over 30-50 particles were randomly chosen for diameter measurement. Such measurement was repeated at least twice for each batch.

3.1.3.2 Drug Encapsulation Efficacy

Encapsulation efficiency (EE) is the percentage of drug encapsulated over the total amount of drug added during fabrication. It is calculated using actual drug loading over theoretical drug loading (see formula below). Approximately 5 mg of particles were weighed and dissolved in 1 ml of DCM. 5 ml methanol was then added to precipitate out the polymer. Polymer precipitates were centrifuged and the supernatant was drawn for HPLC analysis. Drug concentration was measured by Agilent 1100 HPLC system with Agilent Poroshell 120 column. The mobile phase was 80% methanol and 20% water with a flow rate of 0.7 mL/min. Column temperature was 35°C, and the retention time for rapamycin was 6.8 - 7.2 min. Concentrations of rapamycin were measured at 278 nm. All measurements were taken in triplicates.

$$\text{Encapsulation efficiency (EE \%)} = \frac{\text{Actual drug loading}}{\text{Theoretical drug loading}} \times 100\% =$$

$$\frac{\text{Amount of encapsulated drug}}{\text{Weight of particles} - \text{Amount of encapsulated drug}} \div$$

$$\frac{\text{Amount of total drug}}{\text{weight of total polymer}} \times 100\%$$

3.1.4 *In vitro* Drug Release Study

3.1.4.1 Rapamycin Degradation Study

For drug degradation studies, rapamycin was dissolved in DPBS buffer with 5% DMSO (solubilizing agent) at seven different known concentrations and kept at 37°C, protected from light. Samples were drawn at 0, 24, 48, 72 and 96 hr and measured directly by HPLC to determine amount of non-degraded rapamycin. Calibration curve was performed using the same medium, and all measurements were done in triplicate.

3.1.4.2 Rapamycin Liquid Chromatography–Mass Spectrometry

Intact and degraded rapamycin was identified by Liquid Chromatography–Mass Spectrometry (LCMS). Rapamycin was dissolved in methanol at high concentration (1 µg/ml) and low concentration (0.1 µg/ml). A batch of freshly prepared samples and a batch of 3-day-old samples were analyzed by LCMS: MicrOTO-QII (Bruker, US) MS coupled to an Dionex Ultimate 3000 RSLC system (Thermo Fisher Scientific, US). HPLC column was Poroshell 120 EC-C18 column (4.6x100 mm, 2.7 µm, Agilent). The mobile phase consisted of 80% methanol and 20% water. The system operated at a flow-rate of 0.7 ml/min. The injection volume of sample is 1 µL. The electrospray parameters from literature was adapted [130]. The mass analyzer was focused on the sodium adduct ion $[M+Na]^+$ of rapamycin (m/z 936.6).

3.1.4.3 Long Term Release Study *In vitro*

4 mg of particles were weighed and dispersed in a release medium (10 ml DPBS buffer). All samples were maintained in a 37°C shaking incubator (orbital shaker incubator, model: LM-570RD). Release medium was collected daily up to 30 days or at the end of the release, whichever was earlier. Drug in the collected release medium was extracted using 1.5 ml DCM. DCM was allowed to fully evaporate under vacuum, and the extracted drug was re-dissolved into 1 ml methanol and vialled for HPLC. Degraded rapamycin elutes at 3.9 min and 4.6 min, and intact rapamycin elutes at 6.7 - 6.8 min. Intact rapamycin content was quantified against standard by HPLC. All measurements were done in triplicates.

3.2 *In vitro* Assessment of Rapamycin Microparticle Formulations

3.2.1 Materials

The human breast adenocarcinoma cell line MCF-7 was a gift from Biomedical Sciences Institutes of Agency for Science, Technology and Research (A*star). The Jurkat cells were a gift from Prof. Su I-Chin (School of biological science, Nanyang Technological University). Dulbecco's Minimum Essential Medium (DMEM), the Connaught Medical Research Laboratories (CMRL) medium, Hank's balanced saline solution (HBSS), penicillin-streptomycin and glutamine were purchased from Gibco (Singapore). Fetal bovine serum (FBS) was purchased from PAA Laboratory (Singapore). The Roswell Park Memorial Institute (RPMI) 1650 medium was obtained from Biowest (Singapore). The WST-8 assay for cell viability was carried out with the cell counting kit solution (CCK-8) from Dojindo Molecular Laboratories Inc. (Kumamoto, Japan). The 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer was purchased from First BASE laboratory (Singapore). RIPA Lysis buffer was purchased from Sigma. Tecan microplate reader (Infinite® 200 PRO) was used for plate

reading. Collagenase Type V, sodium chloride, potassium chloride, calcium chloride and Magnesium dichloride are purchased from Sigma-Aldrich. All items were used as received.

3.2.2 Effect of Rapamycin Microparticles on MCF-7 Cell Proliferation

MCF-7 cells were seeded at 100-150 cells/mL in 96-well plates in DMEM supplemented with 10% FBS, 2mM L-glutamine, and 1% penicillin-streptomycin. After 24 hr incubation within a 5% CO₂ incubator at 37°C to allow cell attachment, cells were incubated together with rapamycin-loaded particles (equivalent to 100 nM dose) constituted in PBS for 96 hr. Pre-releasing particles were added to cells at day 0, 4, 12, 19 and 23, with at least four replicates at each time point. Subsequently, the cells were subjected to the WST-8 assay [118], to quantify the proportion of cells that remained viable after transient exposure to rapamycin-loaded particles.

The CCK-8 kit utilizes the water-soluble tetrazolium salt WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) in measuring NADH production, resulting from the dehydrogenase activity of viable cells. The subsequent reduction of WST-8 by viable cells produces an orange-colored formazan product with an absorbance at 450 nm. 100 µL of a 10% v/v CCK-8 solution in clear DMEM was mixed and added to each well. No washing was carried out to avoid damage or loss of cells. After incubation for 1 hr at 37°C in a 5% CO₂ incubator, absorbance reading was measured at 450 nm using an Infinite 200TM microplate reader (Tecan Inc., Maennedorf, Switzerland). Due to the limited cell proliferation space in the plate, the incubation time was limited to four days. Thus, the study was adjusted to have multiple time points of particles at different phase of release.

MPs were incubated four days with fixed amount of cells. CCK-8 reading was taken at the beginning (D0) and end of incubation (D4) and the difference is calculated as cell proliferation result (i.e. D4-D0). Cells incubated with MPs (i.e. D4_R-D0_R) proliferated less than cells in blank medium (i.e. D4_{NC}-D0_{NC}), thus the difference was calculated as the effect of inhibition. To aid comparison, all values were normalized with the cells in blank medium (D4_{NC}-D0_{NC}). When it's above 0%, the inhibitory effect of released rapamycin is proven.

Formulation's effect of inhibition on MCF-7 cell proliferation:

$$\frac{(D4_{NC} - D0_{NC}) - (D4_R - D0_R)}{D4_{NC} - D0_{NC}} \times 100\% =$$

$$100\% = \frac{D4_R - D0_R}{D4_{NC} - D0_{NC}} \%$$

3.2.3 Long Term Effect of Rapamycin Microparticles on Jurkat Cell Proliferation

Jurkat cells were cultured in RPMI 1650 medium supplemented with 0.3 g/L L-Glutamine, 5% FBS and 1% penicillin-streptomycin at 37°C and 5% humidified CO₂. Jurkat cells were seeded at a density of 1x10⁵ cells/mL and were incubated with either free rapamycin or rapamycin MP (1.5 nM or 3 nM) to compare the inhibition efficacy. Untreated Jurkat cells and Jurkat cells treated with blank MP were used as controls. Two separate time-point experiments of different durations were carried out: 14-days (cell added on day 0) and 20-days (cells added on day 10). Cell viability was assessed via two methods: one was trypan blue (Sigma-Aldrich) exclusion assay whereby viable cells are counted in a hemocytometer, The other was CCK-8 metabolic assay in which 100 µL of cell suspension from each condition was transferred into a 96-well plate and supplemented with 10 µL of CCK solution. After three hours incubation in the dark at 37°C, absorbance was measured at 450 nm on a microplate reader (Biotek, Synergy H1). Duplicate wells were done for each experiment and the experiment was repeated three times.

Results were presented as means with standard deviation ($\bar{x} \pm \sigma$) for three separate experiments. For comparison across groups, data were analyzed by Student's *t* test using Graphpad. Differences between groups statistically significant at $P < 0.05$.

3.2.4 *Ex vivo* Assessment of Rapamycin Microparticle Impact on Rodent Islets

3.2.4.1 Islets Isolation from Rodent

C57BL6 and BALB/c mice were purchased from the Jackson Laboratories (Bar Harbor, ME). All experiments were approved by the local animal ethics committees at SingHealth Academia and SingHealth institutional Animal Care and Use Committee. Islets donors (BALB/c) and recipients (C57BL6) mice were used at age of 16-21 weeks.

Murine islets were isolated by a well-established technique [131] using an enzyme solution of 0.5 mg/ml collagenase type V (Sigma) in HBSS. Briefly, mice were sacrificed under general anesthesia, the abdomen was opened, and the pancreas were exposed and injected with the enzyme solution through the main bile duct until full distension was achieved. The pancreatic tissue was then surgically removed and immersed in the enzyme solution. Digestion was performed during a 5-6 min long incubation at 37°C, with gentle shaking, after which enzyme kinetics were sharply slowed by addition of cold HBSS supplemented with 10% FBS. Mechanical disruption of the digested pancreatic tissue was achieved by repeated passages through 14G needles, and tissue was then filtered through a 450 μm metal mesh. Islet purification was obtained by centrifugation at 2000 rpm for 15 min on discontinuous Euro-Ficoll gradients (1.110 g/ml, 1.096 g/ml, 1.069 g/ml and 1.037 g/ml), and routinely provided islets of purity greater than 90%.

Before transplantation, islets were cultured overnight at 37°C, 5% CO₂, in CMRL medium supplemented with 10% fetal calf serum, 2 mmol/l l-glutamine, 100 µg/ml peniciline streptomycin, and 25 mM HEPES buffer (CMRL-10).

3.2.4.2 Islet Glucose Stimulated Insulin Secretion (GSIS) Assay

Aliquots of freshly isolated murine islets were cultured in 35-cm tissue culture dishes, containing 2 ml of CMRL-10 in the absence and presence of rapamycin-loaded microparticles, or blank microparticles or rapamycin (20 nM). After 24 hr incubation, islets were removed and washed twice in RPMI medium. Three to five mice islets from each sample were transfered into a 12-well plate, and starved in 1ml of low-glucose buffer, containing 3 mM glucose, 125 mM NaCl, 5.9 mM KCl, 2.56 mM CaCl₂, 1.2 mM MgCl₂, 25 mM HEPES and 0.1% (wt%) BSA (Ca-10 buffer), at 37°C for 1.5 – 2 hr. After starvation, the islets were transferred to fresh 0.5 ml low-glucose buffer for 30 min incubation. Then, the islets were removed and washed 3 times with 1 ml fresh low-glucose buffer. Islets were transferred to 0.5 ml high-glucose buffer, containing 16 mM glucose in Ca-10 buffer, incubating for 30 min. Upon finish, islets were removed and lysed with RIPA Lysis buffer (ThermoFisher). Supernatant from 30 min incubation of both low-glucose and high-glucose buffer were collected for insulin quantification by Mouse Insulin ELISA (Mercodia) and normalized by number of islets.

3.3 *In vivo* Transplantation of Rapamycin Microparticle and Islets

3.3.1 Material

Bovine Serum Albumins (BSA) was purchased from Sigma (Singapore). Anterior chamber cannula (angled, 45°C, 0.50 X 22 mm, 25G) and lacrimal cannula (curved, 0.45 x 28 mm, 26G) were purchased from Beaver-Visitec International (MA, US). Hamilton syringe

(GASTIGHT®#1001, model 1710TLL, 100 µL), tubing, motorized syringe. Viscotears® liquid gel was purchased from Alcon (Novatis).

3.3.2 Particle Preparation Prior to Surgery

Required amount of microparticle was weighted and sterilized under UV lamp (UVP, Thermo Scientific) at 252 nm for 20-30 min. The rest of microparticles were stored in -20°C fridge for future usage.

3.3.3 Transplantation of Pancreatic Islets and Microparticles to the ACE

Immediately before transplantation, good quality islets were handpicked under the microscope and divided in aliquots of 10-50 islets per recipient mice. Recipient mice were put under general anesthesia induced by isoflurane inhalation (isoflurane, Baxter, IL, USA). Then islets were transferred from culture media to sterile PBS and were aspirated into a blunt anterior chamber cannula connected to a 1-ml Hamilton syringe (Hamilton) via 0.4-mm polythene tubing (Portex Limited). To obtain post-operative analgesia, TobraDex (Tobramycin, Dexamethasone Ointment, Alcon) was applied on the eye. Under a stereomicroscope, cornea was punctured at a position close to the sclera at the bottom part of the eye with a 27G needle and took great care not to damage the iris and to avoid bleeding. Next, particles, which was pre-suspended in 5% BSA in PBS (filtered) buffer, was aspirated using lacrimal cannula (Beaver-Visitec, USA). The cannula was gently inserted and the particles were slowly injected into the anterior chamber, settling onto the iris. The islets were then injected into the anterior chamber with a similar manner using cannula from the window made by earlier incision. After injection, cannula was carefully withdrew, leaving the mouse lying on its side before awakening. The mouse quickly recovered and showed no signs of stress or irritation from the manipulated eye.

3.3.4 *In vivo* Imaging of Islets Transplanted to the ACE

At designated time points after transplantation (Figure 5.3), mice were anesthetized with a 40% oxygen and a ~2% isoflurane mixture and placed on a heating pad. The mouse head is restrained with a stereotaxic headholder (SG-4N, Narishige) and positioned the eye containing the engrafted islets facing upwards. The eyelid was carefully pulled back to hold the eye gently at the coreneoscleral junction with a pair of tweezers attached to a UST-2 Solid Universal Joint (Narishige). The tips of the tweezers were covered with a single piece of polythene tubing, creating a loop between the two tips. This arrangement permitted a flexible but stable fixation of the head and eye without causing damage or disrupting the blood circulation in the eye. For imaging, an upright Leica TCS SP8 DM6000 CFS confocal microscope (Leica Microsystems) and lasers for two-photon excitation [132], together with long-distance water dipping lenses (Leica HXC APO 10X0.3w) is used for imaging. Filtered saline or Viscotears® Liquid Gel (Alcon, US) were used as immersion liquid. Confocal LSM imaging is performed employing the minimum required laser-power and scan-time necessary. No signs of photo-damage in islet cells was observed during the work with this experimental model. To de-noise the images captured with confocal LSM, wavelet filtering was used [86] and contrast enhancement is applied for display purposes to the images. Leica Confocal Software (version 3.2.1.9702), and ImageJ were used to process images.

3.3.4.1 Islet Rejection and Survival Quantification

The 3D volume of islet was measured based on confocal images taken at designated time points using ImageJ [133, 134]. Briefly, the images were filtered using 3D median method with proper radius. The islet object was segmented from the filtered image using carefully set thresholds. The volume was calculated based on the segmented image over multiple Z-stacks at a step size of 3 μm . Volume measure on day 3 was set as the maximum volume used to normalize the volume change.

3.4 Drug-loaded Janus Particle Formulation Development

3.4.1 Materials

Glibenclamide and tolbutamide were purchased from Alfa Aesar, Germany. PLGA (PLGA 50:50, IV=0.2) polymers were obtained from Corbion Purac. PCL (MW 10 kDa), PVA (87-90% hydrolyzed, MW 30-70 kDa) and Trypan Blue (powder) were purchased from Sigma Aldrich. Lidocaine (base form) was obtained from Sigma. Dulbecco's PBS (without Ca and Mg, sterile) was bought from GE Healthcare. Methylene chloride (Tech Grade) was purchased from Aik Moh Paints & Chemicals Pte Ltd. Rhodamin B ($\geq 95\%$, HPLC) and coumarin 6 (98%) was purchased from Sigma Aldrich. Same rapamycin from section 3.1.1 was used.

3.4.2 Drug-loaded Janus Particle Fabrication

One-step oil-in-water emulsion solvent evaporation method was used to prepare drug-loaded core-shell or the Janus particles. PLGA and PCL compositions were dissolved in 3ml methylene chloride according to 10% weight-to-volume ratio (w/v). The polymer weight corresponding to each mass ratio used was specified in Table 3.1. The loading of glibenclamide, tolbutamide, trypan blue, rapamycin and lidocaine per formulations was either 2% or 10% weight-to-volume ratio (w/v). This mixture was stirred until thorough dissolution of both loading agent and polymer. The water phase was 0.5% (w/v) PVA dispersed in deionized water. Subsequently, emulsification was conducted at an oil-to-water ratio of 0.012, using overhead stirring at 400 rpm for three hours at room temperature and in absence of light. To wash away excess PVA and other impurities, the particles were vortexed and rinsed with deionized water for a total of five cycle. Water was removed by lyophilization to obtain the dry microparticles. Samples were stored in -20°C freezer for storage

Table 3.1 Weight of PLGA and PCL for each mass ratio used for fabrication. The size exclusion chromatogram measured M_n and M_w of PLGA, PCL polymer

Name	M_n	M_w	Density (g/mL)	Mass Ratio 20:10	Mass Ratio 19:11	Mass Ratio 15:15
PLGA	8715.7	13092.3	1.34	200 mg	190 mg	150 mg
PCL	12758.3	19118.0	1.15	100 mg	110 mg	150 mg

3.4.3 Interfacial Tension Measurement

Phase 1 (PLGA in DCM), Phase 2 (water with 0.5% PVA) and Phase 3 (PCL in DCM) were prepared separately according to the concentration used in fabrication. The interfacial tension of Phase 1 in Phase 2 was measured by pendent drop method using drop shape analysis from contact angle measuring system Contact Angle DataPhysics (OCA 15Pro). A droplet of Phase 1 was immersed in Phase 2, and the droplet shape was balanced in by gravity and surface tension. The build-in software measures droplet dimension and calculates the interfacial tension based on Young's Laplace relationship. Same steps were repeated for the interfacial tension between Phase 2 and Phase 3. The densities of Phase 1, 2, 3 were measured separately for the interfacial tension. A different approach was used to measure the interfacial tension between Phase 1 and Phase 3. Two standard solution cyclohexane and water were used to measure its contact angle with PLGA and PCL films. The interfacial energy was calculated based on the contact angle. Detailed calculation was shown in Table 6.3 (Section 6.2.3). All measurements were triplicates.

3.4.4 Particle Characterization

Freshly fabricated particles were observed under light microscope (Olympus CX21, 10x lens). Optical images were taken from eyepiece

using camera. Microparticles morphology, cross-section and size were analyzed using SEM with the same technique described in section 3.1.3.1. To differentiate PLGA from PCL polymer under SEM, particles were treated with acetone after mounted onto carbon tape. The acetone treatment dissolves PLGA in a short time, leaving PCL intact.

3.4.4.1 Confocal Raman Microscope

Characterization and distribution of polymer and drug glibenclamide were performed using Confocal Raman Microscope (CRM). Samples were mounted on a double sided tape and secured onto glass slides on the stage. To reduce background noise, measurements were done near the top surface of the particle. Microscopy system from WITe Instruments Corporation (Savoy, IL), a diode laser 785nm excitation) and a single monochromator (Acton) employing a 300 groove/mm grating and a thermoelectrically cooled 1024 by 128 pixel array CCD camera (Andor Technology) was used. Using the “Raman Spectral Imaging” mode of the instrument, Raman spectrums were obtained by scanning the sample at each polymer region for 180 scans each time. For presentation of the confocal Raman data, images are generated based on the unique properties of the spectra (i.e. integration of a Raman band, band height intensity, or band width). Typical laser power was set as 100 mW. All experiments were conducted with a plan achromat objective, 20x, Numerical Aperture 0.4 (Nikon). An average spectra was calculated using the software build-in algorithm.

3.4.4.2 Laser Scanning Confocal Microscope

A Zeiss LSM 800 inverted Laser Scanning Confocal Imaging System (Carl Zeiss, Oberkochen, Germany), equipped with Diode laser and Airyscan 32-GaAsP array Super-Resolution detector were used to investigate the structure of the microparticles. All confocal fluorescence pictures were taken with a 20x objective. The software used for the CLSM imaging was ZEN (blue edition) system. The imaging was performed with dry microparticles or particle dispersions in glycerol to

prevent their collapse during sample imaging. The laser was adjusted in the green/red fluorescence mode, which yielded two excitation wavelengths at 488 and 561 nm. Green and red fluorescence images were obtained from two separate channels, with the option of a third picture from the transmitted light detector.

3.4.5 *In vitro* Drug Release Study

5 mg of glibenclamide-loaded microparticles were suspended in vials containing 50 mL DPBS solution. Triplicates (n=3) were prepared for each polymer formulation and drug-free microparticles formulation was included as control for the release study. For up to 30 days, the samples were kept in an incubator with a rotating speed of 50 rpm and maintained at 37°C to simulate normal body condition. At a pre-determined time points, 10 mL of the supernatant was collected from the samples and replaced with a fresh medium of same volume to maintain the overall volume of release as well as the sync condition. The glibenclamide inside the 10 ml release medium was extracted using methylene chloride, dried under vacuum, and dissolved using methanol for HPLC analysis. The HPLC analysis method used 55% methanol and 45% water as mobile phase with 1ml/min flow rate. The drug retention time was 8 min.

3.5 References

- [86] S. Speier, D. Nyqvist, M. Kohler, A. Caicedo, I. B. Leibiger, and P. O. Berggren, "Noninvasive high-resolution in vivo imaging of cell biology in the anterior chamber of the mouse eye," *Nat Protoc*, vol. 3, no. 8, pp. 1278-86, 2008.
- [118] J. Li, S. G. Kim, and J. Blenis, "Rapamycin: one drug, many effects," *Cell Metab*, vol. 19, no. 3, pp. 373-9, Mar 4 2014.
- [128] W. L. Lee, C. Loei, E. Widjaja, and S. C. J. Loo, "Altering the drug release profiles of double-layered ternary-phase microparticles," *Journal of Controlled Release*, vol. 151, no. 3, pp. 229-238, May 2011.
- [129] M. P. A. Lim, W. L. Lee, E. Widjaja, and S. C. J. Loo, "One-step fabrication of core-shell structured alginate-PLGA/PLLA microparticles as a novel drug delivery system for water soluble drugs," *Biomaterials Science*, vol. 1, no. 5, pp. 486-493, 2013.
- [130] G. I. Kirchner *et al.*, "Simultaneous on-line extraction and analysis of sirolimus (rapamycin) and ciclosporin in blood by liquid chromatography–electrospray mass spectrometry," *Journal of Chromatography B: Biomedical Sciences and Applications*, vol. 721, no. 2, pp. 285-294, 1999/01/22/ 1999.
- [131] R. Bottino *et al.*, "Transplantation of allogeneic islets of Langerhans in the rat liver: effects of macrophage depletion on graft survival and microenvironment activation," (in eng), *Diabetes*, vol. 47, no. 3, pp. 316-23, Mar 1998.
- [132] D. Nyqvist *et al.*, "Donor Islet Endothelial Cells in Pancreatic Islet Revascularization," *Diabetes*, vol. 60, no. 10, pp. 2571-2577, 2011.
- [133] T. Boudier and J. Ollion, "3D ImageJ Suite.," *ImageJ Documentation Wiki*, 2012.
- [134] J. Ollion, J. Cochennec, F. Loll, C. Escude, and T. Boudier, "TANGO: a generic tool for high-throughput 3D image analysis for studying nuclear organization," (in eng), *Bioinformatics*, vol. 29, no. 14, pp. 1840-1, Jul 15 2013 .

4 Development of Controlled Releasing Microparticle Formulation for Preserving the Anti-cancer and Immunosuppressive Properties of Rapamycin¹

4.1 Introduction

Rapamycin is one of the most commonly used immunosuppressive agent. It inhibits the mammalian target of rapamycin (mTOR) signal pathway making it a promising anti-cancer agent at the same time. One setback of rapamycin, however, is its highly hydrophobic nature leading to poor water solubility [135, 136]. The physicochemical property of this, otherwise potent, drug poses a challenge to deliver it via the conventional administration route of oral or injection [137]. Furthermore, rapamycin is known to degrade rapidly in an aqueous environment [107, 138]. Its degradation accompanies the opening of its macrolide structure, which alters the binding sites therefore losing its activity [106, 139, 140].

Encapsulation, therefore can not only overcome the solubility related limitation of rapamycin, but also provide a means to control and sustain its release [111, 141], thus maintaining the minimum dosage required for adequate therapy [142]. Furthermore, encapsulation can entrap water-sensitive drugs within a hydrophobic polymer matrix to limit their exposure to the aqueous environment [143].

Several attempts to deliver rapamycin using particles have thus been reported. However, stability of rapamycin delivered through these delivery systems has neither been explored nor discussed extensively. Despite the known instability of rapamycin, currently available literature rarely reports on the functionality of rapamycin after its

release. For a highly unstable drug like rapamycin, it is essential to know whether the encapsulated drug remains intact over the delivery period, and if not, to propose strategies to maintain its bioactivity. This is especially crucial when *in vitro* experiments are translated to more complex *in vivo* studies, where the preservation of unstable drugs becomes critical in clinical applications [144].

In this chapter, the aim is to develop a drug delivery system (DDS) that allows for a sustained delivery of bioactive rapamycin and to evaluate its effect on cancer and immunological cell lines. First, the instability of rapamycin in Dulbecco's phosphate buffer saline (DPBS) was established. Subsequently, rapamycin was encapsulated into FDA-approved biodegradable polymeric particles of various formulations (using single or two polymers) and their release profiles were investigated. Lastly, the bioactivity of released rapamycin was measured using breast cancer cell (MCF-7) and human lymphocyte cells (Jurkat) in terms of its anticancer and immunosuppressive properties. Comparison of the release profile and cell response was assessed for future translational studies.

¹Reproduced from [Y. L. Fan, H. W. Hou, H. M. Tay, W. M. Guo, P. O. Berggren, and S. C. Loo, "Preservation of Anticancer and Immunosuppressive Properties of Rapamycin Achieved Through Controlled Releasing Particles," AAPS PharmSciTech, Mar 01 2017.] with permission from Springer.

4.2 Results

4.2.1 Rapamycin Released from Microparticle Formulations

Rapamycin was encapsulated into seven different microparticle (MP) formulations using an emulsion evaporation method with encapsulation efficiencies (EE) close to 100% (Table 4.1). These values were consistent with the higher values reported in the literature due to the hydrophobic nature of rapamycin that allows strong interactions with the hydrophobic polymer [123]. All the formulations had similar loading values of rapamycin (1.3% w/w by HPLC) and particle diameter (100 μm on average). The SEM images (Figure 4.1) showed that the MPs had smooth surface morphology, and cross-sectional images revealed a mono-layered structure for single polymer MP and a double-layered structure for MP composing of two polymers. For the latter, PCL/PLGA21 MP (Figure 4.1C) revealed a thicker shell than PCL/PLGA11 MP (Figure 4.1D), when the weight ratio of PCL increased from 50% to 66.7%. This suggested that PCL (Figure 4.1G) formed the shell, which was consistent with previous reports [128]. It is noteworthy that, unlike other formulations, PCL MP (single polymer) presented a porous structure with numerous 1-2 nm size pores (Figure 4.1H) that were randomly distributed throughout the particle.

The effect of PLGA polymer molecular weight and end-group on rapamycin release is shown in Figure 4.2. Negligible amount of rapamycin was released from PLGA-HMW MP whose MW was the highest among all PLGA used in this study. This was because for degradation-controlled releasing polymers, significant polymer degradation has to precede drug release. The different onset of rapamycin release between PLGA-LMW (Figure 4.2B) and PLGA (Figure 4.2A) can therefore be explained from its MW difference (Table 4.1). Rapamycin release was also significantly altered when different end-groups were present in the polymer, i.e. PLGA-Acid (Figure 4.2B). Based on polymer end-groups, PLGA-LMW (ester-terminated) was

observed to have a slower release compared to PLGA-Acid (acid-terminated), even though the former had a lower MW value (Table 4.1). Release rates are therefore also dependent on the end-groups in PLGA. The higher amount of release was due to the high water uptake for acid end-capped polymers that promotes drug diffusion. In addition, polymers with acidic end-groups accelerate degradation via autocatalysis.

Table 4.1 Microparticle formulations with its diameter, drug loading and encapsulation efficiency. Values listed are the average of two independent batch of particles in triplicates.

Formulation	Polymer M _w	Diameter ± SD (µm)	Drug Loading	Encapsulation Efficiency ± SD
PLGA-HMW	153000	85 ± 20	1.33%	102.6% ± 4.4%
PLGA	11427.5*	87 ± 27	1.33%	95.77% ± 1.15%
PLGA-LMW	9950.2*	90 ± 27	1.33%	103.94% ± 1.12%
PLGA-Acid	7,000-17,000	77 ± 28	1.33%	101.79% ± 3.38%
PCL	14000	122 ± 28	1.33%	103.73% ± 1.1%
PCL/PLGA21	-	143 ± 28	1.33%	88.53% ± 1.61%
PCL/PLGA11	-	98 ± 25	1.33%	92.89% ± 6.37%

* Values were measured using GPC. The rest of molecular weights were obtained from supplier

It has been reported that double-layered particles reduce burst release and promote sustained delivery [129]. Here, the effect of double-layered MP on rapamycin release was also investigated. PCL and PLGA polymers were blended at ratios of 1:1 (PCL/PLGA11) and 2:1 (PCL/PLGA21), and both formulations showed a release rate in between that of pure PCL (rapid release) and pure PLGA (negligible release). The formulation with a higher PCL content (PCL/PLGA21)

displayed a relatively more rapid release rate (Figure 4.2C). Blending PCL with PLGA slightly accelerated the release as compared to pure PLGA-HMW in PLGA.

Figure 4.1 SEM images of microparticle cross section and surface morphology. A single uncut microparticle was shown on the top right corner for better illustration of the surface morphology. A) PLGA, B) PLGA-Acid, C) PCL/PLGA21, D) PCL/PLGA11, E) PLGA-HMW, F) PLGA-LMW, G) PCL, H) Pores on PCL particle. All particles were solid, spherical particles. Pure polymer yielded single layer structure while blend of polymer resulted in double layer structure

Figure 4.2 Cumulative release of rapamycin from different microparticle. The microparticles are PLGA-HMW (■), PLGA (■), PLGA-LMW (□), PLGA-Acid (◆), PCL (▲), PCL/PLGA21 (△) and PCL/PLGA11 (●). To aid comparison, all release profiles were grouped into A) degradation controlled release, B) effect of polymer MW and end-cap group and C) effect of polymer blend (means $\pm$ SD, n=3).

4.2.2 MP Formulations Preserve Bioactivity of Rapamycin

The degradation rate of free rapamycin under *in vitro* conditions was investigated (Figure 4.3A). Rapamycin of varying concentrations (1.0-25 $\mu\text{g/ml}$) was exposed to DPBS over 72 hr. At low solution concentration (1 $\mu\text{g/ml}$), free rapamycin was no longer detectable after 24 hr. Higher concentrations (≥ 4 $\mu\text{g/ml}$) showed that $\sim 70\%$ of rapamycin was degraded after 24 hr. At 48 hr, only 10% of rapamycin remained intact, and was fully degraded by 72 hr. A degradation peak can be identified in HPLC chromatographs (Figure 4.4A). The MS analysis confirmed the intact rapamycin HPLC peak is at 6.6-6.7 min retention time. The corresponding degradation rate constants were

calculated from the trend line (Figure 4.5), whereby all four concentrations had an average value of $55.4 \pm 1.7 \mu\text{g}/(\mu\text{l} \cdot \text{hr})$.

The amount of intact rapamycin that was released from the MP formulations at 72 hr was thus monitored (Figure 4.3B). PLGA-HMW MP displayed the best results whereby 100% of the encapsulated rapamycin remained intact. PLGA-Acid MP had the second highest amount of intact rapamycin (80.40%). If the amount of released rapamycin was taken into account, this value would have increased to 91.5%. PCL MP preserved only 64.1% of rapamycin, probably due to the highly porous structure of PCL MP. Nevertheless, contrary to free un-encapsulated rapamycin (Figure 4.3B), rapamycin encapsulated in MP were well preserved in all seven formulations.

Figure 4.3 Degradation of rapamycin in aqueous condition and MP. A) Degradation of free rapamycin at seven concentrations (25 $\mu\text{g/ml}$ to 1 $\mu\text{g/ml}$) over 72 hr (means $\pm$ SD, $n=3$). After 72 hr, all free rapamycin was fully degraded. B) Measured amount of intact rapamycin encapsulated in PLGA-

HMW over 30 days at the same condition of release study. C) Amount of intact rapamycin inside all formulation MP after 72 hr. All formulation showed > 70% intact rapamycin was remained inside each microparticle.

Figure 4.4 Chromatography and mass spectrometry profiles of Rapamycin. A) HPLC Chromatograph of rapamycin HPLC peak at retention time (RT) 6.7 – 6.8 min was intact rapamycin peak. HPLC peak at RT 3.87 min appeared only in degraded rapamycin sample, therefore it was degraded rapamycin peak. B) Rapamycin released from microparticle *in vitro*. Peak at RT 6.4 min were intact rapamycin peak. Multiple peaks at RT 2-4 min were degraded rapamycin peaks as they degrade over different time. C) Mass Spectrum of Rapamycin. MS peak at 936.44 was corresponding to rapamycin (914.14) with an added sodium ion (22.9). D) Residue rapamycin from microparticles *in vitro* release. Only intact rapamycin peaks (RT 6.4 min) were observed

$$\ln Y_x = \ln Y_0 + 0.05545x$$

Figure 4.5 Degradation rate of rapamycin in aqueous condition.

Degradation rate of rapamycin was calculated by the exponential relationship of rapamycin amount (HPLC area under curve) vs time x . Y_0 is initial amount of rapamycin. The correlation equation is $\ln Y_x = \ln Y_0 + 0.05545x$. Concentration of rapamycin solution tested are 25 µg/ml; 18 µg/ml; 12 µg/ml; 8 µg/ml.

Formulation PLGA-HMW MP was subsequently selected to understand the stability of encapsulated rapamycin over a month, without any interference of rapamycin release. The amount of intact rapamycin in PLGA-HMW MP (Figure 4.3C) remained close to 100% for up to 30 days, with the residue amount of rapamycin at approximately 95%. This suggests that encapsulating rapamycin into delivery systems, such as MP, could preserve its bioactivity. This result was further confirmed as the degraded rapamycin peak was not observed in HPLC when analyzing for residue rapamycin (Figure 4.4).

4.2.3 Inhibition Efficacy of Released Rapamycin on Cell Lines

When rapamycin degrades, it loses its binding site and therefore its ability to suppress cell proliferation. The inhibition efficacy of released rapamycin from MP on breast cancer cell MCF-7 proliferation was thus assessed (Figure 4.6). PLGA MP showed extremely low inhibition effect ($\leq 5\%$) on MCF-7 cells for the initial 15 days. This agrees with the 0% rapamycin released till day 10 (Figure 4.2A). For a mid-term period (i.e. day 10 to day 16), the amount of released rapamycin from PLGA MP increased. Eventually it reached a peak at day 23. Likewise, the inhibitory effect on MCF-7 cells increased substantially (36.71% on day 19-23), which is in good agreement with the *in vitro* release profile. Formulation PLGA-Acid MP showed the highest cell proliferation inhibition efficacy among all formulations (Figure 4.6B). During the initial first 15 days, the inhibition efficacy was as high as 30% - 40% on average. However, it fell in the mid-term to below 20%. Such a trend matched that of its *in vitro* release profile (Figure 4.2B). For PCL MP formulation, inhibition efficacy on MCF-7 cell was the highest (28.5%) at day 0-4, which subsequently decreased to about 4%, as predicted from its diffusion controlled release profile (Figure 4.2C). All these results demonstrated a good correlation between rapamycin release and cell inhibition. The same experiment was conducted on other formulations (PLGA-LMW, PCL/PLGA11 and PCL/PLGA21), which yielded similar results (Figure 4.6). The correlation of MP *in vitro* release profile and cellular response confirmed the bioactivity of released rapamycin.

Figure 4.6 Inhibition efficacy of rapamycin microparticle on breast cancer cell (MCF-7) proliferation over 30 days. A) PLGA, B) PLGA-Acid, C) PCL. D) PLGA-LMW, E) PCL/PLGA11, F) PCL/PLGA21 *In vitro* released amount of rapamycin at corresponding time point was plotted on the right y-axis (means $\pm$ SD, n=4).

As PLGA-Acid MP and PLGA MP showed the highest release profile among all formulations, they were selected to investigate the impact on Jurkat cell proliferation against equivalent dosage of free rapamycin. The fold change of cell numbers based on metabolic activity was measured when incubating Jurkat cells with the two MP at two different dosages of rapamycin (1.5nM and 3nM). Cells incubated with blank MP displayed similar proliferation as healthy Jurkat cells (control) (Figure 4.7), indicating no inhibitory effect at both dosages. In the first 5 days, free rapamycin reduced Jurkat cell growth at both 1.5nM and 3nM (Figure 4.7, left panel). However, the fold change increased to 18.6 in mid-term period of release and became similar to that of the healthy Jurkat cells (20.9). This indicated that free rapamycin suppressed cell growth for only 5 days. At the 1.5nM and 3nM dosage, the fold change of both PLGA-Acid MP and PLGA MP were significantly lower in comparison to free rapamycin at all-time points (Figure 4.7). In addition, both formulations suppressed cell growth for 20 days, equivalent to four times the duration of free rapamycin. This clearly

demonstrates that the rapamycin sustained release from MP had high efficacy and longer duration in inhibiting Jurkat cell growth.

Figure 4.7 Proliferation of Jurkat cell with microparticle formulation PLGA (■), PLGA-Acid (◆), free drug rapamycin (●), blank microparticle (●) and Healthy (●, dash line) as the control. Fold change was calculated using CCK in log 2 for A) 1.5nM and B) 3nM dosage (means $\pm$ SD, n = 3). * P < 0.05 against blank.

4.3 Discussion

The multifunctional drug, rapamycin, has an increasing importance in the rapidly-growing area of cancer research as it works synergistically with other chemotherapeutic drugs, such as doxorubicin or cyclophosphamide [145]. Rapamycin is water sensitive owing to the hydrolysis at the ester bond [106]. The degradation rate of rapamycin in DPBS (Figure 4.3A), as reported for the first time here, revealed how rapidly rapamycin degrades when exposed to an aqueous medium. Encapsulating rapamycin into hydrophobic polymers as drug delivery carriers could therefore overcome the instability of rapamycin. Microparticle formulations also provide controlled and sustained release capabilities, in addition to protecting the drug. The results (Figure 4.3B

and 3C) confirmed the ability of MP in preserving rapamycin. It was speculated that the shielding effect provided by the polymers restricts the interaction of rapamycin with the aqueous environment, thus prolonging the bioactivity of any water unstable drugs, in this case, rapamycin. At the same time, the major drawbacks of cancer treatment such as toxicity to healthy tissue and severe side-effects can be potentially minimized with the help of biodegradable carriers that provide controlled and sustained release capabilities. It was demonstrated that rapamycin can be encapsulated using FDA-approved biopolymer PLGA and PCL at high loading efficiencies (Table 4.1), and a library of these formulations was developed with different release onset period, rates and profile.

Different formulations showed different release profiles and kinetics, whereby pure PCL and PLGA formulations showed diffusion-controlled and degradation-controlled release mechanisms, respectively. By increasing the polymer MW, i.e. PLGA-LMW and PLGA, the onset of rapamycin release can be delayed. This unique feature can be useful when a delayed release is required. For instance, APF530 is a delayed release formulation of granisetron, which showed better health-related quality of life in terms of chemotherapy induced nausea and vomiting [146]. Various end-groups also affected release rates, with the acid end-groups displaying a more rapid release. Blending PCL with PLGA gave rise to double-layered MPs whereby a higher PCL content predictably gave faster release rates. Theoretically, it is therefore possible to customize the release onset period, kinetics and profile by varying the polymer MW, type, end-groups, and MP composition.

Living cells provide immediate reaction to rapamycin that has been released. Two types of cell lines were selected to evaluate the bioactivity (as an immunosuppressant and as an anti-cancer drug) of released rapamycin. Blank MP showed no inhibition effect on MCF-7 cell (data not shown). In contrast, all rapamycin MP displayed various

levels of inhibition efficacies (Figure 4.6) that matches the respective rapamycin MP release profiles (Figure 4.2), indicating the direct inhibitory effect of rapamycin on MCF-7 cell proliferation. The incubation time of rapamycin MP with Jurkat cells was set to be long enough to determine the difference between the free drug and MP in cell growth experiments. Clearly, rapamycin MP were able to suppress cell growth for up to 20 days, equivalent to four times the duration of the free drug (Figure 4.7). Zhao et al reported antiproliferative effects of rapamycin on Jurkat cells for up to 3 days of incubation duration [147]. Similarly, another study, assessing the effect of rapamycin carrier on immune cells, incubated T-cells for up to 4 days [123]. Such durations may be too short for any rapamycin effect on cell proliferation. On the contrary, the results shown here confirmed the long-acting effect of rapamycin through a MP formulation. MP not only protected the drug but also provided sustained cellular responses. MP therefore provide a means to tune the release of rapamycin, whilst preserving the bioactivity of hydrolytically unstable drugs.

The application of rapamycin MP can be explored in various areas where a sustained release of rapamycin is desired. For example, the effect of free rapamycin in inhibiting breast cancer cell proliferation was evaluated at different dosages [145]. A low dosage of rapamycin significantly blocks breast cancer cell proliferation whereas high dosages may be cytotoxic. Furthermore, the continuous fusion of rapamycin was shown to be more effective than a low bolus dosage [148]. The MP formulations developed in this thesis can be used to deliver rapamycin in a continuous manner, and at lower dosages over a significantly longer duration than free drug. Clinically, direct injection of rapamycin also has its difficulty, whereby the concoction for rapamycin injection often consist of polymer surfactant (carboxymethyl cellulose and/or polyethylene glycol), which makes the solution viscous and difficult to inject [149-152]. Besides that, trace amounts of organic solvent cannot be avoided if the solution is prepared by diluting from a concentrated

organic solution with rapamycin. With the appropriate MP formulation, rapamycin, in its particulate form, is much easier to inject and leaves no trace of organic solvent that could potentially harm tissues. With additional benefits of drug preservation, high encapsulation efficiency and longer delivery duration, rapamycin MP is overall a superior form of delivery.

4.4 Summary

Free drug rapamycin was shown to degrade rapidly under aqueous conditions. However, such a degradation was minimized after encapsulation in MP. A library of original MP formulations was developed and shown to preserve and sustain the release of rapamycin for up to 30 days. Various approaches of modification demonstrated the ability to tune rapamycin release profile to fit different applications. Incubation of rapamycin MP with two different cell lines clearly confirmed the sustained release of rapamycin and improved efficiency compared to free drug. Thus, sustained delivery of rapamycin and its derivatives can potentially lead to improved treatment of renal cancer carcinoma, breast cancer and progressive neuroendocrine tumors of pancreatic origin. It can also act as an immunosuppressive agent in autoimmune diseases and neurodegenerative diseases.

4.5 References

- [106] A. R. Oyler *et al.*, "Forced degradation studies of rapamycin: identification of autoxidation products," *J Pharm Biomed Anal*, vol. 59, pp. 194-200, Feb 5 2012.
- [107] G. M. Ferron and W. J. Jusko, "Species Difference in Sirolimus Stability in Humans, Rabbits, and Rats," *Drug Metabolism and Disposition*, vol. 26, pp. 83-84, 1998.
- [111] N. K. Varde and D. W. Pack, "Microspheres for controlled release drug delivery," (in English), *Expert Opinion on Biological Therapy*, Review vol. 4, no. 1, pp. 35-51, Jan 2004.
- [123] S. Jhunjhunwala *et al.*, "Controlled release formulations of IL-2, TGF-beta1 and rapamycin for the induction of regulatory T cells," *J Control Release*, vol. 159, no. 1, pp. 78-84, Apr 10 2012.
- [128] W. L. Lee, C. Loei, E. Widjaja, and S. C. J. Loo, "Altering the drug release profiles of double-layered ternary-phase microparticles," *Journal of Controlled Release*, vol. 151, no. 3, pp. 229-238, May 2011.
- [129] M. P. A. Lim, W. L. Lee, E. Widjaja, and S. C. J. Loo, "One-step fabrication of core-shell structured alginate-PLGA/PLLA microparticles as a novel drug delivery system for water soluble drugs," *Biomaterials Science*, vol. 1, no. 5, pp. 486-493, 2013.
- [135] P. Simamora, J. M. Alvarez, and S. H. Yalkowsky, "Solubilization of rapamycin," *International Journal of Pharmaceutics*, vol. 213, no. 1-2, pp. 25-29, 2001.
- [136] R. T. Abraham, J. J. Gibbons, and E. I. Graziani, "Chapter 17 - Chemistry and Pharmacology of Rapamycin and Its Derivatives," in *The Enzymes*, vol. Volume 27, N. H. Michael and T. Fuyuhiko, Eds.: Academic Press, 2010, pp. 329-366.
- [137] P. Limited. (27 Oct 2015, 19 Feb). *Rapamune Summary of Product Characteristics*
- [138] F. Streit *et al.*, "Sensitive and specific quantification of sirolimus (rapamycin) and its metabolites in blood of kidney graft

- recipients by HPLC/electrospray-mass spectrometry," (in eng), *Clin Chem*, vol. 42, no. 9, pp. 1417-25, Sep 1996.
- [139] A. W. Thomson, H. R. Turnquist, and G. Raimondi, "Immunoregulatory functions of mTOR inhibition," (in English), *Nature Reviews Immunology*, Review vol. 9, no. 5, pp. 324-337, May 2009.
- [140] D. A. Holt *et al.*, "STRUCTURE-ACTIVITY STUDIES OF NONMACROCYCLIC RAPAMYCIN DERIVATIVES," (in English), *Bioorganic & Medicinal Chemistry Letters*, Article vol. 3, no. 10, pp. 1977-1980, Oct 1993.
- [141] A. Fahr and X. Liu, "Drug delivery strategies for poorly water-soluble drugs," (in eng), *Expert Opin Drug Deliv*, vol. 4, no. 4, pp. 403-16, Jul 2007.
- [142] B. Tyler *et al.*, "Local delivery of rapamycin: a toxicity and efficacy study in an experimental malignant glioma model in rats," *Neuro Oncol*, vol. 13, no. 7, pp. 700-9, Jul 2011.
- [143] R. Dinarvand, N. Sepehri, S. Manoochehri, H. Rouhani, and F. Atyabi, "Polylactide-co-glycolide nanoparticles for controlled delivery of anticancer agents," *Int J Nanomedicine*, vol. 6, pp. 877-95, 2011.
- [144] D. T. Leong and K. W. Ng, "Probing the relevance of 3D cancer models in nanomedicine research," *Advanced Drug Delivery Reviews*, vol. 79–80, pp. 95-106, 12/15/ 2014.
- [145] S. Chan, "Targeting the mammalian target of rapamycin (mTOR): a new approach to treating cancer," *Br J Cancer*, vol. 91, no. 8, pp. 1420-4, Oct 18 2004.
- [146] I. Schnadig, E. Braun, M. Mosier, R. B. Geller, and S. Lee, "Effect of APF530 on health-related quality of life (QOL) and other chemotherapy-induced nausea and vomiting (CINV) end points: Phase III MAGIC trial [abstract]," vol. 34, ed. Journal of oncology practice: 2016 American Society of Clinical Oncology Annual Meeting 2016.
- [147] Y. M. Zhao, Q. Zhou, Y. Xu, X. Y. Lai, and H. Huang, "Antiproliferative effect of rapamycin on human T-cell leukemia

- cell line Jurkat by cell cycle arrest and telomerase inhibition," *Acta Pharmacol Sin*, vol. 29, no. 4, pp. 481-8, Apr 2008.
- [148] M. Guba *et al.*, "Dosing of rapamycin is critical to achieve an optimal antiangiogenic effect against cancer," *Transpl Int*, vol. 18, no. 1, pp. 89-94, Jan 2005.
- [149] R. Gallo *et al.*, "Inhibition of intimal thickening after balloon angioplasty in porcine coronary arteries by targeting regulators of the cell cycle," (in eng), *Circulation*, vol. 99, no. 16, pp. 2164-70, Apr 27 1999.
- [150] V. M. Rivera *et al.*, "Long-term regulated expression of growth hormone in mice after intramuscular gene transfer," (in eng), *Proc Natl Acad Sci U S A*, vol. 96, no. 15, pp. 8657-62, Jul 20 1999.
- [151] S. R. Kimball, L. S. Jefferson, H. V. Nguyen, A. Suryawan, J. A. Bush, and T. A. Davis, "Feeding stimulates protein synthesis in muscle and liver of neonatal pigs through an mTOR-dependent process," (in eng), *Am J Physiol Endocrinol Metab*, vol. 279, no. 5, pp. E1080-7, Nov 2000.
- [152] H. Hackstein, T. Taner, A. J. Logar, and A. W. Thomson, "Rapamycin inhibits macropinocytosis and mannose receptor-mediated endocytosis by bone marrow-derived dendritic cells," (in eng), *Blood*, vol. 100, no. 3, pp. 1084-7, Aug 1 2002.

5 Local Release of Rapamycin by Microparticles Delays Islet Rejection within the Rodent Anterior Chamber of the Eye – An *In Vitro* and *In Vivo* Study

5.1 Introduction

Diabetes is a debilitating disease with high morbidity [1]. Poor glycemic control results in long-term complications such as blood vessel damage, nerve damage, kidney failure and cardiovascular disease from hyperglycemia [5]. Diabetes stems from the inability of the body to supply insulin to meet the metabolic demand. As a result, exogenous insulin supplementation is required for Type-1 diabetic and ‘C-peptide low’ Type-2 diabetic patients to maintain glucose homeostasis. Nonetheless, insulin injection has life-threatening side-effects such as acute hypoglycemia risk and chronic unnatural swings in blood glucose level [153]. As such, islet transplantation has emerged as a promising treatment for diabetes as it overcomes the deficient or inadequate insulin secretion in the most natural of ways without any of the associated side-effects seen with exogenous insulin [9, 11, 131].

Islet transplantation is now routinely done in many diabetic centers [12] and improvements to the protocol now see islets injected through hepatic portal vein site to the liver [95, 131]. However, little is known about islet graft function upon transplantation into the hepatic portal system. Low oxygen tension, sheer physical stress within the hepatic portal system and the induction of inflammation lead to significant islet dysfunction and perhaps even loss [154-157]. To compensate for this loss, many more islets need to be transplanted into a patient putting a real strain on donor adequacy. The ACE, as a novel site for islet transplantation, has unique advantages over the currently used hepatic

portal system. The ACE is a less invasive transplant site, and its relatively enclosed environment makes localized immunosuppression feasible [21]. The iris has a high concentration of blood vessels, which enables fast vascularization of the grafted islets, minimizing islet hypoxia and death. Besides, a transparent cornea allows easy imaging access to the islet grafts thereby enabling non-invasive, single-cell resolution, longitudinal imaging of the islet grafts [15]. Islets being transplanted to ACE have been reported to behave similarly to endogenous islets and islet grafts at this site have been shown to reduce insulin dependence in diabetic non-human primates significantly [21].

A further advantage of clinically transplanting islet into the ACE lies in its potential for local immunosuppression. Prior rodent studies have shown that an immune response towards the islet graft in the ACE starts approximately 7 days post-transplantation. By 14 to 21 days post-transplantation, the islet grafts are completely rejected, *sans* immunosuppression [15]. To keep the grafts from being rejected by the host, and to prevent cases of spurious autoimmunity sometimes seen in islet transplantation, lifelong systemic doses of immunosuppressive agents are required for the recipient patient.

Clinical pancreatic islet transplantation is not only limited by donor scarcity but also by recipient compatibility. For immunosuppression convenience, islet transplantation is carried out in patients who have received, or who are concurrently receiving, a kidney transplant [12]. A recipient's ability to be on lifelong systemic immunosuppression is a key determinant for transplant suitability. Immunosuppressive drugs circulate throughout the body and pose numerous challenges, not least being a compromised immune system to defend against a myriad of common infections. In addition, the presence of immunosuppressive agents in off-target tissues results in undesirable side-effects such as peripheral oedema and kidney dysfunction [158, 159]. To reduce patient burden, drug-load and undesirable side-effects, immunosuppressive

drug delivery need to be engineered better for prolonged duration, localized effect with tunable efficacy.

In this chapter, we report that sustained release of an effective dose of rapamycin (a widely used immunosuppressive agent for islet transplantation [101]) over 30 days was achieved based on microparticles formulation developed in Chapter 4. This microparticle formulation is safe and it does not impede islet function *in vitro*. When co-transplanted together with rodent islets into the ACE of an allogeneic recipient, these microparticles significantly delayed the graft rejection process *in vivo*. Furthermore, the rapamycin dose achieved locally through our microparticle formulation is significantly lower as compared to the current systemically delivered clinical dose, yet the therapeutic effect achieved was the same between the two. Results here suggest that the local delivery of immunosuppressive agents through bioengineered microparticles is an effective clinical strategy to prevent islet rejection and yet mitigate challenges posed by such biologically harsh agents. We envisage this procedure will relieve patients from the damage of systemic immune suppression, in the meanwhile, improve glycemic control with reduced insulin dependence and avoid hypoglycemic episodes.

5.2 Results

In order to determine if microparticles can be employed to release rapamycin in an effective dose, the abilities of FDA-approved biodegradable polymer PCL and PLGA of encapsulating and releasing rapamycin was accessed to establish the *in vitro* release profile. It was shown that the release of rapamycin was not adquet when the molecular weight of PLGA and PCL were high [160]. Hence, low molecular weight PCL and PLGA were selected for microparticle formulation because of their characteristics generally exhibiting higher release.

5.2.1 Effective Dosing of Rapamycin Released from Biodegradable Microparticles *in vitro*

In order to evaluate the ability of microparticle to release rapamycin, particle morphology, including surface smoothness, cross-section and pores was closely examined. Scanning electron microscope showed PCL microparticle had certain concaved area on the surface (Figure 5.1A). Multiple micron to submicron size pores were randomly distributed at the surface as well as the cross-section. These small pores penetrate from the surface to the core of particle (Figure 5.1A), creating multiple channels to facilitate water uptake and drug diffusion. As a result, the release of rapamycin in phosphate buffer (PBS) medium surged to 0.13 μg per day on day 1 (Figure 5.1B). Afterwards, the daily release dose decreased gradually over time and stopped at day 30. Rapamycin loaded PLGA microparticle, on the other hand, displayed solid nonporous spherical structure (Figure 5.1A). It started to release rapamycin only after 10 days in the PBS medium. The daily release dose kept increasing from day 10 to day 18. After reaching the peak value of 0.197 μg per day, the daily release decreased until below detection limit at day 30. Hence, the effective duration, where daily released dose is higher than the required dose (20nM, 5.2ng/day, Table 5.1), is different for the two types of microparticle. The duration for PCL microparticle is 15 days from the beginning, while the effective duration for PLGA microparticle starts from day 10 to day 30. Therefore, a mixture of both types of microparticle could extend the effective duration.

Figure 5.1 Rapamycin microparticles and its release *in vitro*. Surface morphology and cross-sectional views of rapamycin in PCL microparticle (A, left column) and rapamycin in PLGA microparticle (A, right column) under scanning electron microscope (SEM). Daily released rapamycin amount measured from individual microparticle (B) showed PCL (■) released drug immediately while PLGA (●) had a 10-day delay in release onset. Mixture of these two microparticles in 1:1 showed a sustained release over 30 days (▼) at a daily dose greater than 20 nM (●) (mean $\pm$ SD, n=3) in PBS (C) and balanced salt buffer (D).

Table 5.1 Mass of rapamycin calculated from mice ACE volume & target concentration of 20nM

Target Concentration	Rapamycin M _w	Volume* per Day	Mass
20 nM	914.2 daltons	5.9 μ l x 2 times/hr x 24 hr = 283.2 μ L	5.2 ng

*The volume of aqueous humor in ACE is 5.9 μ l. And it is replenished completely every 30-40 mins. Hence we estimated the volume of aqueous humor per hour equals to 5.9 μ l x 2 times/hr

To meet the required dose for entire 30 days, a mixture of both PCL and PLGA microparticle at 1:1 ratio was tested for release in phosphate buffer (PBS) (Figure 5.1C). The release profile of the microparticle

mixture displayed two peaks at day 0 and day 18, which corresponds to the maximum daily release rate observed in individual release profiles (Figure 5.1B). The proposed hypothesis was confirmed when the mixture released rapamycin at a daily dose higher than the required dose of 20nM throughout the 30 days. To ensure the targeted dose can be achieved in animal work, we repeated the release study in another buffer medium: balanced salt solution (BSS). BSS is an intracocular irritation solution with a composition similar to the aqueous humor solution [161]. Therefore, microparticle release profile in BSS will provide a better prediction of the actual release *in vivo*. To ensure the accuracy of *in vitro* release profile of rapamycin, we compared the daily release dose of microparticle mixture in PBS and BSS. The time point of daily release peak was same, and the cumulative release and daily release dose at each time point had similar value between PBS and BSS, except that fluctuation of daily release dose was less in BSS.

Despite the immunosuppressive capability, it has been shown previously that rapamycin plays the deleterious roles on the islets [108, 162]. To evaluate the toxicity of microparticle and rapamycin dose encapsulated on islets function, glucose-stimulated insulin secretion (GSIS) of islets was conducted (Figure 5.2). Islets were incubated with rapamycin microparticle mixture (1:1 ratio), blank microparticle mixture and the equivalent amount of free drug for 24 h before the GSIS test. GSIS from the islets treated with blank microparticle was no significantly different from the non-treated islets (NT), indicating that microparticle vehicle has no toxic effects to the islets. Furthermore, rapamycin at 20nM dose did not reduce the fold change in terms of glucose stimulation index (Figure 5.2B), indicating the selected dose will not be compromise islets functionality. Similarly, rapamycin microparticles did not show any reduction effect on the islets insulin secretion ability.

Figure 5.2: Effect of rapamycin microparticles and blank microparticles in glucose-stimulated insulin secretion (GSIS) *in vitro* of islets. A) Aliquots of 3-5 hand-picked islets were culture in RPMI-1640 medium in the absence of rapamycin (NT) and in the presence of 20nM rapamycin microparticle (Rapa MP), blank microparticle (Blk MP) and 20nM free drug (Control) for 24 h, followed by shifting glucose concentration of culture media from 3mM to 16mM. Thirty minutes later, aliquots (100ul) of media were sampled for insulin content assay using ELISA. *P < 0.05. **P < 0.005. □, basal, ■, induced. B) Fold change of insulin level were compared across the different treatments stated in A). Difference measured was not significant (ns). Bars indicate mean $\pm$ SEM.

5.2.2 Rapamycin Microparticles Delay Islet Graft Immune Rejection *in vivo*

To determine the effect of rapamycin microparticles is suppressing islet rejection *in vivo*, rapamycin-releasing microparticles were co-transplanted with islets into the right ACE of an allogeneic recipient mouse. Concurrently, blank, control microparticles were co-transplanted with similar number of islets obtained from the same mouse into the left ACE of the recipient mouse. Subsequently, both transplanted islets and microparticles were longitudinal monitored up to 40 days by bright-field and confocal microscopy technique [15]. The notion of ACE being an immune privileged site was broken when T-cell infiltration was seen on

post-transplantation Day 7. Afterwards, T-cells increasingly infiltrated around and within the islet grafts post [15].

Without the protection of rapamycin, the islet number in the eye that received blank microparticles rapidly dropped within the first 14 days of transplantation (Figure 5.3A, first row). By post-transplantation Day 17, all islets graft visually disappeared from the left ACE with blank microparticles, suggesting complete islet graft-host immune rejection. In comparison, the islets within the ACE of the right eye (rapamycin-releasing microparticle) remained visually present up to post-transplantation Day 30 (Figure 5.3A, second row). This *in vivo* observation was consistent with rapamycin microparticle numbers as low as 20 (Figure 5.4). The data suggests that rapamycin-releasing microparticles are able to protect islets graft from immune rejection in a localized milieu of the ACE and the amount of microparticles required is small. The safety of these biodegradable polymer microparticles was observed with increased microparticle opaqueness which suggests polymer degradation [163].

Figure 5.3 Effect of blank and rapamycin microparticles on transplanted islet grafts *in vivo*. A) Images of mouse eyes transplanted with allogeneic islets (yellow circle) and blank microparticles (first row, Blank) or rapamycin microparticles (second row, Rapamycin). With the blank microparticle, visible islets number decreased gradually during the first 14 days and disappeared fully on Day 17. In the eye transplanted with rapamycin-loaded microparticles, the grafted islets were present throughout the 30 days, without any distinguishable decrease of islet volume visually. Scale bar: 500 μ m. B) Change in the average islet volume with blank particles (●) vs. the islet volume with rapamycin microparticles (■). Data based on 5-9 islets/time point from 5 eyes with rapamycin microparticle and 3-10 islets/time point from 3 eyes with blank microparticles. Results presented as means $\pm$ SEM * $P < 0.05$ ** $P < 0.005$. Horizontal red line marks the 70% islet volume position as a benchmark indicates rejection onset C) Survival curves of islet grafts in the ACE based on volume (Blank microparticle: n=9; Rapamycin microparticle: n=9).

Figure 5.4 Effect of microparticle number on islets rejection. Among all the eye transplanted with microparticles, the smallest number of particle transplanted was 20. The 3 islets transplanted was present throughout the 30 days of monitoring, which suggests islets rejection can be delayed with even a small number of particles.

Next, by quantifying changes in islet volume, we were able to determine the relative progression of islet rejection. When the islet size is reduced by $\geq 30\%$ of the original imaged size (at post-transplantation Day 3), the islets are arbitrarily considered as totally rejected [15]. In our hands, the volume of islets in the left eye with blank microparticle decreased by more than 30% between Day 7 to Day 10 (Figure 5.3B). By post-transplantation Day 14, the islet volume remaining in the left ACE was approximately 20% of that when imaged at initial time point. This result is consistent with the previous reported visual inspection under stereomicroscope. In contrast, islets with rapamycin microparticle had less than 30% volume reduction till Day 20. The decrease of volume after Day 20 had only a mild slope compared to the blank microparticles. This indicated that the drug was still present, having preserving effects on the islets. At each time point, the difference of islets volumes between blank and rapamycin microparticles were significant, hence the islets preserving effect of rapamycin microparticles was evident. We further validated the finding with survival curve, based on individual islets rejection (Figure 5.3C). The islets with rapamycin microparticle survived longer than the islets with blank microparticle. When an adequate number of rapamycin microparticles ranging between 20-50 were transplanted, the invariable loss of grafts function caused by allograft rejection was postponed.

5.3 Discussion

The high risk of systemic immunosuppression has limited the clinical adaption of islet transplantation as well as other organ transplantations. The drawbacks of systemic immunosuppression therapy include, but are not limited to: 1) use of high dose for suppressing whole-body immune response, 2) subjecting patient to risk of other diseases, especially infectious disease, 3) intolerant side effects and 4) compromise on quality of life [164]. Local immunosuppression can protect the transplanted organs with lower dose, while minimizing the above drawbacks. In addition, islets transplantation at ACE is shown to have better islet vascularization and higher survival rate compared to other transplantation sites [13, 21, 83]. Therefore, microparticles releasing an immunosuppressive agent to ACE were transplanted together with islets to establish a proof-of-concept of local immunosuppression for islets allografts. In this chapter, microparticle mixture was successfully synthesized employed to deliver rapamycin for 30 days above the minimum required dosage. After transplantation in mouse model, the islets with rapamycin microparticles were present for a significantly longer time, while islets with blank microparticles (control) were rejected within the first two weeks. This indicates the islets can be locally protected from rejection by a sustained delivery of immunosuppressive agent.

A lot of background work has been carried out to determine suitable criteria for the rapamycin carrier. Microparticle was the selected drug carrier because of its flexibility and versatility. It could maintain its integrity during the transplantation procedure. Nanoscale drug carrier has higher tendency of burst release [165], therefore micro-scale is more suitable for long-term delivery. A quick trial of microparticles with different sizes ($<20\text{ }\mu\text{m}$, $50\text{-}100\text{ }\mu\text{m}$ and $>200\text{ }\mu\text{m}$) showed that particles with size of $50\text{-}100\text{ }\mu\text{m}$ is the easiest to handle with the transplant tool kit. Hence, a library of different microparticles encapsulated with rapamycin were developed, at average size of $100\text{ }\mu\text{m}$ (Table 4.1).

Besides, the selected immunosuppressive agent, rapamycin, is highly hydrophobic and water-insoluble [135, 136]. These properties imposed a challenge in developing the microparticle formulation. Desired dose could be met only when low molecular weight polymer were used [160]. Among the formulations developed, the PLGA and PCL microparticles delivered rapamycin higher than minimum required dose – 20 nM per day (Figure 5.1). However, the individual releasing period is shorter than 30 days. Hence, a mixture of both were used to meet both criteria: duration and dose. Besides that, the potential toxicity of mixture formulation was eliminated by batch incubation (GSIS) study with islets (Figure 5.2) before the microparticle formulation was finalized. Rapamycin was reported to reduce pancreatic β -cell function and insulin sensitivity in murine islets at both low dose (32.8 nM) [166] and high dose (109.4 nM) [167]. However, the dose we used is only one fifth of the highest dose. This could be the reason that no toxic effect of rapamycin was observed in our study.

After optimizing the microparticle formulation, allogenic islets and microparticles were successfully transplanted into the recipient mice ACE. One of the advantage of using ACE as transplantation site is that longitudinal observation is possible without invasive method. The observation not only monitored the amount of islets present in ACE, but also tracked the microparticle movement and change of microparticle in physical appearance and mass. During the post-transplantation observation, islet grafts were attached to iris with negligible movement. But microparticles were observed to locate towards the edge of the iris. Slight relocation of microparticles was seen at few time points. However, these movements do not affect any visual ability of host mice based on their behavior in conscious state. Based on prior study of DBA/2 (H-2d) mouse islets in C57BL/6 (H-2b) mice, the islets rejection happened between day 7 to 14 and islet volume rapidly decreased to 50% on day 14 when natural immune response occurred [15]. The islets with blank microparticle showed the rejection time between post-

transplant Day 7 to 10, same as the previously reported time (Figure 5.3A). In addition, the islet volume decreased to a similar value as reported results on post-transplant Day 14 (Figure 5.3B). On the other hand, islets with rapamycin microparticle showed lower degree of volume reduction and were visually present till Day 30. This suggests that rapamycin microparticle strongly suppressed the immune response inside ACE. Only when it was reaching the end of release period (post-transplant Day 30), islets rejection started. Furthermore, rejected islets are at various distance with microparticles. Hence, the distance between islets and microparticles has no effect on the rate of rejection. Therefore, the microparticles may have infused the entire ACE with rapamycin, which caused a uniform effect on the local immune system. Furthermore, pigmentation on microparticles increased gradually during the 40 days post transplantation optical observation. The degree of pigmentation varies between different eyes, where in certain ACEs, the microparticles were fully pigmented. This pigmentation caused difficulty in the visual counting of microparticles as well as the backscattering measurement. The degradation of microparticle in ACE become evident when the entire microparticle swelled and become fully opaque towards the end of release period. This phenomena is the same in release study *in vitro* condition. Aqueous humor, the body fluid that fills ACE, is constantly replenishing itself over time [92]. Hence, the degraded by-product of polymers could get excreted naturally, and would not accumulate to induce pH change and affect islets function.

By now, it was demonstrated that islet allografts received prolonged immunosuppression with a minimized dosage to enhance their survival and function with lowered side effects by implanting rapamycin microparticle together with islets. The success of this model demonstrates the advantages of local immunosuppression and opens the possibility of transplanting islets to ACE as metabolic therapy for diabetes. Using this approach, the deleterious effect of chronic immunosuppression and cytotoxicity to grafted islets can be minimized and the risk of infection post transplantation is also significantly

lowered. Besides, this model has additional potential beyond this model. The microparticle carrier system is customizable in terms of dose and duration. Delivery longer than 30 days is highly feasible by tailoring the polymer degradation time. Microparticle system is versatile to load multiple drugs, such as diabetes drug or sensing agent. Doing so could expand its functionality and transform ACE to be a site of interest in islets biology study.

5.4 Summary

From the library of rapamycin microparticle formulation developed, suitable microparticle combinations were co-transplanted with allogenic islets into rodent ACE. The result of microparticle releasing rapamycin in the ACE and delaying rejection onset of islets graft is promising. It is one of the key steps paving the way for the islet transplantation technologies towards a practical clinical application. Further modification of the microparticle design could bring in additional function to enhance the therapeutic effect. One possible way is to delivery dual drugs for immunosuppression and insulin secretion. Clinically, multifunctional microparticle would be significantly useful to co-transplant with islets as a treatment for T1D patients.

5.5 References

- [1] WHO. (2017, 06 Feb). *The top 10 causes of death-The Fact Sheet*. Available: <http://www.who.int/mediacentre/factsheets/fs310/en/>
- [5] J. E. Fradkin, "Confronting the urgent challenge of diabetes: an overview," *Health Aff (Millwood)*, vol. 31, no. 1, pp. 12-9, Jan 2012.
- [9] A. M. Shapiro *et al.*, "International trial of the Edmonton protocol for islet transplantation," (in eng), *N Engl J Med*, vol. 355, no. 13, pp. 1318-30, Sep 28 2006.
- [11] L. Jansson and P. O. Carlsson, "Graft vascular function after transplantation of pancreatic islets," (in English), *Diabetologia*, Review vol. 45, no. 6, pp. 749-763, Jun 2002.
- [12] A. M. J. Shapiro, M. Pokrywczynska, and C. Ricordi, "Clinical pancreatic islet transplantation," *Nat Rev Endocrinol*, Review vol. advance online publication, 11/11/online 2016.
- [13] J. Lau *et al.*, "Implantation site-dependent dysfunction of transplanted pancreatic islets," *Diabetes*, vol. 56, no. 6, pp. 1544-50, Jun 2007.
- [15] M. H. Abdulreda *et al.*, "High-resolution, noninvasive longitudinal live imaging of immune responses," *Proc Natl Acad Sci U S A*, vol. 108, no. 31, pp. 12863-8, Aug 2 2011.
- [21] V. L. Perez *et al.*, "The anterior chamber of the eye as a clinical transplantation site for the treatment of diabetes: a study in a baboon model of diabetes," *Diabetologia*, vol. 54, no. 5, pp. 1121-6, May 2011.
- [83] E. Cantarelli and L. Piemonti, "Alternative transplantation sites for pancreatic islet grafts," (in eng), *Curr Diab Rep*, vol. 11, no. 5, pp. 364-74, Oct 2011.
- [92] M. Aihara, "Aqueous Humor Dynamics in Mice," *Investigative Ophthalmology & Visual Science*, vol. 44, no. 12, pp. 5168-5173, 2003.

- [95] S. Merani and A. M. Shapiro, "Current status of pancreatic islet transplantation," *Clin Sci (Lond)*, vol. 110, no. 6, pp. 611-25, Jun 2006.
- [101] T. Berney and A. Secchi, "Rapamycin in islet transplantation: friend or foe?," (in eng), *Transpl Int*, vol. 22, no. 2, pp. 153-61, Feb 2009.
- [108] M. C. Fabian, J. R. Lakey, R. V. Rajotte, and N. M. Kneteman, "The efficacy and toxicity of rapamycin in murine islet transplantation. In vitro and in vivo studies," (in eng), *Transplantation*, vol. 56, no. 5, pp. 1137-42, Nov 1993.
- [131] R. Bottino *et al.*, "Transplantation of allogeneic islets of Langerhans in the rat liver: effects of macrophage depletion on graft survival and microenvironment activation," (in eng), *Diabetes*, vol. 47, no. 3, pp. 316-23, Mar 1998.
- [135] P. Simamora, J. M. Alvarez, and S. H. Yalkowsky, "Solubilization of rapamycin," *International Journal of Pharmaceutics*, vol. 213, no. 1-2, pp. 25-29, 2001.
- [136] R. T. Abraham, J. J. Gibbons, and E. I. Graziani, "Chapter 17 - Chemistry and Pharmacology of Rapamycin and Its Derivatives," in *The Enzymes*, vol. Volume 27, N. H. Michael and T. Fuyuhiko, Eds.: Academic Press, 2010, pp. 329-366.
- [153] T. D. Control and C. T. R. Group, "The Effect of Intensive Treatment of Diabetes on the Development and Progression of Long-Term Complications in Insulin-Dependent Diabetes Mellitus," *New England Journal of Medicine*, vol. 329, no. 14, pp. 977-986, 1993.
- [154] N. V. Evgenov *et al.*, "In Vivo Imaging of Immune Rejection in Transplanted Pancreatic Islets," *Diabetes*, 10.2337/db06-0484 vol. 55, no. 9, p. 2419, 2006.
- [155] G. Miao *et al.*, "Dynamic Production of Hypoxia-Inducible Factor-1 α in Early Transplanted Islets," *American Journal of Transplantation*, vol. 6, no. 11, pp. 2636-2643, 2006.
- [156] X. Zheng *et al.*, "Acute hypoxia induces apoptosis of pancreatic [beta]-cell by activation of the unfolded protein response and

- upregulation of CHOP," *Cell Death Dis*, vol. 3, p. e322, 06/14/online 2012.
- [157] M. D. Stegall, K. J. Lafferty, I. Kam, and R. G. Gill, "Evidence of recurrent autoimmunity in human allogeneic islet transplantation," *Transplantation*, Article vol. 61, no. 8, pp. 1272-1274, 1996.
- [158] R. P. Robertson, "Islet transplantation as a treatment for diabetes - a work in progress," (in eng), *N Engl J Med*, vol. 350, no. 7, pp. 694-705, Feb 12 2004.
- [159] B. Hirshberg, "Lessons learned from the international trial of the edmonton protocol for islet transplantation," (in eng), *Curr Diab Rep*, vol. 7, no. 4, pp. 301-3, Aug 2007.
- [160] Y. L. Fan, H. W. Hou, H. M. Tay, W. M. Guo, P. O. Berggren, and S. C. Loo, "Preservation of Anticancer and Immunosuppressive Properties of Rapamycin Achieved Through Controlled Releasing Particles," (in eng), *AAPS PharmSciTech*, Mar 01 2017.
- [161] A. S. Huang, C. Mohindroo, and R. N. Weinreb, "Aqueous Humor Outflow Structure and Function Imaging At the Bench and Bedside: A Review," *J Clin Exp Ophthalmol*, vol. 7, no. 4, Aug 2016.
- [162] A. D. Barlow, M. L. Nicholson, and T. P. Herbert, "Evidence for rapamycin toxicity in pancreatic beta-cells and a review of the underlying molecular mechanisms," *Diabetes*, vol. 62, no. 8, pp. 2674-82, Aug 2013.
- [163] T. D. Farahani, A. A. Entezami, H. Mobedi, and M. Abtahi, "Degradation of poly(D,L-lactide-co-glycolide) 50 : 50 implant in aqueous medium," (in English), *Iranian Polymer Journal*, Article vol. 14, no. 8, pp. 753-763, Aug 2005.
- [164] K. I. Rother and D. M. Harlan, "Challenges facing islet transplantation for the treatment of type 1 diabetes mellitus," *Journal of Clinical Investigation*, vol. 114, no. 7, pp. 877-883, 2004.

- [165] W. H. De Jong and P. J. A. Borm, "Drug delivery and nanoparticles: Applications and hazards," *International Journal of Nanomedicine*, vol. 3, no. 2, pp. 133-149, 06/ 2008.
- [166] N. Zhang *et al.*, "Sirolimus Is Associated With Reduced Islet Engraftment and Impaired β -Cell Function," *Diabetes*, vol. 55, no. 9, pp. 2429-2436, 2006.
- [167] M. C. Fabian, J. R. T. Lakey, R. V. Rajotte, and N. M. Kneteman, "The Efficacy And Toxicity Of Rapamycin In Murine Islet Transplantation: In Vitro And In Vivo Studies," *Transplantation*, vol. 56, no. 5, pp. 1137-1141, 1993.

6 One-step Synthesis of Novel Drug Carrier, Janus Particle, Encapsulating Diabetes Drug for Control Release²

6.1 Introduction

With the promising result generated from rapamycin-releasing microparticles, we sought to extend the function of bioengineered microparticles in the clinical application of islet transplantation. One possible way is the modification of microparticle carrier to include diabetes drugs which promotes islet insulin secretion. Besides protecting graft islets from immune rejection, these microparticles could enhance the chance of insulin independence with fewer islets grafts. Following this direction, new microparticle were explored to allow versatility and multi-functional delivery [23, 24, 129, 168]. The Janus particle (JP) is one example of a structurally-unique, yet promising multi-drug carrier [10].

The concept of JP was first proposed by the Nobel laureate de Gennes in the 1990s [169]. It has several distinct advantages over other particulate delivery systems, because architecturally it allows for a compartmentalized encapsulation of drugs. Such particles feature segregated, anisotropic compartments on two sides of an individual particle. In addition, it can provide other value-adding features such as bioimaging, by incorporating imaging agents in a separate compartment for combinatorial theranostic application [10]. We are interested in the ability of JP to co-deliver multiple drugs by distinct physicochemical properties in segregated compartments, allowing independent but controllable release of different drugs simultaneously.

Over the decades, tremendous effort has been channeled to optimize the synthesis process for large-scale production of JP with specific

functionalities [170-172]. Common approaches such as toposelective surface modification [173-175], template-directed self-assembly [176, 177] and controlled surface nucleation [178-180] can provide precise morphological and structural control of the JP, but they suffer in scalability [171, 172]. More recent techniques were thus developed to synthesize polymeric JP using microfluidic devices [181, 182] and through the electro-hydrodynamic jetting strategy [183]. The former produces particles of limited size range and may require an additional step for polymer crosslinking, while the latter is applicable only to polymers that are conductive. In contrast, the phase separation method, e.g. emulsion evaporation, is generally recognized as the most feasible method for scalable production of JP owing to its economical set-up and relatively simple process [184]. The key challenge of this method, however, is the ability to control simultaneous phase separation of polymers in the dynamic colloidal system to consistently yield JP of the desired architectural design. Although several recent studies have demonstrated the possibility of generating multi-dimensional JP using biodegradable polymers through the emulsion evaporation approach, there have been no reports where drugs were included into the fabrication process [185, 186]. In fact, attempts to encapsulate drug molecules into JP through emulsion have not met with much success [187]. In most cases, the addition of a drug molecule appeared to alter the initial structure of the JP, of reasons yet not known [187]. This suggests a complex relationship between the drug and polymers during particle fabrication that interfered with the formation of JP. There is therefore a need to determine the relationship between drug and polymer, in order to devise an empirical strategy to consistently generate structurally intact drug-loaded JP through the emulsion phase separation technique.

In this study, we approached the question by scoping the work using two different FDA-approved biodegradable polymers, PLGA and PCL, as well as several drugs as our model system. Instead of encapsulating the drug using a blank JP formula, we synthesized the JP in the presence

of the drug using the emulsion evaporation method and systematically investigated the factors that affect JP formation. It was found that both the weight ratio of PLGA to PCL and the net charge of the drug (i.e. glibenclamide, rapamycin and lidocaine) were critical in governing the switch between anisotropic (i.e. JP) and core-shell (i.e. non-JP) structure. This further highlights the importance of drug-polymer interaction in the JP fabrication process. Specifically, the charge of the drug appeared to modulate the interfacial tension and spreading coefficient of the polymers that prime the formation of JP. These formulation principles can thus serve as a theoretical framework for the generation of drug-loaded JP.

²Reproduced from [Fan, Y. L.[†], Tan, C. H.[†], Lui, Y. S., Zudhistira, D., and Loo, S. C. J.*, “Mechanistic Formation of Drug-encapsulated Janus Particles through Emulsion Solvent Evaporation,” *RSC Adv.*, 2018, **8**, 16032] with permission from The Royal Society of Chemistry.

6.2 Results

6.2.1 Glibenclamide Drives Janus Particle Formation at a Specific Polymer Weight Ratio

Microparticles consist of biodegradable polymers, i.e. PLGA and PCL, at different weight ratios, as well as the model drug glibenclamide, were synthesized using a one-step oil-in-water emulsion solvent evaporation technique. Without glibenclamide, microparticles with core-shell structures, i.e. double-layered, were consistently generated regardless of the polymer weight ratio (Figure 6.1.A4 to A6). Similarly, JP were not obtained at polymer weight ratios less than 20:10, e.g. 10:20 and 15:15, when glibenclamide was present (Figure 6.1.A1 and A2). In contrast, a bi-compartmental JP structure was observed at the polymer weight ratio 20:10 with glibenclamide (Figure 6.1.A3), suggesting an intricate relationship between glibenclamide and the polymers at this weight

ratio. The bi-compartmental microparticles were generally characterized with a distinct, hemispheric Janus structure where both PLGA and PCL polymers appeared to occupy almost an equal volume of the particle (Figure 6.1.B1), consistent with JP morphology synthesized by other methods [30]. The anisotropic characteristic of JP was evidenced by acetone treatment [31], which resulted in the dissolution of the PLGA component, leaving the PCL compartment with pox-like surfaces (Figure 6.1.B2). Glibenclamide was found distributing in both compartments of JP as indicated by the confocal Raman microscopy (CRM) (Figure 6.2). Quantitative microscopy image analysis further revealed that more than 90% of the particles yielded were JP with mean diameter of 104.5 μm and standard deviation of 38 μm (Figure 6.3).

Figure 6.1 Microparticles fabricated at different PLGA/PCL weight ratios in the presence or absence of glibenclamide. Cross-sectional views of glibenclamide-loaded (A1-A3) and blank (A4-A6) microparticles under scanning electron microscope (SEM). Microparticles were fabricated at different PLGA/PCL weight ratios (wt:wt), i.e. 10:20 (A1 and A4), 15:15 (A2 and A5), and 20:10 (A3 and A6). Surface morphology of an intact, glibenclamide-loaded microparticle characterized with Janus structure (B1) and SEM image of that Janus particle after acetone treatment (B2). Scale bar: A1-A3, A5, 10 μm; A4, A6, 20 μm; B1-B2, 50 μm.

Figure 6.2 Distribution of glibenclamide in a Janus particle. The localization of glibenclamide in a Janus particle was detected using Raman spectroscopy. The Raman spectra of PCL polymer (PCL), PLGA polymer (PLGA), PCL compartment of a glibenclamide-loaded Janus particle (JP-PCL+GLN), PLGA compartment of a glibenclamide-loaded Janus particle (JP-PLGA+GLN) and pure glibenclamide (GLN) are shown (top to bottom). Glibenclamide is characterized with unique Raman shifts at 1150 cm^{-1} caused by sulphonyl stretching vibrations.

Figure 6.3 Size distribution of Janus particle. Three batch of drug-loaded PLGA/PCL particles were fabricated. The yield of Janus particle in each batch are: $92.5 \pm 4.6\%$; $96.6 \pm 2.8\%$ and $95.1 \pm 0.6\%$. The average particle diameters (μm) are 106.7 ± 54.4 ; 96.7 ± 30.7 and 110.1 ± 28.8 .

6.2.2 Negative Charge of Glibenclamide Determines JP Formation at 20:10 Polymer Weight Ratio

To gain a further insight into the relationship between glibenclamide and microparticle formation, different concentrations of glibenclamide were supplemented in the fabrication process whilst the PLGA/PCL polymer weight ratio was maintained at 20:10 (Figure 6.4). While core-shell (i.e. non-JP) particles were dominant in the absence of glibenclamide, more than 90% of the microparticles were JP when glibenclamide at 2% (wt:wt) was used (Figure 6.4A and B). A higher concentration of glibenclamide, i.e. 10% (wt:wt), however, neither altered the Janus structure nor significantly increased the JP yield, i.e. $96.6 \pm 0.9\%$ vs. $92.5 \pm 0.5\%$ (Figure 6.4C). Interestingly, it was also shown that the negatively charged glibenclamide could be functionally substituted with other negatively charged drugs such as tolbutamide (Figure 6.5.A1) or a negatively charged agent like trypan blue (Figure 6.5.A2). However, a neutral drug, e.g. rapamycin (Figure 6.5.A3) or a positively charged drug, e.g. lidocaine (Figure 6.5.A4), did not yield any JP. This suggests a charge-dependent selectivity in JP formation. To further confirm the impact of negative charge on JP formation, the relationship between the charge density of glibenclamide at different pH values, and its yield was quantified (Figure 6.5B). The amount of JP yielded was found positively correlated with the negative charge density of glibenclamide (Figures 6.5.B4 and Figure 6.6). For example, at pH 6, 83.4% of glibenclamide were expected to carry a negative charge and that resulted in more than 92% of microparticles with a Janus structure (Figure 6.5.B1). However, the JP yield decreased to 71% at pH 4 and JP disappeared entirely at pH 2, where the negative charge densities of glibenclamide were reduced to 11.4% and 0.1%, respectively (Figure 6.5.B2 and B3).

Figure 6.4 Microparticles fabricated with different concentrations of glibenclamide. Light microscopy (left column) and scanning electron microscopy (SEM)(right column) images of microparticle fabricated with PLGA and PCL at the polymer weight ratio 20:10 (wt:wt), in presence of glibenclamide at concentrations ranging from 0% (A) to 2%(B) to 10% (C). Yields of Janus particle for were 92.5 ± 0.5 % (B) and $96.6 \pm 0.9\%$ (C). Scale bar, 100 μm

Figure 6.5 Microparticles fabricated at the PLGA/PCL weight ratio 20:10 in the presence of drugs with different charges (A) or charge densities (B). Scanning electron microscopy (SEM) images of PLGA/PCL microparticles fabricated in the presence of drugs with different charges, i.e. tolbutamide (negatively charged)(A1), trypan blue (negatively charged)(A2), rapamycin (non-charged)(A3) and lidocaine (positively charged)(A4). Light microscopy images of PLGA/PLCA microparticles fabricated with glibenclamide at different, negatively charged densities, i.e. 83.4%, 11.2% and 0.1%, generated at pH 6.0 (B1), pH 4.0 (B2) and pH 2.0 (B3), respectively. The relationship between pH, negatively charged density of glibenclamide and

the yield of microparticles with Janus structure (B4). Scale bar: A1, B1-B3, 100 μm ; A2, 50 μm ; A3-A4, 20 μm .

Figure 6.6 The charge status of glibenclamide at different pH. The pKa value of glibenclamide is 5.3. Thus, the molecule carries one negative charge at pH 6 (A). When pH decrease to 4 and 2, the molecule become neutral (B). The percentage of negatively charged molecule was calculated by classic Henderson-Hasselbalch equation at pH 6, 4 and 2.

6.2.3 Glibenclamide Alters the Interfacial Tensions in the Emulsion System

The interfacial tensions (γ) between different phases in an emulsion system determine the spreading coefficients (S) between phases [32]. This plays a key role in shaping the particle morphology, as S would influence the degree of polymer engulfment during phase separation [33, 34]. Thermodynamically favored polymer engulfment can be predicted by the spreading coefficient, S, which is calculated based on interfacial tension, γ , between all the phases according to Eqn. 1. By Harkin's definition, a positive spreading coefficient leads to spreading of phase i on phase j [32]. In this study, the emulsion system consisted

of three different phases namely, the oil phase PLGA/DCM (Phase 1), the water phase PVA (Phase 2) and the second oil phase PCL/DCM (Phase 3).

$$S_i = \gamma_{jk} - (\gamma_{ij} + \gamma_{ik}) \quad (1)$$

Based on the spreading coefficient, there are three possible configurations, shown below:

$$\text{Full engulfment 1):} \quad S_1 < 0, S_2 < 0, S_3 > 0 \quad (2)$$

$$2): \quad S_1 > 0, S_2 < 0, S_3 < 0 \quad (2)$$

$$\text{Partial engulfment:} \quad S_1 < 0, S_2 < 0, S_3 < 0 \quad (3)$$

$$\text{No engulfment:} \quad S_1 < 0, S_2 > 0, S_3 < 0 \quad (4)$$

Figure 6.7 Graphic abstract of different polymer engulfment and the Janus particle formation.

In this study, it is hypothesized that the negatively-charged glibenclamide may modulate the polymer interaction only at a specific range of PLGA/PCL weight ratios, leading to JP formation. To verify our hypothesis, the initial γ and the corresponding S in different emulsion systems were determined (Table 6.1), using the pendent drop method [35] and the Owens-Wendt-Kaelble approach [36] (Table 6.3). Without glibenclamide, at PLGA/PCL weight ratio of 20:10, all

interfacial tensions differed markedly from each other and the spreading coefficients, i.e. $S_1 > 0$, $S_2 < 0$ and $S_3 < 0$, met the full engulfment criteria, resulting in core-shell microparticles (Table 6.1 and Figure 1.A6). When weight ratio decreased to 15:15, the interfacial tension of PLGA/DCM and PVA increased ($\gamma_{12} = 6.72$), while the value of PCL/DCM and PVA decreased ($\gamma_{23} = 5.93$). Despite the change, full engulfment condition was still maintained. In contrast, the interfacial tension between PLGA/DCM and PVA ($\gamma_{12} = 7.04$) was found comparable to that of PCL/DCM and PVA ($\gamma_{23} = 7.05$) when 2% of glibenclamide (w/w) was included at PLGA/PCL weight ratio of 20:10 (Table 6.1). Accordingly, all the spreading coefficients were estimated to be negative, with S_1 (-0.64) nearly equivalent to S_3 (-0.67) but significantly less negative than S_2 (-13.43). This satisfies the partial engulfment requirement for JP formation (Table 6.1 and Figure 6.1.A3). Such drug modulation effect, however, was different when positively-charged-lidocaine and neutral-rapamycin were included at PLGA/PCL weight ratio of 20:10. Under such circumstances, the value of interfacial tensions, γ_{12} (i.e. 5.78, 3.75) and γ_{23} (i.e. 6.62, 8.60) was no longer the same as blank polymer but fulfilled the full engulfment criteria (i.e. $S_1 = 0.19$ or $S_3 = 0.27$), resulting in the formation of core-shell microparticles (Table 6.1 and Figure 6.1.A2).

Besides polymer engulfment, the contact angle θ_i between any two phases at the 3-phase equilibrium joint part of Janus (Figure 6.7) can be calculated by the extended theory in geometrical prediction by Torza and Mason. [34] Thus, the contact angle θ_2 between the interfacial tension γ_{12} and γ_{23} can be calculated by Eqn.5.

$$\cos \theta_i = \frac{\gamma_{jk}^2 - \gamma_{ij}^2 - \gamma_{ik}^2}{2\gamma_{ij}\gamma_{ik}} \quad [5]$$

For example, glibenclamide loaded PLGA/PCL JP calculated value of $\cos \theta_2$ is - 0.9957, thus θ_2 is 175° , which is very close to 180° (Figure 6.1.B1). The predicted contact angle from fabricated JP matched with the experimental results: a JP hemisphere.

Table 6.1 Interfacial tensions and spreading coefficients at different PLGA/PCL weight ratios

PLGA/PCL ratio*	Drug charge ^s	Interfacial tension [†]			Spreading coefficient [‡]			Predicted [#]	Observed
		γ_{12}	γ_{23}	$\gamma_{13}^{\wedge}$	S_1	S_2	S_3		
15:15	n.a.	6.72	5.93	0.65	-1.44	-12.00	0.14	Core-shell	Core-shell
15:15	negative	4.38	6.43	0.65	1.40	-10.16	-2.70	Core-shell	Core-shell
20:10	n.a.	6.69	10.56	0.65	3.22	-16.60	-4.53	Core-shell	Core-shell
20:10	negative	7.04	7.05	0.65	-0.64	-13.43	-0.67	Janus	Janus
20:10	neutral	5.78	6.62	0.65	0.19	-11.74	-1.50	Core-shell	Core-shell
20:10	positive	3.75	8.60	0.65	4.20	-11.70	-5.50	Core-shell	Core-shell

Table 6.2 Interfacial tensions and spreading coefficients at PLGA/PCL weight ratio 19:11

PLGA/PCL ratio*	Drug charge ^s	Interfacial tension [†]			Spreading coefficient [‡]			Predicted [#]	Observed
		γ_{12}	γ_{23}	$\gamma_{13}^{\wedge}$	S_1	S_2	S_3		
19:11	n.a.	5.40	5.64	0.65	-0.41	-10.39	-0.89	Janus	Janus
19:11	neutral	6.50	6.04	0.65	-1.11	-11.89	-0.19	Janus	Janus
19:11	negative	5.82	5.06	0.65	-1.41	-10.23	0.11	Core-shell	Core-shell
19:11	positive	4.85	4.28	0.65	-1.22	-8.48	-0.08	Janus	Janus

*Each emulsion system consists of three phases, i.e. Phase A: PLGA/DCM; Phase W: PVA (Water) and Phase B: PCL/DCM. The weight proportion of each polymer used in the fabrication is indicated. PVA: Poly (vinyl alcohol); DCM: Dichloromethane.

^sDrugs with different charges were included in the fabrication, i.e. glibenclamide (negatively charged), rapamycin (neutral) and lidocaine (positively charged). n.a.: not applicable.

[†]Interfacial tensions between PLGA/DCM and PVA (γ_{12}) as well as PCL/DCM and PVA (γ_{23}) were determined using the pendant drop method. Meanwhile, the interfacial tension between PLGA/DCM and PCL/DCM (γ_{13}) was estimated based on the surface energy of PLGA and PCL according to the Owens-Wendt method (Table 6.3). The interfacial tension is expressed in mN/m.

[^]Phase 1 and 3 are miscible at the beginning. Therefore the interfacial tension between the solid state of PLGA and PCL was used to represent the final form as it gave more accurate morphology prediction. [188] It is assumed the interfacial tension at the solid state remained same after low level drug addition [189].

[‡]The spreading coefficient (S) is calculated based on Harkin's equation [190], $S_i = \gamma_{jk} - (\gamma_{ij} + \gamma_{ik})$. The spreading coefficient is expressed in mN/m.

[#]According to the classic spreading and partial wetting theory [191] – Core-shell: $S_1 < 0, S_2 < 0, S_3 > 0$ or $S_1 > 0, S_2 < 0, S_3 < 0$; Janus: $S_1 < 0, S_2 < 0, S_3 < 0$ and separate particles: $S_1 < 0, S_2 > 0, S_3 < 0$.

Table 6.3 Calculation of interfacial tension between PLGA and PCL in DCM system

	Cyclohexane-PLGA	Water-PLGA	Cyclohexane-PCL	Water-PCL
Contact Angle (°)*	10.10±0.83	65.58±0.87	9.49±0.92	65.47±1.73
Surface Energy (mN/m) \$	38.47		39.12	
Interfacial Energy (mN/m) †	0.65			

*Contact angles of two standard probe liquids (water and cyclohexane) were measured against the polymer film using Contact Angle DataPhysics OCA15Pro system at room temperature (25°C). PLGA and PCL films were prepared by dissolving the polymers in dichloromethane (DCM). The polymer mixture was casted on a glass slide and the solvent was dried under vacuum for sufficient time to ensure total evaporation of the organic solvent.

§ According to the general equation of interfacial tension proposed by Owens and Wendt (below), The surface energy (γ_L), dispersive (γ_L^d) and polar component (γ_L^p) are well known for the probe liquid. Based on the above equation, the dispersive (γ_L^d) and the polar component (γ_L^p) of PLGA and PCL could be obtained, and their surface energy was calculated as $\gamma_S = \gamma_S^d + \gamma_{LS}^p$.

$$1 + \cos \theta = 2 \left[\frac{\sqrt{\gamma_S^d \gamma_L^d}}{\gamma_1} + \frac{\sqrt{\gamma_S^p \gamma_L^p}}{\gamma_1} \right]$$

†The difference between two solids' surface energy is their interfacial energy.

6.2.4 Modulation of Interfacial Tensions by Polymer Weight Ratio Allows the Formation of Lidocaine- and Rapamycin-loaded Janus Particles

Besides net charge of the drug, interfacial tensions also changed with polymer weight ratios (Table 6.1). It was found that at a weight ratio of 19:11, JP were obtained even in the absence of drugs (Figure 6.8.A). At this weight ratio, the addition of rapamycin and lidocaine (Figure 6.8.C and D) also yielded JP. However, this time, the encapsulation of glibenclamide resulted in core-shell microparticles, due to more positive spreading coefficients that shift away from partial engulfment. The subsequent interfacial tension and spreading coefficient measurements

(Table 6.2) agreed with this hypothesis. With a newly adjusted weight ratio of 19:11, the interfacial tensions of both PLGA/DCM – PVA ($\gamma_{12}=5.40$) and PCL/DCM – PVA ($\gamma_{23}=5.64$), without any drug, now favor the partial engulfment condition. The addition of the neutral rapamycin increased the interfacial tensions at both interfaces ($\gamma_{12}=6.50$, $\gamma_{23}=6.04$), that decrease the spreading coefficient to meet the partial engulfment condition. Although positive lidocaine decreased the interfacial tensions, its corresponding decrease resulted in a situation that fulfilled the partial engulfment. In summary, the formation of JP is a thermodynamically driven process that is determined by the interfacial tensions of the polymer solutions, which are influenced by the charge of the drug and the polymer weight ratio. Interfacial tensions that result in negative spreading coefficients favor the formation of anisotropic JP.

Figure 6.8 Microparticles fabricated in the absence or presence of drugs with different charges at the PLGA/PCL weight ratio 19:11. Scanning electron microscopy (SEM) images of PLGA/PCL microparticles fabricated, at the specific polymer weight ratio 19:11, without drug (A) or with drugs carrying different charges, i.e. glibenclamide (negatively charged) (B), rapamycin (non-charged) (C) and lidocaine (positively charged) (D). Scale bar: A, D, 20 μm; B, 10 μm; C, 50 μm.

6.2.5 Janus Particles Demonstrate Greater Control over Burst Release of Glibenclamide Compared to the Core-shell Particles

The difference of JP and core-shell particles in terms of *in vitro* releasing glibenclamide (GLN) was studied. Figure 6.9 displays the 30 days release profiles of PLGA monolayer particle with 10% glibenclamide, PCL monolayer particle with 10% glibenclamide, PLGA/PCL JP with 10% glibenclamide and PLGA/PCL core-shell particle with 10% glibenclamide. PCL particle showed up to 80% burst release in first 5 days, and is the earliest to reach a plateau of maximal release. PLGA particle showed linear release in first 20 days, with an increase of release rate at day 20 - 30 period. This increase corresponds to PLGA polymer MW degradation duration [192]. Both JP and core-shell particle suppressed the burst release at the beginning, however JP showed a better suppression effect. After 20 days, both JP and core-shell particles showed increase of release rate, sharing the same characteristics of PLGA monolayer particle. Except the PCL monolayer particle, all three particles did not reach 100% release during the 30 days period. The residue amount of glibenclamide was analyzed. Highest amount of glibenclamide remained inside JP, followed by core-shell particle. This further suggests JP retain glibenclamide better.

Figure 6.9 *In vitro* release profile of glibenclamide (GLN).10% (w/w) drug loading and particle size were kept same for all particles (n=3, mean $\pm$ SD).

6.3 Discussion

Among the various techniques for JP fabrication, the single-step emulsion technique is most cost-effective and easily scalable approach. Several attempts have been reported in the literature to achieve this anisotropic JP structure by manipulating different process parameters [37], surfactant [27] and solvent [38]. However, effect of drug in the JP fabrication is poorly understood [10]. In this thesis, drug loaded JP was successfully fabricated using one-step emulsion evaporation technique. During the method development, two factors: polymer weight ratio and the net charge of a drug, were found to be attributing to polymer phase separation to produce JP. The effect of these two factors can be explained on the change of interfacial tension (IFT) and spreading efficiency, a classic model describes the controlled polymer phase separation in emulsion system.

6.3.1 Janus Particle Hemisphere Forms When Polymer Phase Shows Equivalent Interfacial Tension

JP formation based on the phase separation method, without any drug encapsulation, has been well described according to the spreading and partial wetting principle [190, 191, 193]. Here, we extended the application of this thermodynamic rule to synthesizing JP with different drug loads. Regardless of the type of drug molecules incorporated or the polymer weight ratio used, the Janus structure (i.e. the partial engulfment configuration) was achieved only when all three spreading coefficients in an emulsion system were determined negative or whenever $|\gamma_{12} - \gamma_{23}| < \gamma_{13}$ (Tables 6.1 and 6.2). However, unlike the acorn-, snowman- or the dumbbell-shape JP commonly synthesized in the absence of drug molecules^[172], microparticles fabricated in this study were uniquely characterized with two equal hemispheres, which is designated here as the JP hemisphere (Figures 6.1.3 and Figure 6.7). The different JP morphology is likely due to the varying degrees of partial engulfment during phase separation [191, 194]. Such JP hemisphere morphology is also in line with the prediction by Torza and Mason (Figure 6.7). According to the Equation 5, the contact angle between the two interacting polymers (i.e. θ_2) of a JP hemisphere is approximately 180° , which could only occur when the interfacial tensions between the PLGA-PVA (i.e. γ_{12}) and PCL-PVA (i.e. γ_{23}) are almost equivalent. Comparable interfacial tensions (i.e. $\gamma_{12} \approx \gamma_{23}$) with an absolute difference less than the interfacial tension between the polymers (γ_{13}) are therefore not only critical to drive the JP formation but also governing the JP morphology. Given that multiple drugs would be controlled release differently from a JP, consistent particle surface to area ratio as exemplified by the hemisphere morphology is thus highly desirable.

6.3.2 Interfacial Tension is Susceptible to Change Induced by Drug

The root cause of particle morphology change after drug encapsulation is the effect of drug on IFT at oil/water interface. Our data revealed that the interfacial tensions between the polymers and the water phase (i.e. γ_{12} and γ_{23}) were highly dynamic, and susceptible to changes induced by drug molecules incorporated (Tables 6.1 and 6.2). However, it remains unclear how a drug molecule may alter the interfacial tension and what is the mechanism underscoring the transformation event. Further study indicated that the interfacial tension change was strongly linked with the net charge of a drug molecule, and this might be further influenced by the type and the amount of polymer used in the emulsion system (Table 6.3). For example, addition of the positively charged lidocaine consistently decreased the interfacial tension regardless of the type (i.e. PLGA or PCL) or the amount of polymer (100 to 200 mg) used. In contrast, the negatively charged glibenclamide reduced the interfacial tension only when the polymer (i.e. PLGA or PCL) was in a lower weight proportion (e.g. 100-150 mg). When a higher weight range of polymer was involved (e.g. 150-200 mg), glibenclamide increased the interfacial tension of the respective polymer with the water phase. The neutral drug rapamycin was the only exception that either increased or decreased the interfacial tensions concurrently irrespective to the type and the amount of polymer.

In conjunction with the emulsion theory described above, we propose here a model to define the drug-induced interfacial tension change based on the interfacial charge density attributed to the drug-polymer interaction (Figure 6.10). In this model, it is hypothesized that a drug molecule interacts with a polymer at a specific molar ratio and that alters the surface hydrophobicity of the polymer. The electrical charge of the drug molecule causes the polymer to become more polar or hydrophilic and thus, reducing the interfacial tension of the polymer

with the water phase in the emulsion system. In fact, a similar role by a positively charged surfactant DMBA in lowering the oil-in-water interfacial tension has been reported [195]. The charge density of a polymer, as conferred by the interacting drug molecule, will be proportional to the amount of polymer used in the emulsion. While higher charge density may reduce the hydrophobicity of a polymer, accumulation of the like (e.g. negative charge) repels especially during phase separation when the drug-polymer concentrates. This is thermodynamically unfavorable. A consequence of such is the rise of the interfacial tension as in the case when high proportion of PLGA (i.e. 190 and 200 mg) or PCL (i.e. 150 mg) was mixed with the negatively charged glibenclamide (Table 6.3). In line with our hypothesis, lower amount of PLGA (i.e. 150 mg) or PCL (i.e. 100 and 110 mg) may chelate lesser glibenclamide, sufficiently to reduce the polymer hydrophobicity and the interfacial tension, and yet to reach the repulsive threshold. Since both PLGA and PCL are slightly polar with multiple carbonyl functional groups displaying partial negative charge (δ^-), interaction between the positively charged lidocaine with either polymer is thermodynamically favorable. This may therefore explain for the constant reduction in the interfacial tension when lidocaine was added to either polymer regardless of the polymer weight. Rapamycin, on the other hand, as an example of neutral drugs is not expected to affect the polymer hydrophobicity per se. Nonetheless, regardless of the amount of the polymer, supplementation of rapamycin appeared to increase or decrease the interfacial tension of both polymers with the water phase simultaneously, differed from the modulation effects mediated by the charged drug molecules. It is therefore likely that the highly hydrophobic rapamycin may have altered the polymer property and behavior distinctively, beyond the concept of charge density proposed here.

Although this charge density-driven model remains to be attested by other polymer and drug combinations, it provides the first molecular

insight into the possibilities of how a drug molecule may influence the behavior of a polymer in an emulsion system and thus, driving the formation of different microparticles, including both core-shell and Janus particles. The findings in this study clearly suggest that it is possible to encapsulate any drug molecule into a Janus structure using different polymer combinations by modulating the interfacial tensions between the polymers and the water phase. Our later model further submits that this can be achieved by interfacial charge density adjustment through tuning of the net charge of drug molecule (e.g. by altering the emulsion pH) or the amount of polymer. It is important to determine next the extent to which a drug molecule of interest may influence the interfacial tension for different types of polymers at various polymer concentrations. Such outcomes will be critical to provide a quantitative measurement or a standard to which degree each interfacial tension should be adjusted to become compatible for JP formation. Similarly, questions if multiple drugs can be incorporated to a JP based on the same principle, and if there is any drug-drug interaction that may influence the outcome should be addressed accordingly to broaden the applicability of this novel strategy.

Besides thermodynamic factors, other kinetic factors may dominate the microparticle formed in emulsion system as polymer chain movement is not perfectly free. This limitation is reflected in JP fabricated in this thesis. The poxy island formed by PLGA on top of PCL region proves such polymer movement tendency (Figure 6.1C). The reason of PLGA did not form a layer lies in the kinetic factors, where particle morphology is heavily affected by polymer precipitation rate. Kinetically, the movement of PLGA chain is restricted by PCL, thus PLGA at the inner core was not able to fully move out by the time of polymer precipitation [43]. Thus the core-shell structure particle (Figure 6.1.B2) showed PLGA is distributed on the surface of particle in pox form.

Figure 6.10 The proposed model for drug-induced interfacial tension change. The interfacial tension is referred to the energy cost per unit area associated with creation of an interphase between the polymer (i.e. PLGA or PCL dissolved in DCM) and the water phase. Both PLGA and PCLs are slightly polar with multiple carbonyl groups displaying partial negatively charge (δ^-) when exposed to the water. The lesser hydrophobic the polymer surface is, the better the interaction between polymer and water, and hence the lower the interfacial tension. Drug molecules, either positively or negatively charged, interact with the polymer at specific molar ratios, causing the polymer to become more hydrophilic and thus, reducing the interfacial tension at low polymer weight. At high polymer concentration, more drug molecules are expected to interact with the polymer chains, resulting in higher interfacial charge density. This is thermodynamically favorable for interaction between positively charged drug and the polymer due to the opposite attracts. However, it is unfavorable for negatively charged drug interacting with the polymer when the charge density reaches a repulsive threshold due the like repels. Consequently, addition of positively charged drug reduces the interfacial

tension while the negatively charged drug increases the interfacial tension at high polymer concentration.

Table 6.4 Alteration of interfacial tensions by drugs at different polymer weights

PLGA/DCM-PVA (γ_{12})				PCL/DCM-PVA (γ_{23})			
PLGA (mg)*	Drug			PCL (mg)*	Drug		
	Negative	Positive	Neutral		Negative	Positive	Neutral
200	↑	↓	↓	100	↓	↓	↓
190	↑	↓	↑	110	↓	↓	↑
150	↓	-	-	150	↑	-	-

*Each emulsion system consists of three phases, i.e. Phase 1: PLGA/DCM; Phase 2: PVA (Water) and Phase 3: PCL/DCM. Interfacial tensions between PLGA/DCM and PVA (γ_{12}) as well as PCL/DCM and PVA (γ_{23}) were determined using the pendant drop method. The increase or decrease of the interfacial tension (i.e. γ_{12} and γ_{23}), in the presence of drugs, was determined with reference to that of the blank particle (without drug addition) at the respective polymer weight. Drugs used including, i.e. glibenclamide (negatively charged), rapamycin (neutral) and lidocaine (positively charged).

6.3.3 Janus Particle Inhibits Burst Release Better Than Core-Shell Microparticle

Compared to monolayer PCL particle and PLGA particle, Janus particle displayed intermediate release attributed by fast releasing PCL hemisphere and slow releasing PLGA hemisphere. It is interesting that Janus particle showed a lower cumulative release than core-shell particle, which suggests its sustain-release has better inhibition on burst release. The reason of the inhibition of burst release could be the reduced surface to volume area of Janus particle. At the beginning ten days of release period, the PCL polymer contributes to majority of drug release. Janus particle, due to its unique morphology, has half the surface area over the same volume to release drug. Thus the cumulative release is reduced to about 50% at the early time points. The difference in cumulative release of core-shell and Janus was maintained in later stage of release may be caused by the same reason. As a drug carrier,

Janus particle certainly has its unique advantages in burst release inhibition and long-term delivery. Furthermore, Janus particle can release multiple drugs independently and simultaneously from each compartment. The control delivery can be achieved from configuration modification, less dependent on drug property in comparison to core-shell particle or multilayer particle.

With proper modification, we achieved a controlled phase separation of PLGA and PCL with model drugs encapsulated: glibenclamide (hydrophobic), rapamycin (highly hydrophobic) and lidocaine (hydrophilic). Our findings potentially provide a possible explanation to the reported disappearance of the Janus structure after drug loading [44], and a direction of strategies to overcome the problem without complicating the process. This enhances the chance of large scale production and commercial application of JP in pharmaceuticals and biomedicine. With the know-how of JP synthesis of different drugs, we are excited to move this work further for dual-drug encapsulation and release from JP. With the positive results obtained in earlier chapters, the potential of this novel form of dual-drug delivery as a treatment to islet transplantation in ACE is high. Unfortunately, due to the time limit of this dissertation, this part of the work will be part of the future work.

6.4 Summary

With the success of encapsulating the three model drugs carrying different net charge, it is proved that emulsion method can be used to synthesis Janus particle drug carrier in one-step, with high yield and precise control of morphology. The weight ratio of PLGA/PCL polymer was varied systematically to obtain the optimum polymer phase separation for Janus structure. The process of development identified an unexpected factor by which the net charge carried by the drug played a key role in altering polymer interaction. The findings offer a new perspective, which is the presence of drug could alter or change completely the particle morphology by influencing the interaction of polymers in emulsion system. Based on that, both core-shell particle and Janus particle were fabricated using the same technique, but with a simple change of condition. Our findings may thus serve as an important foundation for subsequent JP studies or fabrications based on the selected polymers. The one step synthesis method allows biodegradable and drug loaded Janus particle production in large scale with great commercialization potential.

6.5 References

- [23] W. L. Lee, E. Widjaja, and S. C. Loo, "One-step fabrication of triple-layered polymeric microparticles with layer localization of drugs as a novel drug-delivery system," *Small*, vol. 6, no. 9, pp. 1003-11, May 7 2010.
- [24] W. L. Lee and S. C. Loo, "Revolutionizing drug delivery through biodegradable multilayered particles," *J Drug Target*, vol. 20, no. 8, pp. 633-47, Sep 2012.
- [129] M. P. A. Lim, W. L. Lee, E. Widjaja, and S. C. J. Loo, "One-step fabrication of core-shell structured alginate-PLGA/PLLA microparticles as a novel drug delivery system for water soluble drugs," *Biomaterials Science*, vol. 1, no. 5, pp. 486-493, 2013.
- [168] C. L. Ventola, "The Nanomedicine Revolution: Part 1: Emerging Concepts," *Pharmacy and Therapeutics*, vol. 37, no. 9, pp. 512-525, 2012.
- [169] P.-G. de Gennes, "Soft Matter (Nobel Lecture)," *Angewandte Chemie International Edition in English*, vol. 31, no. 7, pp. 842-845, 1992.
- [170] S. Jiang, Q. Chen, M. Tripathy, E. Luijten, K. S. Schweizer, and S. Granick, "Janus particle synthesis and assembly," *Adv Mater*, vol. 22, no. 10, pp. 1060-71, Mar 12 2010.
- [171] A. Perro, S. Reculosa, S. Ravaine, E. Bourgeat-Lami, and E. Duguet, "Design and synthesis of Janus micro- and nanoparticles," *Journal of Materials Chemistry*, vol. 15, no. 35-36, p. 3745, 2005.
- [172] A. Walther and A. H. Muller, "Janus particles: synthesis, self-assembly, physical properties, and applications," *Chem Rev*, vol. 113, no. 7, pp. 5194-261, Jul 10 2013.
- [173] L. Carbone and P. D. Cozzoli, "Colloidal heterostructured nanocrystals: Synthesis and growth mechanisms," *Nano Today*, vol. 5, no. 5, pp. 449-493, // 2010.
- [174] J. Choi, Y. Zhao, D. Zhang, S. Chien, and Y. H. Lo, "Patterned Fluorescent Particles as Nanoprobes for the Investigation of

- Molecular Interactions," *Nano Letters*, vol. 3, no. 8, pp. 995-1000, 2003/08/01 2003.
- [175] Z. Bao *et al.*, "Toward Controllable Self-assembly of Microstructures: Selective Functionalization and Fabrication of Patterned Spheres," *Chemistry of Materials*, vol. 14, no. 1, pp. 24-26, 2002/01/01 2002.
- [176] Y. Xia, Y. Yin, Y. Lu, and J. McLellan, "Template-Assisted Self-Assembly of Spherical Colloids into Complex and Controllable Structures," *Advanced Functional Materials*, vol. 13, no. 12, pp. 907-918, 2003.
- [177] D. Wang and H. Mohwald, "Template-directed colloidal self-assembly - the route to 'top-down' nanochemical engineering," *Journal of Materials Chemistry*, 10.1039/B311283G vol. 14, no. 4, pp. 459-468, 2004.
- [178] S. Reculosa *et al.*, "Hybrid Dissymmetrical Colloidal Particles," *Chemistry of Materials*, vol. 17, no. 13, pp. 3338-3344, 2005/06/01 2005.
- [179] T. Teranishi, Y. Inoue, M. Nakaya, Y. Oumi, and T. Sano, "Nanoacorns: Anisotropically Phase-Segregated CoPd Sulfide Nanoparticles," *Journal of the American Chemical Society*, vol. 126, no. 32, pp. 9914-9915, 2004/08/01 2004.
- [180] A. Pfau, R. Sander, and S. Kirsch, "Orientational Ordering of Structured Polymeric Nanoparticles at Interfaces," *Langmuir*, vol. 18, no. 7, pp. 2880-2887, 2002/04/01 2002.
- [181] I. U. Khan *et al.*, "Microfluidic conceived drug loaded Janus particles in side-by-side capillaries device," *Int J Pharm*, vol. 473, no. 1-2, pp. 239-49, Oct 1 2014.
- [182] S. Yang *et al.*, "Microfluidic synthesis of multifunctional Janus particles for biomedical applications," *Lab Chip*, vol. 12, no. 12, pp. 2097-102, Jun 21 2012.
- [183] A. C. Misra, S. Bhaskar, N. Clay, and J. Lahann, "Multicompartmental particles for combined imaging and siRNA delivery," *Adv Mater*, vol. 24, no. 28, pp. 3850-6, Jul 24 2012.

- [184] J. Hu, S. Zhou, Y. Sun, X. Fang, and L. Wu, "Fabrication, properties and applications of Janus particles," *Chemical Society Reviews*, 10.1039/C2CS35032G vol. 41, no. 11, pp. 4356-4378, 2012.
- [185] X. Huang, Q. Qian, and Y. Wang, "Anisotropic Particles from a One-Pot Double Emulsion Induced by Partial Wetting and Their Triggered Release," *Small*, vol. 10, no. 7, pp. 1412-1420, 2014.
- [186] X. Cao, W. Li, T. Ma, and H. Dong, "One-step fabrication of polymeric hybrid particles with core-shell, patchy, patchy Janus and Janus architectures via a microfluidic-assisted phase separation process," *RSC Advances*, 10.1039/C5RA16504K vol. 5, no. 97, pp. 79969-79975, 2015.
- [187] F. S. Romanski, J. S. Winkler, R. C. Riccobene, and M. S. Tomassone, "Production and characterization of anisotropic particles from biodegradable materials," *Langmuir*, vol. 28, no. 8, pp. 3756-65, Feb 28 2012.
- [188] A. L. Tasker, J. P. Hitchcock, L. He, E. A. Baxter, S. Biggs, and O. J. Cayre, "The effect of surfactant chain length on the morphology of poly(methyl methacrylate) microcapsules for fragrance oil encapsulation," *Journal of Colloid and Interface Science*, vol. 484, pp. 10-16, 12/15/ 2016.
- [189] E. J. Pollauf and D. W. Pack, "Use of thermodynamic parameters for design of double-walled microsphere fabrication methods," *Biomaterials*, vol. 27, no. 14, pp. 2898-906, May 2006.
- [190] W. D. Harkins and A. Feldman, "FILMS. THE SPREADING OF LIQUIDS AND THE SPREADING COEFFICIENT," *Journal of the American Chemical Society*, vol. 44, no. 12, pp. 2665-2685, 1922/12/01 1922.
- [191] S. Torza and S. G. Mason, "Three-phase interactions in shear and electrical fields," *Journal of Colloid and Interface Science*, vol. 33, no. 1, pp. 67-83, 1970/05/01 1970.

- [192] H. K. Makadia and S. J. Siegel, "Poly Lactic-co-Glycolic Acid (PLGA) as Biodegradable Controlled Drug Delivery Carrier," *Polymers*, vol. 3, no. 3, pp. 1377-1397, 08/26 2011.
- [193] Y. C. Chen, V. Dimonie, and M. S. El-Aassei, "Theoretical aspects of developing latex particle morphology," *Pure and Applied Chemistry*, Article vol. 64, no. 11, pp. 1691-1696, 1992.
- [194] Y. Wang *et al.*, "Emulsion Interfacial Synthesis of Asymmetric Janus Particles," *Macromolecules*, vol. 44, no. 10, pp. 3787-3794, 2011/05/24 2011.
- [195] H.-Y. Kwon, J.-Y. Lee, S.-W. Choi, Y. Jang, and J.-H. Kim, "Preparation of PLGA nanoparticles containing estrogen by emulsification–diffusion method," *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, vol. 182, no. 1–3, pp. 123-130, 6/30/ 2001.

7 General Conclusion and Future Work

7.1 Perspective

In this dissertation, a biodegradable microparticle capable of drug delivery system was rationally designed and synthesized. It was then employed to control delivery of an immunosuppressant drug (rapamycin) and insulin release promotion drug (glibenclamide) in the Anterior Chamber of the Eye (ACE). The literature survey, in this regard, was focused on understanding the limitation of current islet transplantation in ACE and subsequently the current landscape of research in this field. FDA-approved polymers (PLGA and PCL) were selected to be the main material because of their excellent control in drug dosage and duration, biocompatibility with tissue, flexibility in design configuration and simplicity in transplantation for use in future clinical application [111]. This project is the first to create a sustained and local release in ACE environment using novel microparticle system. It gives the advantage of limiting the strong adverse effect caused by drug at the site of action.

This research work limits the studies to particulate type of carrier in micro size on account of their high efficiency in encapsulating both hydrophilic and hydrophobic drugs. It is highly feasible to achieve long term release (up to six months) using these type of carriers. The protective effect of polymeric carrier on highly unstable drugs might also contribute to the long term release. The animal model was limited to mice as the immune rejection of islets graft inside mice ACE model has been well-studied.

7.2 Summary of Results

This thesis investigated the key aspects of a product from conception to realization, ranging from formulation, delivery, transplantation, and animal trial. The specific conclusions derived from this dissertation are:

7.2.1 Rapamycin Microparticle Formulation Development

- a) The rapid degradation of rapamycin in aqueous medium was first understood through the degradation profile study. The microparticle system developed for rapamycin preserved the drug for a duration that is ten times longer in comparison to the free drug. In addition, the entrapped rapamycin was bioactive throughout the delivery period, which affirms the advantages of microparticle as a drug carrier.
- b) A library of rapamycin microparticle formulations were developed to tune the release over a 30 day period. Different means to modulate the release onset, release rate and period were evaluated. A deeper understanding of how different factors impact rapamycin release was built upon all the release profiles. Furthermore, the release rate was tuned for different application requirement. This is particularly important for multifunctional drugs like rapamycin.
- c) The inhibition effect of the formulations on cell proliferation showed trend similar to the release profiles. The incubation duration of lymphocyte cell (Jurkat Cell) and formulations was extended to 20 days, to measure the long-acting effect of rapamycin microparticle. The inhibition duration of the top two formulations (i.e. PLGA and PCL microparticle) were four times longer than an equivalent amount of free drug. The working dosage of rapamycin microparticle is much lesser yet had a better effect on cell. The sustained release of bioactive rapamycin from the formulations and the potential of improved therapeutic effect was affirmed.

7.2.2 Co-transplant Rapamycin Microparticle with Islets to ACE in Rodent Model

- a) The rapamycin microparticles were co-incubated with isolated mice islets for glucose-stimulated insulin secretion (GSIS) test.

The concern of rapamycin having a deleterious effect on the islets was not observed. The rapamycin microparticle showed no toxicity effect and did not reduce the ability of islets responding to glucose stimulation.

- b) The selected rapamycin microparticle formulations were successfully transplanted together with allogeneic islets to mice. This is a new ocular surgery procedure carried on rodent model and it is the first time that a biodegradable polymer particle was put into ACE.
- c) In the model reported here, the allogenic grafts with blank microparticles were completely rejected 18 days after transplantation, which is the same rejection time frame from prior studies in ACE without any drug carrier. This proves the materials selected as drug vehicle does not interfere with the biological response in the host.
- d) The onset of islet rejection in grafts co-transplanted with rapamycin-releasing microparticles was delayed by approximately 10 days, as compared to grafts with blank microparticles. The allogeneic islet grafts with rapamycin releasing microparticles were preserved much longer with some graft surviving more than 30 days after transplantation. This demonstrated the ability of the microparticles to release rapamycin in a sustained manner in ACE, and the released rapamycin was actively able to suppress the allogeneic islet rejection locally through using micro-scale drug carrier.

7.2.3 One-step Synthesis of Drug-loaded Janus Particle, a Novel Microparticle System for ACE

- a) One-step synthesis technique was established for Janus Particle (JP) encapsulating various type of drug based on emulsion evaporation approach. A hemisphere type JP was fabricated successfully with two-equal volume compartments. This hemisphere type JP has well-defined surface to volume ratio for

both compartments and hence provides better controlled-release in biomedical applications when individual drug is released from separate compartments.

- b) A series of investigations revealed that the formation of drug-loaded JP is thermodynamically-driven. The formation of JP is strongly governed by the emulsion interfacial tensions in accordance with the classic spreading and partial wetting principle. Such interfacial tensions were amenable, highly dependent on the interfacial charge density attributed to both drug and the polymer used. The change of interfacial tension under the influence of drug carrying different type of charge was studied in detail. It was identified as the reason for the difficulty in encapsulating drugs in JP without losing the Janus structure.
- c) Another key factor attributed to JP formation is the effect of polymer weight ratio. The interfacial tension analysis revealed that both weight ratio of polymers (i.e. PLGA and PCL) and the net charge of drug molecules (i.e. glibenclamide, tolbutamine, rapamycin and lidocaine) were critical to determine the type and the yield of microparticles. Hence, a model was proposed to provide a mechanistic insight into the fabrication of drug-loaded JP and paves the way for large-scale production of JP using a simplified, single-step emulsion solvent evaporation strategy.

7.3 Novel Contribution

This thesis significantly advances an unconventional application area of micro-scale drug carriers' potential in diabetes treatment. The results and observations reported in the thesis demonstrate that a carefully designed formulation could address the challenge of a highly promising diabetes treatment – islet transplantation. A procedure of co-transplanting living organism islets and synthetic biodegradable microparticles was established. We envisage this procedure will relieve patients from damage caused by systemic immune suppression, while improving glycemic control with reduced insulin dependence. In addition, the flexibility of the formulation configuration, efficiency in

drug-loading and customizable sustained release of the drug holds great potential for future development.

7.4 Recommendations for Future Directions

Based on the results and conclusions of this thesis, the following recommendations are made for future study in order to expand the knowledge on this subject:

a) **Test Rapamycin Microparticle in Non-human Primate (NHP)**

Model: Leveraging on the successful result in rodent model, testing the rapamycin release from microparticles in non-human primates receiving allogeneic non-human primate islets would be the next stage in animal trial. The islet physiology and pathology may differ from species to species [196]. For example, cytoarchitecture of human islets showed β -cell is not clustered as mice islets [197]. The arrangement of endocrined cells, including β -cell, has functional implication for islet cell function. Hence, it is important to expand the evaluation to NHP model for validation and discrepancy study.

b) **Longitudinal Observation of Localized Treatment Effect and Potential Eye Condition Change:**

Investigation of the rapamycin distribution after being released from microparticles will indicate whether any drug travel out from ACE to other part of the body. It is crucial to confirm that the therapeutic effect is localized instead of systemic. Besides that, long-term observation of host mice eye condition should be carried out, focusing on any potential eye infection or other conditions triggered by surgery. Rapamycin itself has anti-inflammatory effect which may be helpful in reducing the chance of eye infection. However, the existence of microparticle, before full degradation, may trigger any negative consequence in the ACE, which is not yet fully studied. Hence,

longitudinal observation (up to 6 month) of post-transplanted ACE is necessary.

c) **Improving the Lifelong Immunosuppressive Regimen:**

Fabrication of rapamycin-releasing microparticles that delivers for 100 days *in vivo* instead of the current 30 days would reduce the frequency of transplantation surgery. The duration of drug release from microparticle is dependent on rate of polymer degradation, the properties of drug encapsulated and its interaction with polymer. Multiple approaches can be employed to extend the delivery period. Firstly, delaying the polymer degradation time can extend the release duration. Secondly, modification of microparticle design (size, monolayer, multilayer) can modulate the release. Besides, other effective immunosuppressive agent such as AntiCD 145 (widely used as antihypertensive and vasodilator agents in non-human primates to achieve long-term graft survival rates) [198] can be used for microparticle encapsulation.

d) **Multi-drug Delivery for Therapeutic Effect More than Immunosuppression:**

Leverage on the newly designed Janus Particle (JP) that allows for co-release of rapamycin with exenatide or rapamycin with Sulfonylurea/GABA, so as to not only preserve but to also enhance islet function *in vivo*. The unique structure of JP could provide dual drug release from each compartment without interfering with each other. Therefore it is very useful when a combined therapy of small molecule drugs (hydrophobic) and biopharmaceutics (extreme hydrophilic) is required as the materials used for each compartment can be changed to suite the compatibility requirement. Delivering the multiple agents in ACE enables close and live observation of engrafted islets' response to different stimulating factors. How islets interact with various drug-loaded microparticles can be observed longitudinally up to single-cell resolution [86]. Studying

the live response from islets *in vivo* condition under different stimulation may reveal some insights of *in situ* islet functionality.

- e) **Encapsulate and Deliver Biosensor Cells:** The most challenging application to explore is to encapsulate and deliver biosensor cells into ACE. Biosensor cells are useful tools to evaluate hormone secretion in real time. These cells express specific G Protein Coupled Receptor (GPCR) that binds different hormones secreted by islets, such as glucagon and somatostatin. Upon binding of the hormone, the biosensor cell depolarizes, triggering an influx of calcium ions through voltage-gated calcium channels, which is proportional to the secreted hormone concentration [199]. Therefore, by measuring intracellular calcium dynamics by fluorescence microscopy *in vivo*, we can establish and monitor the secretory activity of different islet cells. A new synthesis method to encapsulate generic biosensor cell in microparticle systems needs to be developed. If needed, other encapsulation options such as scaffold in tissue engineering can be considered. Vascular endothelial growth factor (VEGF) may be delivered simultaneously with biosensor cell transplantation to facilitate vessel growth [200]. Upon successful encapsulation, the sensing ability will first be evaluated in cell culture conditions. Based on *in vitro* results, the biosensor cell encapsulation system will be transplanted into mice ACE for *in vivo* evaluation. These biosensor cells will provide feedback on *in situ* islets and gave more insights regarding islets function and survival.

7.5 References

- [86] S. Speier, D. Nyqvist, M. Kohler, A. Caicedo, I. B. Leibiger, and P. O. Berggren, "Noninvasive high-resolution in vivo imaging of cell biology in the anterior chamber of the mouse eye," *Nat Protoc*, vol. 3, no. 8, pp. 1278-86, 2008.
- [111] N. K. Varde and D. W. Pack, "Microspheres for controlled release drug delivery," (in English), *Expert Opinion on Biological Therapy*, Review vol. 4, no. 1, pp. 35-51, Jan 2004.
- [196] R. Arrojo e Drigo, Y. Ali, J. Diez, D. K. Srinivasan, P.-O. Berggren, and B. O. Boehm, "New insights into the architecture of the islet of Langerhans: a focused cross-species assessment," *Diabetologia*, journal article vol. 58, no. 10, pp. 2218-2228, October 01 2015.
- [197] O. Cabrera, D. M. Berman, N. S. Kenyon, C. Ricordi, P.-O. Berggren, and A. Caicedo, "The unique cytoarchitecture of human pancreatic islets has implications for islet cell function," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 7, pp. 2334-2339, 2006.
- [198] K. L. Kover, Z. Geng, D. M. Hess, C. D. Benjamin, and W. V. Moore, "Anti-CD154 (CD40L) prevents recurrence of diabetes in islet isografts in the DR-BB rat," (in eng), *Diabetes*, vol. 49, no. 10, pp. 1666-70, Oct 2000.
- [199] A. I. Laskowski and K. F. Medler, "Sodium-calcium exchangers contribute to the regulation of cytosolic calcium levels in mouse taste cells," *J Physiol*, vol. 587, no. Pt 16, pp. 4077-89, Aug 15 2009.
- [200] L. Deveza, J. Choi, and F. Yang, "Therapeutic Angiogenesis for Treating Cardiovascular Diseases," *Theranostics*, vol. 2, no. 8, pp. 801-814, 2012.