Bioactivity and controlled release studies of ranibizumab from polymeric carriers

Chua, Hui Yee

2018

Chua, H. Y. (2018). Bioactivity and controlled release studies of ranibizumab from polymeric carriers. Doctoral thesis, Nanyang Technological University, Singapore.

<http://hdl.handle.net/10356/73341>

<https://doi.org/10.32657/10356/73341>

NANYANG
TECHNOLOGICAL
UNIVERSITY

**BIOACTIVITY AND CONTROLLED RELEASE
STUDIES OF RANIBIZUMAB FROM POLYMERIC
CARRIERS**

CHUA HUI YEE
Interdisciplinary Graduate School
NTU Institute for Health Technologies

2018

**BIOACTIVITY AND CONTROLLED RELEASE
STUDIES OF RANIBIZUMAB FROM POLYMERIC
CARRIERS**

CHUA HUI YEE

**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

Acknowledgements

The author would like to express her utmost gratitude to the following people without whom the completion of the thesis would not be possible.

My heartfelt thanks to my supervisor Prof Subbu Venkatraman whom had provided close guidance and support for this project. I am very thankful for the opportunities provided, his patience and timely advise. I would also like to thank my co-supervisor Prof Peter Preiser whom had offered insightful discussion towards biological aspect of the project, encouragement as well as his concern for my personal well being. I am also very thankful to the clinician mentor Prof Tina Wong for her guidance, encouragement, time and advice she had generously provided. Additionally, I am also very grateful to Dr Rupesh Agrawal for the valuable inputs and guidance he had provided in and beyond the meetings despite his busy schedule.

I would like to extend my gratitude towards the lab members: Dr Yuan Siang, Dr Yee Shan, Dr Xu Wen, Dr Scott, Dr Jay, Xin Ying, Ramya, Abhijit, Jing Ni, Dulcia Meghali, Yang Fei, Su Yin, Anastasia, Jean-Marc, Pei Qi and Welly for the constant advice, concern, encouragement and laughter shared. These are the people I interact with on a daily basis and have provided me with a much-needed helping hand one way or another contributing to my growth in this journey. Additionally, I would also like to thank the administrative staff from IGS, NITHM and MSE including Suriani, Fiona, Seok Fen, Ellen, Charmaine, Sandy, Kin Sheng, Kek Koon and Nelson always going the extra mile to help facilitate the students needs.

I am especially grateful for the support, courage and understanding from my parents, and friends to always being by my side to lend a listening ear and supportive shoulders in my pursuit of postgraduate studies. This journey had certainly not been an easy one. I would not have been able to make it through alone. Their presence and support had given lifted me and given me the strength to push through.

Table of Contents

Acknowledgements.....	i
Table of Contents.....	ii
Tables.....	vi
Figures.....	vii
Abbreviations.....	xi
Abstract.....	xiii
 1. Introduction.....	 1
1.1. Background.....	1
1.2. Problem statement.....	2
1.3. Hypothesis.....	3
1.4. Objectives	3
1.5. Overview.....	4
1.6. Findings and outcomes	5
 2. Literature Review.....	 6
2.1. Pathology of age-related macular degeneration.....	6
2.1.1. Classification.....	6
2.1.2. Role of VEGF in CNV	7
2.1.3. Treatment options: From past to present	8
2.1.4. Current and potential administration method	10
2.2. Sustained drug delivery of VEGF antagonist	11
2.2.1. Overview of drug delivery	11
2.2.2. Sustained drug delivery in the eye	11
2.2.3. Limitations of current VEGF antagonist release	12
2.3. Techniques to fabricate PLGA particles	13
2.3.1. Solvent emulsion evaporation.....	13
2.3.2. Other fabrication techniques	15
2.4. Drug release	17
2.4.1. Issues in protein drug delivery	17
2.4.2. Hydrolytic degradation	19
2.5. Stability of proteins.....	19
2.5.1. Ranibizumab quantification	19

2.5.2. Intrinsic ranibizumab stability	20
2.5.3. Barriers to quantification of bioactive VEGF antagonist released	20
2.6. Summary	25
3. Materials and Methods.....	27
3.1. Materials	27
3.2. Methods.....	27
3.2.1. Cell culture.....	27
3.2.2. Protein characterization	28
3.2.3. Ranibizumab storage study	30
3.2.4. Particle preparation	30
3.2.5. Bioactivity studies of ranibizumab after emulsification	32
3.2.6. Characterization of ranibizumab loaded PLGA particles	33
3.2.7. Statistical analysis	36
4. Characterization of ranibizumab.....	37
4.1. Introduction.....	37
4.2. Effects of VEGF and ranibizumab on HUVECs proliferation	37
4.3. Stability of ranibizumab.....	39
4.3.1. Ranibizumab stability in PBS	39
4.3.2. Recovery of ranibizumab in various buffers.....	40
4.3.3. Desorption of ranibizumab from storage tubes.....	41
4.4. Ranibizumab bioactivity during emulsion	42
4.4.1. Effect of PVA amounts in the internal water phase during WOW NP emulsion.....	42
4.4.2. Effect of PVA amounts in the internal water phase during WOW MP emulsion	44
4.4.3. Comparing ranibizumab recovery for three emulsion techniques	45
4.4.4. Effect of structural integrity of ranibizumab after emulsion	45
4.5. Discussion	46
4.6. Summary	51
5. Incorporation of ranibizumab into PLGA particles	52
5.1. Introduction.....	52
5.2. Fabrication of PLGA particles	52

5.2.1. Effect of PVA concentration on the size of PLGA NPs	53
5.2.2. Effect of PVA concentrations on the size of PLGA MPs	54
5.2.3. Effect of PLGA concentration on the size of SOW MPs.....	55
5.3. Ranibizumab loading into PLGA particles	57
5.3.1. Characterizing ranibizumab loaded PLGA particles	57
5.3.2. In vitro release of ranibizumab from three emulsion methods	59
5.4. Modification of SOW MP	60
5.4.1. Effect of particle size on release kinetics of SOW MP.....	60
5.4.2. Effect of polymer nature on the release kinetics of SOW MP.....	61
5.5. <i>In vitro</i> degradation of ranibizumab encapsulated particles	63
5.5.1. Comparisons of the three fabrication methods	63
5.5.2. Comparisons of the effect of size.....	66
5.5.3. Comparisons of the effect of polymer physiochemical properties	68
5.6. Ranibizumab distribution within SOW MP and WOW MP	69
5.7. Release of SOW MP in different buffers	70
5.8. Discussion	72
5.9. Summary	77
6. Modulating ranibizumab release from WOW MP with sodium chloride.....	79
6.1. Introduction.....	79
6.2. Effect of NaCl concentration on morphology of PLGA particles	80
6.3. Ranibizumab loading into WOW MP with various NaCl concentrations.....	82
6.3.1. Characterizing ranibizumab loaded WOW MPs.....	82
6.3.2. In vitro release of ranibizumab from WOW MPs.....	83
6.4. <i>In vitro</i> degradation of WOW particles emulsified with various NaCl concentrations.....	86
6.5. Fabrication of SOW MP NaCl.....	88
6.5.1. Characterizing ranibizumab loaded SOW MP NaCl	88
6.5.2. <i>In vitro</i> release of ranibizumab from SOW MP NaCl	90
6.6. <i>In vitro</i> degradation of ranibizumab encapsulated SOW MP NaCl.....	91
6.7. Effect of ranibizumab VEGF binding ability on incubation with NaCl	92
6.8. Discussion	93
6.9. Summary	98

7. Conclusions.....	100
8. Recommendations.....	105
8.1. Dosing perspective.....	105
8.2. In vivo testing for the suppression of AMD with sustained release	106
8.3. Further improvements to the drug delivery system	107
8.3.1. Increase bioactivity of the released ranibizumab from PLGA MP	
NaCl	107
8.3.2. Further suppression in the burst release of ranibizumab	108
8.3.3. Increase loading of ranibizumab in a bulk implant.....	110
9. Reference	111

Tables

Table 3.1 Summary of buffer components used in storage study	30
Table 3.2 Summary of fabricated PLGA particles.....	31
Table 5.1 Characteristics of respective fabrication techniques examined include: Size of fabricated particles, E.E., and bioactivity calculated at the end of the release.	58
Table 5.2 Effect of different polymer concentration on the size, E.E. and bioactivity of fabricated SOW MP with reference to 20% SOW MP.	61
Table 5.3 Effect of polymer hydrophobicity on the size and E.E. of fabricated SOW MP with reference to SOW 0.4A.....	62
Table 6.1 Characteristics of respective fabrication techniques examined include: Size of fabricated particles, E.E., and bioactivity calculated at the end of the release.	83
Table 6.2 Fit of release from SOW MP Mid NaCl to various release models. Rate of release (k) and correlation function (R^2) was calculated.	84
Table 6.3 Characteristics of respective fabrication techniques examined include: Size of fabricated particles, E.E., and bioactivity calculated at the end of the release.	90

Figures

Figure 2.1 Illustration depicting CNV progression into the retina affecting vision [27].	7
Figure 2.2 Role of VEGF leading to the cascade of events associated with angiogenesis [8].	8
Figure 2.3 Factors affecting the solidification rate and consequently the encapsulation efficiency during solvent emulsion evaporation [62].	14
Figure 2.4 Chemical structure of PLGA polymer and its degradation byproducts after hydrolysis.	19
Figure 2.5 Illustration for the protein behavior during WOW where the red and green portions indicate hydrophobic and hydrophilic parts of a protein (A) Protein in the oil phase. (B) Organic solvent interface. (C) Non encapsulated proteins. (D) Oil-water interface. (E) Water channels in the particles. (F) Pores in particles [24].	21
Figure 4.1 (A) Effects of VEGF concentration on HUVEC proliferation presented in terms of fold increase of cell number at specified VEGF concentration normalized to cell number at 0 ng/mL VEGF treatment. (B) Effects of ranibizumab concentration on HUVEC proliferation presented in terms of fold increase of cell number at specified ranibizumab concentration co-incubated with 12.5 ng/mL VEGF normalized to cell number at 0 ng/mL ranibizumab treatment. Categories with p-value<0.05 are indicated with *, categories with p-value<0.01 are indicated with **, categories with p-value<0.001 are indicated with *** and categories with p-value<0.0001 are indicated with ****.	38
Figure 4.2. Percentage ranibizumab recovery in PBS as measured by ELISA after storage at 37 °C for 24 hours. (A) In the microgram concentration range between 5- 40 µg/mL; P= 0.9. (B) In the nanogram concentration range between 6.25- 50 ng/mL; P< 0.001. P values indicate significant difference compared to fresh ranibizumab.	39
Figure 4.3 Percentage ranibizumab recovery in various buffers (a) PBS; P< 0.001 (b) Intrinsic buffer; P= 0.6 (c) Serum buffer; P= 0.17 (d) BSA buffer; P< 0.05 as measured by ELISA in the concentration range between 6.25- 50 ng/mL when stored at 37°C for 24 hours. P values indicate significant difference compared to fresh ranibizumab.	40
Figure 4.4 Recovery of ranibizumab loss due to storage in PBS through flushing receptor containers with intrinsic buffer over 10 days. Recovery is measured in terms of bioactive ranibizumab as measured with ELISA.	41
Figure 4.5 Effect of PVA concentration on total and bioactive ranibizumab recovery during the nanoparticle emulsification process in the absence of PLGA polymer. 1% PVA was found to be optimal for total and bioactive recovery of ranibizumab. Categories with p-value<0.05 are indicated with *, categories with p-value<0.01 are indicated with **, categories with p-value<0.001 are	

	indicated with *** and categories with p-value<0.0001 are indicated with ****.	43
Figure 4.6	Effect of PVA concentration on total and active ranibizumab recovery during the microparticle emulsification process in the absence of PLGA polymer. 1% PVA was again found to be optimal for total and bioactive recovery of ranibizumab. Categories with p-value<0.05 are indicated with *, categories with p-value<0.01 are indicated with **, categories with p-value<0.001 are indicated with *** and categories with p-value<0.0001 are indicated with ****.	44
Figure 4.7	Effect of three emulsion methods: WOW NP; WOW MP; SOW MP on Ranibizumab recovery in the absence of PLGA polymer. WOW NP and WOW MP was stabilized by 1% PVA. No significant loss in bioactivity of the ranibizumab during the respective fabrication methods was observed. Hence, these three methods are suitable for future loading studies.	45
Figure 4.8	Coomassie-stained SDS-PAGE gel of ranibizumab after various particle fabrication emulsions. No aggregation or fragmentation of the ranibizumab was observed.	46
Figure 5.1	The effect of PVA concentration in the internal aqueous phase on the size of WOW NP. (A) As visualized under SEM. (B) Size distribution of the particles in terms of diameter. Categories with p-value<0.05 are indicated with *, categories with p-value<0.01 are indicated with **, categories with p-value<0.001 are indicated with *** and categories with p-value<0.0001 are indicated with ****.	54
Figure 5.2	The effect of PVA concentration in the internal aqueous phase on the size and morphology of WOW MP. (A) As visualized under SEM. (B) Size distribution of the particles in terms of diameter.	55
Figure 5.3	The effect of polymer concentration on the size of SOW MP. (A) As visualized under SEM. (B) Size distribution of the particles in terms of diameter.	57
Figure 5.4	Summary of ranibizumab after release, as a percentage of the drug used during encapsulation- including percentage active ranibizumab released, calculated percentage of non active ranibizumab released and percentage of residual ranibizumab. These three characteristic sums up to give the encapsulation efficiency of each system.	58
Figure 5.5	(A) In vitro cumulative percentage release profile of bioactive ranibizumab from WOW NP, WOW MP and SOW MP over a period of at least 15 days as measured by ELISA. WOW NP and WOW MP demonstrated a burst release followed by a plateau. With SOW MP a sustained release of 15 days was observed. (B) In vitro daily release release amounts of ranibizumab from WOW NP, WOW MP and SOW MP over a period of at least 15 days. WOW NP and WOW MP demonstrated a burst release followed decreased release amounts for 7 days. With SOW MP a burst release was observed followed by a sustained release of 15 days.	60

Figure 5.6 In vitro cumulative percentage release profile of ranibizumab from smaller sized SOW MP over a period of at least 15 days.	61
Figure 5.7 The hydrophobicity of the polymer used to fabricate SOW MP was examined. (A) In vitro cumulative percentage release profile of ranibizumab from SOW MP made with different hydrophobicity over a period of at least 15 days. (B) Size and surface morphology difference was visualized under SEM at 100X for SOW 0.4A and SOW 0.4E.	62
Figure 5.8 In vitro degradation of ranibizumab loaded PLGA particles fabricated with different techniques characterized by (A) Molecular weight change and (B) Gravimetric measurements of particles weight loss over time mass.	64
Figure 5.9 SEM micrographs chronicling the degradation of (A) WOW NP, (B) WOW MP, (C) SOW MP at 15,000 X, and 500 X magnification following in vitro degradation up to a period of 30 days.	66
Figure 5.10 In vitro degradation of ranibizumab loaded 10 wt% SOW MP up to a period of 30 days. (A) Characterized by molecular weight change of PLGA polymer. (B) Gravimetric measurements of particles weight loss over time mass. (C) SEM micrographs chronicling particle degradation at 500 X.	67
Figure 5.11 In vitro degradation of ranibizumab loaded SOW MP fabricated with end-capped ester group up to a period of 30 days. (A) Characterized by molecular weight change of PLGA polymer. (B) Gravimetric measurements of particles weight loss over time mass. (C) SEM micrographs chronicling SOW MP 0.4E degradation at 500 X.	69
Figure 5.12 Pure component raman spectra estimates and their associated score images obtained from: (A) Ranibizumab loaded SOW MP. (B) Ranibizumab loaded WOW MP.	70
Figure 5.13 In vitro cumulative percentage release profile of ranibizumab from various buffers including IB at pH 5.5, PBS at pH 5.5, PBS at pH 7.4, PBS at pH 8.5.	71
Figure 5.14 In vitro cumulative percentage release profile of ranibizumab into BSA coated and non BSA coated receptor chambers.	72
Figure 6.1 SEM micrographs of PLGA microparticles prepared with WOW and SOW emulsion methods showing the surface morphology and cross section at 5000X and 1000X magnification respectively. In the WOW emulsion method, NaCl concentration used in the W ₂ phase was varied from 0 to 1000 mM. SOW MP is also used as a reference.	81
Figure 6.2 Summary of ranibizumab after release from WOW MP fabricated with mid and high NaCl concentrations, as a percentage of the drug used during encapsulation- including percentage active ranibizumab released, calculated percentage of non active ranibizumab released and percentage of residual ranibizumab as calculated at the end of release. These three characteristic sums up to give the encapsulation efficiency of each system.	83

Figure 6.3	In vitro cumulative percentage release profile of bioactive ranibizumab from WOW MP fabricated with mid and high concentrations of NaCl over a period 40 days as measured by ELISA (Secondary y axis). WOW MP fabricated without NaCl was used as a reference (Primary y axis). WOW MP fabricated with NaCl in the W ₂ phase followed a trend of minimal burst, slow release followed by second burst after 20 days.	84
Figure 6.4	In vitro daily release release amounts of ranibizumab from WOW MP fabricated with mid and high concentrations of NaCl over a period 40 days. WOW MP fabricated with NaCl in the W ₂ phase showed minimal burst release with release of at least 35 days.	85
Figure 6.5	In vitro degradation of ranibizumab loaded WOW MP fabricated with mid and high concentrations of NaCl up to a period of 30 days. (A) Characterized by molecular weight change of PLGA polymer. (B) Mass loss of PLGA particles (C) SEM micrographs chronicling particle degradation at magnification of 500 X.	88
Figure 6.6	SEM micrographs showing the cross section of (a) SOW MP (b) SOW MP fabricated with NaCl in W ₂	89
Figure 6.7	Summary of ranibizumab after release from SOW MP fabricated with mid NaCl concentrations, as a percentage of the drug used during encapsulation- including percentage active ranibizumab released, calculated percentage of non active ranibizumab released and percentage of residual ranibizumab. These three characteristic sums up to give the encapsulation efficiency of each system.	89
Figure 6.8	In vitro cumulative percentage release profile of bioactive ranibizumab from SOW MP fabricated with NaCl over a period 20 days as measured by ELISA. SOW MP fabricated without NaCl was used as a reference. SOW MP fabricated with NaCl in the W ₂ phase followed the same trend of release as SOW MP.	90
Figure 6.9	In vitro daily release release amounts of ranibizumab from SOW MP fabricated with NaCl over a period 20 days. SOW MP fabricated with NaCl in the W ₂ phase showed a more prolonged release.	91
Figure 6.10	In vitro degradation of ranibizumab loaded SOW MP fabricated with NaCl up to a period of 30 days. (A) Characterized by molecular weight change of PLGA polymer. (B) Mass loss of PLGA particles. (C) SEM micrographs chronicling particle degradation at magnification of 500 X.	92
Figure 6.11	Recovery of ranibizumab measured by ELISA on storage of ranibizumab in NaCl buffer after 4 hours to simulate potential contact during PLGA particle fabrication.	93
Figure 6.12	Schematic diagram depicting the mechanism for solidification of WOW MP with and without NaCl in the external aqueous phase W ₂ [87].	95
Figure 8.1	In vitro cumulative release profile of ranibizumab from SOW MP and hybrid DPPC_SOW MP.	109

Abbreviations

Acquired immune deficiency syndrome	AIDS
Age-related macular degeneration	AMD
Bicinchoninic acid protein assay	microBCA
Bovine serum albumin	BSA
Choroidal neovascularization	CNV
Dichloromethane	DCM
Drug delivery systems	DDS
Encapsulation efficiency	EE
Endothelial cell growth media	EGM-2
Enzyme linked immunoassay	ELISA
Ethylene vinyl acetate	EVA
Fetal bovine serum	FBS
Field emission scanning electron microscope	FeSEM
Fragment antigen-binding	Fab
Fragment crystallizable	Fc
High performance liquid chromatography	HPLC
Human epidermal growth factor	hEGF
Human fibroblast growth factor	hFGF- β
Human umbilical vein endothelial cells	HUVECs
Immunoglobulin G	IgG
Insulin-like growth factor	R ³ -IGF-I
Intrinsic buffer	IB
Intrinsic viscosity	IV
Isoelectric point	pI
Matrix metalloproteinases	MMPs
Microparticle	MP
Monoclonal antibody	mAb
Nanoparticle	NP
Neonatal Fc receptor	FcRn
Phosphate buffer saline	PBS
Platelet-derived growth factor-B	PDGF-B

Poly vinyl alcohol	PVA
Poly-lactide	PLA
Poly(ethylene glycol)	PEG
Poly(lactide-co-glycolide)	PLGA
Retinal pigment epithelium	RPE
Scanning electron microscope	SEM
Size exclusion chromatography	SEC
Sodium dodecyl sulfate polyacrylamide gel electrophoresis	SDS-PAGE
Solid-in-oil-in-oil	SOO
Solid-in-oil-in-water	SOW
Supercritical fluid	SCF
Transmission electron microscopy	TEM
Tumour necrosis factor- α	TNF- α
Vascular endothelial growth factor	VEGF
Vascular endothelial growth factor receptor	VEGFR
Vascular endothelial growth factor receptor 1	VEGFR1
Wash buffer	WB
Water-in-oil-in-water	WOW

Abstract

Age-related macular degeneration (AMD) is the leading cause of irreversible blindness in developed societies. Ranibizumab is an anti-angiogenic agent commonly prescribed to treat AMD. However several issues associated with its frequent intravitreal injections have intensified the need for a sustained delivery system. This thesis aims to explore the feasibility of using synthetic polymer- poly(lactic-co-glycolic) acid (PLGA), as the carrier for sustained ranibizumab delivery, at the same time addressing the common issues encountered while incorporating delicate hydrophilic drug such as ranibizumab inside polymeric carriers using emulsions techniques. Protein instabilities that aroused due to carrier release condition or fabrication process have been evaluated using in-house enzyme linked immunoassay for quantification of bioactive ranibizumab. Significant proteins loss was observed due to adsorption only when the protein content was low (in nanograms range), which could be prevented with the use of appropriate buffers, leveraging on excipients and pH of the buffer. In addition, ranibizumab stability as a result of fabrication stresses was simulated and it was demonstrated that ranibizumab bioactivity was indeed affected during emulsion, where ranibizumab might have denatured at the organic-water interface. This denaturation can be salvaged through the use of polyvinyl alcohol as the emulsifying agent at an optimal concentration of 1% in the first aqueous phase during the water-in-oil-in-water (WOW) emulsion process. Notably, in the solid-in-oil-in-water (SOW) emulsions, the use of solid ranibizumab proteins was found to be stable without the need of emulsification agent. *In vitro* release study demonstrated that particles formulated from SOW possessed relatively lower burst release and ranibizumab released was sustained for 15 days while particles formulated from WOW suffered from burst release with no further release. The sustained release observed from SOW particles was deemed to be diffusion controlled. It is worth to note that the released ranibizumab from SOW particles retained the highest bioactivity, which could be attributed to the reduced interaction between the encapsulated protein and polymer, where the proteins in the interior of the solid protein particle is shielded with less

homogenous drug distribution. The applicability of SOW technique in preserving ranibizumab activity and achieving sustained release was further demonstrated through varying the particle size and the hydrophobicity of the polymer used. Despite suffering from burst release, WOW particles possessed higher encapsulation efficiency an important factor for its translational potential. Porous surface morphology, identified as the main cause for the burst release in WOW particles needs to be addressed. For the first time, non-porous ranibizumab-loaded WOW particles were fabricated through manipulating the osmotic pressure between different phases via varying sodium chloride (NaCl) concentration in the external aqueous phase. These WOW particles fabricated with NaCl in the second aqueous phase (W₂) maintained their typical high drug encapsulation efficiency. Notably, these particles displayed minimal burst release and biphasic profiles for the subsequent release: the initial slow release could be attributed to the diffusional release, while the faster release after 2 weeks was due to bulk degradation that corroborated with mass loss of the particles. The release was improved and continued for 40 days. In conclusion, the use of a biodegradable polymer to incorporate and release bioactive ranibizumab over a period of 40 days was highlighted in this work. This is a significant achievement, and points the way to particulate or implantable systems that can deliver ranibizumab against various conditions, for example AMD and diabetic retinopathy.

1. Introduction

1.1. Background

Age-related macular degeneration (AMD) is one of the causes of irreversible blindness in the developed societies [1-3] and its impact is expected to increase as the population age and life expectancy increase. Exudative AMD, notably choroidal neovascularization (CNV) is identified by abnormal growth of new blood vessels from existing vasculature, growing through gaps the Bruch's membrane progressing between or through the Bruch's membrane and retinal pigment epithelium (RPE). Rapid loss of vision occurs as the vessels grow towards the fovea, disrupting the photoreceptors in the retinal and distorting the images viewed [4]. Despite CNV accounting for about only 10% of AMD cases, it accounts for the majority (80% to 90%) of blindness due to the disease [2].

A variety of methods have been employed to treat AMD. Variable success had been achieved for managing of CNV with non-pharmacological methods such as laser photocoagulation [5], photodynamic therapy and submacular surgery [6]. Meanwhile, various proangiogenic and antiangiogenic factors have been identified to modulate the pathology of CNV. Angiogenic promoters include vascular endothelial growth factor (VEGF), platelet-derived growth factor-B (PDGF-B), tumour necrosis factor- α (TNF- α), integrins, matrix metalloproteinases (MMPs), components of complement cascade, ligand receptor systems[7]. Amongst the proangiogenic and antiangiogenic factors discovered, the most intensively studied is VEGF whose role in induction of angiogenesis and enhancement of vascular permeability have been identified [8]. Inhibition of VEGF culminated in the development of pharmacological intervention method where anti-angiogenic drugs such as pegatanib (macugen[®]), bevacizumab (avastin[®]), ranibizumab (lucentis[®]) and aflibercept (eylea[®]) is delivered to the diseased site. Since its FDA approval in 2006, ranibizumab has been the gold standard for treatment of AMD.

Current treatment of AMD involves administration of VEGF antagonists to the diseased site at the posterior segment of the eye. Apart from drug potency, the method of administration also determines the translational potential of the drug from bench to bedside as it regulates the drug bioavailability and consequently its

therapeutic effects at the diseased site [9]. Existing therapies for wet AMD necessitates monthly or bimonthly invasive intravitreal injections of various VEGF antagonists into the vitreous space. Due to the proximity of the injection site to retina, choroid and RPE, this method maximizes drug concentrations reaching the diseased site with minimal systemic side effects. However, repeated injections are associated with injection related adverse events including increased intraocular pressure, retinal detachment and endophthalmitis [10]. Moreover the tolerability, inconvenience and cost of such frequent injections highlight the need for sustained delivery systems capable of providing therapeutic dosage to the diseased site for an extended period of time to reduce the administration frequency.

With the advancement in biotechnology, various carriers [11-16] have been explored for delivering VEGF antagonists. Among them, synthetic polymers such as poly(lactide-co-glycolide) (PLGA) have been widely used in biomedical applications for their biodegradability, reproducibility and tailorability [17, 18]. For example, one FDA approved ophthalmic application of PLGA is its incorporation in the dexamethasone implant, Ozurdex® [19]. Several efforts involving the use of PLGA carriers in releasing VEGF antagonists have demonstrated promising potentials in treating AMD in different *in vivo* settings [14, 20, 21], highlighting the feasibility of PLGA carriers for sustaining VEGF antagonist release to address the need for frequent VEGF antagonist administration.

1.2. Problem statement

In the event of releasing therapeutic agents from PLGA based particles, solvent emulsion-evaporation techniques are widely used due to low barrier entry [22-24]. This method is not without its limitations especially when more delicate drugs like proteins are encapsulated. Instabilities due to processing methods, formulation choice and incompatible experimental conditions could limit the bioactivity of released ranibizumab. However, therapeutic activity of ranibizumab released from most of the reported drug delivery systems remains largely unknown as the associated *in vitro* release data showed total protein release [14-16, 25], where the biological activity of the released antagonists was not reported. It should be noted

that maintenance of ranibizumab bioactivity is crucial to its pharmaceutical efficacy.

Additionally, while incorporating VEGF antagonists for CNV indication, polymeric particles inevitably suffered from burst release unless a secondary barrier was employed [12, 13, 26]. The additional process complicates the fabrication for ranibizumab carriers, at the same time introducing unknown factors to the drug loading (possible drug loss in the additional process) and protein activity. *Hence, it would be desirable to have a simple yet controllable PLGA-ranibizumab carriers fabrication process, producing carriers with minimal burst release and high drug loading.*

1.3. Hypothesis

It is of utmost importance to address the abovementioned issues so as to achieve protein delivery for AMD treatment sustainably. It is hypothesized that the sustained release of ranibizumab can be realized while achieving sufficient drug loading and retention in bioactivity of ranibizumab in polymeric carriers.

1.4. Objectives

To validate the hypothesis above, the degradable polymer poly (lactide-co-glycolide) or PLGA, is explored as the carrier for releasing ranibizumab sustainably, at the same time addressing the common issues encountered while incorporating proteins inside polymeric carriers, namely protein instability and burst release.

Hence, the first objective of this thesis includes establishing quantification methods to measure bioactive ranibizumab and understanding the basic properties of ranibizumab. This sets the foundation for measuring and maintaining the biological activity of the released VEGF antagonist. To achieve this objective, the following key activities has been conducted:

- To optimize assays and characterization methods to quantitatively and qualitatively analyse ranibizumab ability to bind to VEGF.
- These methods will be used to elucidate sources of destabilization during fabrication and storage. Appropriate strategies will be formulated to reduce its effects.

The secondary objective of this thesis is to identify an optimal fabrication method for bioactive and sustained release ranibizumab release, assessed by methods established in the first objective. The following key activities were conducted:

- To fabricate ranibizumab-loaded PLGA particles without compromising the antigen binding ability of the incorporated VEGF antagonist.
- To quantify the *in vitro* release of ranibizumab from these carriers using the dialysis method and to assess the bioactivity of the released VEGF antagonist.
- To study the mechanisms of the observed drug release profiles, by evaluating *in vitro* degradation of the particles
- To identify PLGA carrier with the best sustained release profile and to explore the use of polymers with different physicochemical properties and their effects on the release kinetics.

The last objective is to apply appropriate strategies based on the mechanisms of release identified in second objective to prolong the sustained release effect. The following key activities was conducted:

- To optimize the fabrication method to obtain the ideal particle identified.
- To elucidate the mechanism of release such that an optimal fabrication method that provides a longer period of sustained release can be identified.

1.5. Overview

This thesis is divided into eight chapters.

In chapter 1, background information on AMD, its pathology and treatment method is summarized forming the basis motivating the need for sustained ranibizumab release. The hypothesis, objectives of the project and strategies to achieve then are discussed. In chapter 2, a review of current literature applicable for general protein drug delivery to more specific VEGF antagonist delivery is summarized. Gaps in the literature for VEGF antagonist delivery are highlighted forming the niche of our study. In chapter 3, experimental methods used in the study is documented in detail. The recurrent quantification methods of Ranibizumab, which will be used throughout the thesis is clearly explained.

The results and discussion of this thesis is presented in chapters 4, 5 and 6. In chapter 4, we seek to understand and characterize the basic properties of ranibizumab. This includes establishing the inhibitory effects of ranibizumab on endothelial cells to validate future quantification study and stability studies of ranibizumab. In chapter 5, the fabrication technique used for encapsulating ranibizumab in PLGA particles was reviewed. This includes looking at the stability of ranibizumab after fabrication, encapsulation efficiency, *in vitro* release profiles and polymer degradation of each individual fabrication technique. In chapter 6, we combine the understanding of ranibizumab encapsulation in PLGA particles from chapter 5 and proposed the usage of osmotic pressure to achieve an ideal encapsulation and sustained release profile. Characterization of the particles fabricated with this technique was also studied. In the final chapter, conclusions were drawn on the work that was investigated and future recommendations were proposed.

1.6. Findings and outcomes

This research led to several novel outcomes:

1. Demonstrating that the process of fabricating the anti-VEGF loaded PLGA nanoparticles plays a significant role in retaining bioactivity, as well as achieving sustained release. The best process was identified to be the solid-in-oil-in-water (SOW) emulsion technique.
2. Elucidating the mechanisms and successfully reducing burst release through employing the use of osmotic gradient in the water-in-oil-in-water (WOW) emulsion technique. PLGA particles of high encapsulation efficiency and sustained release at least 40 days was obtained.

2. Literature Review

2.1. Pathology of age-related macular degeneration

2.1.1. Classification

Aged-related macular degeneration (AMD) is the main cause of irreversible blindness in the developed societies [1-3] and its impact is expected to increase throughout world as the population age and life expectancy increases. Exudative AMD, notably choroidal neovascularization (CNV) is identified by abnormal growth of new blood vessels from existing vasculature, growing through gaps the Bruch's membrane progressing between or through the Bruch's membrane and retinal pigment epithelium (RPE) as illustrated in Figure 2.1. Despite CNV accounting for about only 10% of AMD cases, it accounts for the majority (80% to 90%) of blindness due to the disease [2]. Some of the proposed theories to cause CNV include: inflammatory processes due to drusen accumulation or significant choroidal thickness and choriocapillaries lumina size reduction with age [4]. All of these theories stem from hypoxia, the condition of insufficient oxygen supply. The outer retina, unable to receive sufficient oxygen from the adjacent choriocapillaries triggers molecular regulators such as vascular endothelial growth factor (VEGF) secretion and consequently choroidal neovascular membranes formation. Neovascular membranes are highly permeable, discharging fluid, protein and blood, which eventually cause the RPE to detach from the Bruch's membrane. This condition is exacerbated as the vessels grow towards the fovea resulting in rapid loss of vision and distorting the images viewed as the photoreceptors in the retinal are disrupted [4].

Figure 2.1 Illustration depicting CNV progression into the retina affecting vision [27].

2.1.2. Role of VEGF in CNV

Various proangiogenic and antiangiogenic factors have been identified to modulate the pathology of CNV [7] the most intensively studied is VEGF whose role in induction of angiogenesis and enhancement of vascular permeability have been identified as illustrated in Figure 2.2 [8]. VEGF is a 45 kDa homodimeric glycoprotein that stimulates vasculogenesis- de novo blood vessel formation in an embryo and angiogenesis- neovascularization from existing blood vessels [7, 28]. VEGF is crucial for tissue homeostasis and wound healing but VEGF overexpression can lead to conditions such as tumour growth in cancer, rheumatoid arthritis and neovascular AMD. In humans VEGF-A is the most widely studied for AMD with nine major VEGF-A isoforms derived from alternate post-translational exon splicing, the most widely expressed of all VEGF₁₆₅. VEGF-A exerts its effect on the endothelial cells through two tyrosine kinases, vascular endothelial growth factor receptor 1 (VEGFR1) and VEGFR2 initiating a cascade of intracellular signals triggering extracellular events. The role of VEGFR1 is highly debatable as it is thought to be a “decoy” receptor [8] which sequester and prevents binding of VEGF-A to VEGFR2. VEGFR-2 is believed to be a dominant mitogenic mediator despite its lower binding affinity as compared to VEGFR-1 [8, 29].

Figure 2.2 Role of VEGF leading to the cascade of events associated with angiogenesis [8].

2.1.3. Treatment options: From past to present

A variety of non-pharmacological and pharmacological therapies have been employed to treat AMD. Before the introduction of pharmacological methods for managing AMD, non-pharmacological treatment methods such as laser photocoagulation [30, 31] and submacular surgery [6] enjoyed variable success. Photodynamic therapy with verteporfin marked the beginning of pharmacological therapies via a two-step procedure involving activation with laser ensuing intravenous injection for a more targeted treatment without damaging healthy tissue in the surroundings.

Subsequently, a more specific pharmacological method targeting VEGF-A was introduced [8]. Inhibition of VEGF-A through anti-angiogenic drugs culminated in the development of pegaptanib (macugen®), ranibizumab (lucentis®), aflibercept (eylea®) and bevacizumab (avastin®). Pegaptanib an oligonucleotide aptamer with a high specificity for only VEGF₁₆₅, represents the first targeted VEGF antagonist therapy indicated for treating AMD [30, 32, 33]. However pegaptanib binds to the amino acid within the heparin binding domain of VEGF not present in the breakdown products of VEGF and smaller isoforms [34]. While pegaptanib modestly reduced the rate of vision lost, the improvements in visual benefits was exceeded by the newer recombinant antibodies therapies [35].

The newer VEGF antagonists include: bevacizumab, ranibizumab and aflibercept, are proteinaceous in nature as they are recombinant antibodies or parts of it. Antibodies are Y shape glycoproteins consisting of two light and two heavy chains, each chain having a constant and variable region [36]. Fragment antigen-binding (Fab) region is the variable domain responsible for the antibody's specificity while the fragment crystallizable (Fc) region regulates binding to leukocytes and complement system activation [33]. Antibody fragments are made up of only the Fab fragment. They have shorter half-life and are eliminated more quickly due to the absence of the Fc region and consequently missing protection by neonatal Fc receptor (FcRn) [37]. While prolonged elimination half-life has its advantage in terms of sustained drug delivery, it also increases the extent of systemic exposure.

Bevacizumab a humanized monoclonal antibody (mAb), though primarily developed for cancer to bind to VEGF-A, is used off labeled for CNV indication largely due to its low cost. With a molecular weight of 149 kDa, diffusibility of bevacizumab across the retina layers to reach the choroid was initially a concern [8, 38, 39]. Furthermore, presence of Fc portion in the immunoglobulin G (IgG) could provoke complement mediated or cell dependent toxicity and the longer half-life of IgG molecule increases systemic exposure [27, 38]. To avoid the above concerns ranibizumab was developed. Ranibizumab has been the gold standard for wet AMD treatment, although bevacizumab had been used off-labeled by ophthalmologists.

Ranibizumab a 48 kDa Fab of a recombinant humanized mAb specifically designed for intraocular use against all isoforms of VEGF-A is FDA approved for CNV indication. It binds to all bioactive forms of VEGF-A and consequently reduces downstream permeability and angiogenesis in the endothelial cells. To reduce the number of invasive intravitreal administration, ranibizumab was designed higher binding affinity to VEGF than bevacizumab through a series of recombinant DNA and phage-display selection steps. In bioassays measuring mitogenesis, ranibizumab was 5-20 times more potent than bevacizumab on a molar basis [38, 40].

The newest FDA approved VEGF antagonist - aflibercept is a 115 kDa soluble fusion protein comprising of second Ig domain of human VEGFR1 and third Ig domain VEGFR2 fused with Fc portion of the IgG1 [41]. With improved binding affinity and faster association, aflibercept exhibits higher potency than anti-VEGF antibodies [29]. Even though aflibercept requires less frequent dosing regiment as the presence of the Fc region increases its half-life, concerns remain on the potential correlation between suppression of systemic VEGF elevating chances of adverse events [42, 43]. Due to the above considerations, ranibizumab is still widely used for anti VEGF treatment of CNV indication.

2.1.4. Current and potential administration method

While drug development is crucial, the translational potential of the drug from bench to bedside has to be coupled with the most efficient administrative method to achieve therapeutic concentrations at the diseased site[9]. The administration method determines bioavailability of the drugs at the diseased site and consequently therapeutic effects. Currently known routes for drug administration to the eye include topical, systemic, intraocular and periocular avenues but only some methods can deliver therapeutic concentrations to the retinal tissues.

Some possible routes of delivery to the posterior poles are briefly discussed. Topical drug administration, although a noninvasive delivery method, is met with little success for delivery due to the drug clearance mechanisms and ocular barriers present [44, 45]. Meanwhile, systemically administered drug via intravenous or oral routes is limited by the transport of drugs across the blood retinal barriers and high dosage has to be administered to reach therapeutic levels. Thus, systemic administration of naked drug is the poorest in terms of efficacy and safety. Thirdly, periocular pathways for drug delivery include retrobulbar, peribulbar, subtenon and subconjunctival route. This noninvasive method delivery is safer than intravitreal injections exploiting permeability of sclera for drug diffusion into the retinal [46]. An inadequacy for this method is the low bioavailability of drug impeded by the static anatomical barriers, dynamic clearance mechanisms and outward clearance flow [47]. Lastly, intraocular injection at the suprachoroidal space is another potential route of delivery that obviates the need for macromolecules diffusion through the sclera and inner limiting membrane barrier. However, due to the delicate nature of this injection area a more robust method is needed to for delivery to this space [48].

Currently, intravitreal injection into the vitreous space is the most effective known administration method for various VEGF antagonists. Due to the proximity of the injection site to retina, choroid and RPE, this method maximizes drug concentrations reaching the diseased site with minimal systemic side effects. However, repeated injections are associated with injection related adverse events including increased intraocular pressure, retinal detachment and endophthalmitis [10]. Moreover monthly treatment [49, 50] or monitoring at the ophthalmologist is taxing for the patients and their caregivers. Less frequent dosing schedules can be achieved either with sustained release drug delivery systems (DDS) or drugs modified to increase affinity or reduce clearance.

2.2.Sustained drug delivery of VEGF antagonist

2.2.1. Overview of drug delivery

A clear need and market for a DDS capable of providing therapeutic dosage of ranibizumab to the diseased site, for an extended period of time, to reduce the administration frequency is evident. Ideally, drug delivery system should provide (i) controlled and sustained release at the therapeutic concentration; (ii) increased residence time at the diseased site for continued drug delivery; and (iii) ease of device application to improve patient compliance and comfort.

2.2.2. Sustained drug delivery in the eye

In the market, there are some clinically used devices providing sustained release for the ocular application although it is mostly for diseases in the posterior segment of the eye [51]. Vitrasert® is a nonbiodegradable intravitreal implant used to treat cytomegalovirus retinitis associated with acquired immune deficiency syndrome (AIDS) [52]. It delivers ganciclovir for 5 to 8 months after administration and its release is regulated by poly vinyl alcohol (PVA) and ethylene vinyl acetate (EVA). Retisert® and Iluvien® is another non-biodegradable implant delivering flucinolone acetonide for treating chronic noninfectious uveitis and diabetic macular edema respectively [53, 54]. Both devices provide a sustained release period of up to 3 years. While nonbiodegradable implant provides a more precise control over the release, the surgical implant removal needed entails its associated risks. Meanwhile, Ozurdex® is a biodegradable intravitreal implant made from poly(lactide-co-glycolide acid)

(PLGA) polymer delivering dexamethasone for treatment of macular edema with its effects sustained for up to 6 months [55]. Although most ocular sustained release systems in the market are made up of implants, release can be sustained from (i) implants; (ii) injectable colloidal systems; and (iii) injectable hydrogels where the last two systems are more easily injected.

2.2.3. Limitations of current VEGF antagonist release

The concept of sustained release from VEGF antagonists for this indication has been examined in various carriers including liposomes [14], hydrogels [15, 56] and polymers [11, 13, 16, 20, 21, 25, 57]. Amongst the reported material, poly (dl-lactide-co-glycolide) is widely used for biomedical applications due to its biocompatible, biodegradable and diversity of tunable physicochemical properties [17, 18, 58]. One FDA approved ophthalmic application of PLGA is its incorporation in the dexamethasone implant, Ozurdex® [19] where the pharmacokinetics achieved by this implant is similar to pulse administration of corticosteroids systemically [59].

Although sustained release of VEGF antagonist for CNV indication had been reported, many of these polymeric particles suffered from burst release with very little subsequent release [11-13, 26]. Burst release of VEGF antagonist with these particles were only suppressed when a secondary barrier is used: chitosan nanoparticles within PLGA microparticles [12], poly-lactide (PLA) nanoparticles within PLGA microparticles [13] and PLGA microparticles within hydrogel [26]. Moreover while there have been previous reports of bevacizumab release from PLGA particles in the *in vivo* settings [14, 20, 21], the demonstrated sustained release effect might not be substantial without thorough *in vitro* release studies. Since different animal models and evaluation indicators were used for *in vivo* studies [13, 14, 56], *in vitro* studies allow for more standardized comparison among different carriers and enable more effective analysis for drug carrier optimization. Often even if the *in vitro* release is reported, it is in terms of total protein release [14-16, 25] where the VEGF binding activity of the released antagonists remains vague. To address this gap, enzyme linked immunoassay (ELISA) can be used to quantify VEGF-binding capacity of ranibizumab released *in vitro* hence focusing on the maintenance of its bioactivity during fabrication to ensure pharmaceutical efficacy.

2.3. Techniques to fabricate PLGA particles

Some common methods used to fabricate PLGA particles include: (i) Solvent emulsion evaporation; (ii) Phase separation (coacervation); (iii) Spray drying; and (iv) Supercritical fluid technology.

2.3.1. Solvent emulsion evaporation

Due to the ease of fabrication, solvent emulsion-evaporation is the most common method used to encapsulate hydrophilic drug in PLGA particles. Generally, the aqueous drug solution (W_1) is emulsified in a polymeric solution dissolved in organic solvents (O) such as dichloromethane and ethyl acetate by sonication or high shear homogenize [22, 60, 61]. This emulsion (W_1/O) is added to a stabilizer (usually poly (vinyl alcohol)) containing second aqueous phase (W_2) to form a double emulsion ($W_1/O/W_2$). The organic solvent is removed by evaporation or extraction into second aqueous phase to yield solidified particles. To maximize loading efficiency of the drug, the first W_1/O can be stabilized by addition of surfactants such as polysorbates can be added to both W_1 and O phases respectively to prevent loss of drug through coalescence of W_1 with W_2 [22]. Solvent emulsion evaporation is not without its limitations especially when encapsulating more delicate drugs like proteins. Instabilities due to processing methods, formulation choice and exposure to degradation byproducts will be further elaborated together with strategies to overcome them in Section 2.5.3.1.

High drug encapsulation efficiency into the particles would be ideal to support a longer release period. In the emulsion-based technique, encapsulation efficiency depends on the speed of polymeric precipitation where drug diffusion into the continuous phase is reduced as the polymer solidifies thereby increasing drug encapsulation [62]. Some factors that determine the polymer solidification rate include: (i) Solubility of polymer in organic solvent; (ii) Polymer concentration; and (iii) Water to oil ratio in the primary emulsion. Polymers with higher solubility in the solvent take longer to solidify resulting in lower encapsulation efficiency. The molecular weight, lactide to glycolide ratio and end capped groups are properties of the polymer which affects its solubility in the solvent [63]. Meanwhile, at higher polymer concentrations, solubility reduces leading to faster polymer precipitation and eventually moderating drug loss. Additionally, drug loss by diffusion in the polymer droplets is retarded with the higher viscosity

accompanying the higher polymer concentration. Lastly, the water to oil ratio is critical to the stability of the primary emulsion. Unstable internalization of the water-drug phase results in loss to the external aqueous phase and consequently lowers encapsulation efficiency. Emulsifying agents such as PVA is frequently added to stabilize this primary emulsion [23].

Figure 2.3 Factors affecting the solidification rate and consequently the encapsulation efficiency during solvent emulsion evaporation [62].

To avoid the loss of protein bioactivity due to adsorption and denaturation at the water/oil interface, modification of the emulsion to solid-in-oil-in-water (SOW) has been developed. The procedure remains the same except that solid proteins are now dispersed in the oil phase for encapsulation. Generally a suspension of solid drug or protein will yield a lower encapsulation efficiency in the SOW emulsion than WOW, as the larger solid protein volume may not co-localize with the carrier polymer as much and result in higher loss as it dissolves into the aqueous continuous phase. This difference has also been reported elsewhere [64, 65]. Higher encapsulation efficiency in SOW particles can also be achieved by reducing the initial size of solid protein powder [66]. This can be accomplished by protein micronization through methods such as co-lyophilization with PEG polymer [67, 68], spray drying of proteins [64, 69] and precipitation [70] to obtain solid protein powders.

Nanocarriers can be made from solvent emulsion-evaporation and they offer an edge with at least one dimension smaller than 1000 nm. The benefits of nano size include: optical clarity in the vitreous, size range capable of particulate transport and reduced settling velocity consequently reduced particle clearance [19] explains the tremendous effort in developing nanocarrier based DDS for the posterior segment of the eye. While nano sized particles bring about the above benefits, challenges also stem from its small size. Compared to microparticles, the reservoir for sustained release is significantly lowered consequently lower encapsulation efficiency due to the smaller volume for encapsulation. This problem is even more conspicuous when loading large macromolecular VEGF antagonist such as bevacizumab and ranibizumab with previously reported hydrodynamic radius of 6.3 and 4.2 nm respectively into nanoparticles [71]. Moreover burst release is expected with the close proximity- in the scale of nanometers of the drug to the surrounding environment and the large surface area in the nanoparticles allowing a larger surface for drug to escape [72, 73]. Song et al., had encapsulated vincristine sulfate and quercetin representing hydrophilic and hydrophobic drug respectively and both particles experienced burst release [74]. Although encapsulation of vincristine sulfate and quercetin into PLGA NP managed to prolong the release of both drugs compared to release of pure drug, a burst release followed by a plateau within 24 hours was still reported.

2.3.2. Other fabrication techniques

Building on the emulsion technique, the other techniques for fabricating PLGA particles will be briefly discussed. Phase separation also known as coacervation is a water-oil-oil emulsion where the primary emulsion is added to a nonsolvent phase which the polymer is not soluble to extract the polymer solvent [75]. A large volume of hardening phase where is then added to extract both oil phase for solidification of the particles. This process can be divided into three steps: (i) Coating polymer solution phase separate; (ii) Coacervate adsorbs onto the drug particles; and (iii) Solidification of the microspheres. The final microspheres characteristic depends on the kinetics of solvent removal, which is controlled by formulation and process variables [76]. However, capsules produced in this process are prone to agglomeration and precise control of experimental conditions is required.

Spray drying is made up of three main processes: (i) Atomization; (ii) Drying; and (ii) Collection [77]. The atomizer is used to disperse the feed solution usually an emulsion into a droplet. This atomized droplet dry as it loses its solvent to the preheated drying gas, in a process largely controlled by the temperature of the system. On drying, the separated particles are collected at the bottom of the chamber. Factors such as drying air flow rate, air humidity, inlet temperature, atomizing air flow, feed rate and dispersion solvent are factors to be tuned to control the particles [75, 78]. While spray drying offers a quick, convenient, high throughput fabrication method for mass production of polymeric particles, yield for small batches needs to be improved and drying parameter have to be optimised for temperature sensitive drug [79].

More recently, supercritical fluid (SCF) technology provides an alternative method for formation of porous polymeric particles by SCF extraction from an emulsion. At temperatures and pressures above their critical points, critical fluids behaves as a single phase with intermediate gaseous like diffusivities and liquid liked densities [80, 81]. Carbon dioxide (CO₂) is commonly used as the SCF for pharmaceutical applications as it requires lower critical pressure and temperature. Under high pressure, CO₂ dissolves in the polymer and on rapid pressure drop the dissolved CO₂ expands the polymer inducing pore formation. The high affinity of supercritical CO₂ to organic solvents also reduces residual dichlormethane in PLGA microspheres [80]. Yandrapu et al., has used supercritical CO₂ to encapsulate bevacizumab coated PLA nanoparticles within PLGA microparticle system to offer sustained release of bioactive bevacizumab for 4 months [13]. While SCF CO₂ presents various advantages, agglomeration of particles leading to irregular shapes and the low yield of PLGA needs to be addressed [82].

Despite progress in methods used to fabricate PLGA particles, most of the techniques build on and involves the modification of emulsion techniques. Solvent emulsion evaporation provides a technique where particles can be produced in small batches, low agglomeration and regular shape with basic and readily available equipment. In this thesis modifications of the solvent-emulsion evaporation will be used to encapsulate ranibizumab.

2.4. Drug release

Fredenberg et al., had classified four drug release mechanisms for PLGA particles under diffusion through water filled pores, erosion of polymer, diffusion through polymer and osmotic pumping [83]. Generally protein release in the initial stage is governed by diffusion through water filled pores followed by erosion in the later phases. Protein drug release from polymeric carriers has been limited by issues such as: (i) initial burst release; (ii) incomplete release; and (iii) instability of encapsulated proteins. Instability of encapsulated proteins can arise from processing, formulation and experimental conditions and it will be discussed separately under Section 2.5.

2.4.1. Issues in protein drug delivery

Uncontrolled burst release is undesirable, tantamount to injection of free drug from a therapeutic standpoint. As the onset of PLGA degradation varies from weeks to months, burst release that occurs in the first few days is due to ineffective control over diffusion based release. Burst release can be largely attributed to two possibilities- (i) drug that is loosely associated on the surface or embedded near the particle surface [84]; (ii) morphology of the microparticle allow easy drug release through preformed water channels [85]. Drug accumulates nearer to the particle surface during solidification as it diffuses from the drug containing aqueous phase through the semi-solidified polymer to the continuous phase. As the time the polymer remains in its semi-solidified form of the polymer is extended, more time is available for such diffusion. Hence, the rate of solvent extraction determines the time the polymer stay in its semi-solidified form and subsequently the accumulation of drug near the particle surface. Secondly, the morphology of the microspheres specifically the porosity also plays an important role in regulating the release. Porosity is influenced by the preparation temperature, solvent removal rate and internal aqueous phase volume during solvent extraction [86]. Lastly, some formulation parameters contributing to higher burst release include low molecular weight, lower polymer concentration, more hydrophilic polymer and higher drug loading [62].

One method to control morphology specifically the porosity of the outer sphere skin is by introducing osmotic gradient across the oil phase through the addition of osmotic agents such as sucrose or NaCl [87]. The mechanism where WOW MP

solidification is altered had been proposed. During solidification, exchange of water molecules between the internal and external aqueous phase occurs although limited by the solubility of water in the oil phase. Protein diffusion is restricted due to its size and miscibility while NaCl salt diffusion is also minimal due its low solubility. With the osmotic gradient, water diffuses from the internal aqueous phase to the external continuous phase resulting in a less porous particle due to the lowered internal aqueous volume. Meanwhile, when water is the external continuous phase there is no reduction in the volume of the internal aqueous phase with the absence of osmotic gradient. The internal aqueous phase tend to coalesce as the polymer hardens and condenses resulting in a porous matrix prone to burst release. NaCl increases the polarity of the external continuous phase resulting in reduced solubility of DCM in this phase and slower rate of organic solvent extraction. Eventually, the dispersed phase stays in the transitional semi-solid state for a longer time allowing for more exchange between the two aqueous phases and consequently denser particles are formed. Moreover, not much is known towards the encapsulation of ranibizumab with NaCl in the W₂ phase specifically the concentration of NaCl needed. The NaCl concentration will affect the final morphology of the particles formed. Additionally, NaCl concentration may also affect the stability of ranibizumab during encapsulation or release.

Protein drug release has been limited by incomplete release that was classically reported in many polymeric systems [65, 66, 70, 88]. Park et al. had investigated the non-release of hen egg-white lysozyme [89]. Through a methodical study where varying concentrations of sodium chloride, guanidine hydrochloride, and sodium dodecyl sulfate separately added into the release medium suggested that ionic interaction, non-covalent aggregation and non-specific adsorption between lysozyme and PLGA could be disrupted. Incomplete release has been attributed to the interaction of proteins with polymer but mainly stem from the protein instability arising during polymer degradation. To reduce instabilities, strategies include stabilizing protein during release and mechanisms to avoid protein destabilization due to polymer degradation with some of which discussed in Section 2.5.

2.4.2. Hydrolytic degradation

Beyond the diffusion release phase, drug release from PLGA is controlled by degradation of the particles. PLGA degrades as a result of hydrolytic scission of its ester bonds into shorter oligomers during hydrolysis resulting in a decrease in molecular weight as illustrated in Figure 2.4. Upon sufficient degradation, at a reported molecular weight of 1100 Da, oligomers are water soluble and were able to diffuse out of the microspheres [90]. This process of erosion of the particles leads to a subsequent mass loss as measured by gravimetric methods.

PLGA microparticles frequently experience bulk degradation where the onset of molecular weight loss occurred much earlier than the erosion of the polymers [91, 92]. The rate of bulk degradation is size dependent. As the size of the devices increases, heterogeneous hydrolytic degradation becomes prominent where degradation in the core is more pronounced than that at the surface. Accelerated degradation in the core is attributed to the autocatalytic action of the acidic degradation byproducts where the delay of its removal exacerbates degradation. Acidic byproducts lead to an accompanying drop in pH of the microenvironment [93], whose concomitant effect on the protein stability will be discussed in the Section 2.5. Since there is flexibility in tuning the polymer physicochemical properties by varying factors such as the molecular weight, copolymer composition and polymer end group, degradation rates of PLGA can be controlled [90, 91].

Figure 2.4 Chemical structure of PLGA polymer and its degradation byproducts after hydrolysis.

2.5. Stability of proteins

2.5.1. Ranibizumab quantification

Two different techniques can be used to quantify ranibizumab. ELISA can be used to quantify bioactive ranibizumab as it measures for VEGF binding capable ranibizumab. On the other hand, bicinchoninic acid protein assay (microBCA) can

be used to measure total protein as it quantifies peptide bonds and certain amino acid groups irrespective of activity of the protein.

2.5.2. Intrinsic ranibizumab stability

The intrinsic stability of ranibizumab under physiological pH of 7.4 has been documented [94]. Properties such as melting temperature, conformational change and VEGF binding ability evaluated over a period of 30 days and across an extended range of pH conditions showed that ranibizumab was stable. In addition to maintaining its secondary and tertiary structure, ranibizumab maintained its VEGF binding ability under the mentioned conditions.

2.5.3. Barriers to quantification of bioactive VEGF antagonist released

Injection of drug delivery system capable of releasing VEGF antagonist is similar to pulsed drug administration [59]. Apart from the period of sustained drug release it is equally important for ranibizumab to maintain its pharmaceutical efficacy during release. Stability can be compromised during processing or due to the formulation choice. Moreover choice of experimental conditions can potentially introduce artifacts affecting assessment credibility. Since each protein is unique, protein encapsulation in polymers should follow a well thought out trial and error approach following assessment and analysis of protein's characteristic, eliminating unsuitable methods.

2.5.3.1. Processing instability

Processing effects on the protein instability is narrowed to the destabilizing effects during the fabrication of particles.

Hydrophilic-hydrophobic interface is a detrimental destabilization factor for protein denaturation [95, 96]. Chances for protein exposure to such interface can be found at: the water-organic solvent interfaces formed during emulsion and contact between proteins and their hydrophobic carriers during release into the aqueous receptor buffer. This interface encourages the protein to unravel exposing the buried hydrophobic area to the hydrophobic environments as illustrated in Figure 2.5. One method to reduce exposure of proteins to such interfaces is to lyophilize proteins and encapsulate it in its solid form by solid/oil/water (SOW) or even solid/oil/oil (SOO) emulsification. In fact pure organic solvents do not in itself denature proteins. It is the mixture of organic solvents and aqueous protein

solution that encourages conformational mobility of protein impairing the protein secondary structures supporting denaturation [97].

Figure 2.5 Illustration for the protein behavior during WOW where the red and green portions indicate hydrophobic and hydrophilic parts of a protein (A) Protein in the oil phase. (B) Organic solvent interface. (C) Non encapsulated proteins. (D) Oil-water interface. (E) Water channels in the particles. (F) Pores in particles [24].

Proteins are sensitive to mechanical forces during fabrication such as high pressure extrusion for size reduction or shearing during homogenization process. Shearing increases the exposure of proteins to air/water and water/solvent interfaces aggravating the likelihood of aggregation. Nonetheless its effect is minimal compared to presence of hydrophobic-hydrophilic interface [98].

Proteins are known to be vulnerable at high temperatures, and local temperatures can be raised during emulsification. Higher temperatures provide the protein with the energy to unfold when the interactions such as hydrophobic interactions, electrostatic forces, intermolecular forces and even covalent bonds are overcome. At sufficiently high temperatures, the energy barrier against protein conformational entropy is overcome [99] resulting in the protein losing its conformation and subsequently the ability to perform its function. The effect of shear and temperature stresses can be assuaged by reducing the intensity and exposure time to the shear forces and temperature.

2.5.3.2. *Formulation instability*

Formulation triggered instability refers to the choice of materials such as polymer and excipients that influences protein stability.

Protein concentration is an important consideration for particulate formulations due to losses from adsorption at low concentration and aggregation at high concentrations [100]. Such adsorption may eventually lead to aggregation, denaturation and precipitation all of which impairing the protein's biological activity. It is postulated that once the protein saturates the interface, it shields the remaining proteins from the surface preventing further aggregation [98]. Hence protein loss due to adsorption is especially prominent at lower concentrations because of the higher percentage loss. Some methods to reduce adsorption include: increasing protein concentration, siliconizing the container to reduce adsorption, adding excipients or macromolecules such as bovine serum albumin (BSA) with higher surface activity. On the other spectrum, excessively concentrated protein solutions increase protein contact points resulting in aggregation due to transient exposure of hydrophobic domains [100].

Varsochian et al., identified that more than 90% of bevacizumab activity could be retained when another stabilizer, albumin, is used [20]. That was attributed to the protective ability of albumin during emulsion due to its surfactant-like properties by acting as a sacrificial protein in place of bevacizumab. At high concentrations these sacrificial proteins preferentially compete with the drug of interest at the interface reducing the exposure of bevacizumab. However as these sacrificial proteins themselves have been reported to aggregate at the interface, it might cause immunogenic reaction *in vivo* [66] and is not desirable from a regulatory point of view [101].

Often the polymer choice depends on the targeted release profile and some reported properties impeding stable protein release are discussed. Firstly, the use of hydrophobic polymers has been reported to result in a slower and more incomplete release due to hydrophobic contacts [88]. To counter this, blends of slow degrading hydrophobic poly (lactic acid) PLA and pore forming hydrophilic poly(ethylene glycol) (PEG) was shown to be able to minimise local acid microenvironment while allowing sustained and complete release of BSA [102].

Secondly, when proteins are formulated below its isoelectric point (pI), positively charged proteins can interact with the negatively charged PLGA free carboxyl end groups leading to incomplete protein release. This effect is less prominent when carboxyl end-capped PLGA is used. Thirdly, the polymer used affects the rate of polymer degradation and acidic degradation byproducts generated. PLGA degradation produces acidic byproducts accompanied by a drop in pH. Proteins are prone to aggregation outside its stable pH range. Aggregated proteins will not be released from the carriers henceforth reducing the effectiveness of sustained release. Incorporation of basic salts can be considered to neutralize the acidic byproducts.

A variety of additives such as sugars, surfactants, proteins and amino acids have the propensity to reduce protein aggregation [103]. Sugars such as sucrose and trehalose are hypothesized to stabilize proteins with the preferential hydration theory when water is in excess and water replacement theory when the protein is in the solid state[104]. In the preferential hydration theory, trehalose is ordered around the water molecules, reducing the protein hydration radius and conformational mobility of the protein. In the water replacement theory, trehalose maintains the protein three-dimensional structure forming hydrogen bond with protein in replacement of the lost water. Nonionic surfactants on the other hand reduce protein adsorption, aggregation and unfolding by preferentially adsorbing to such surfaces lowering the system's surface tension. Proteins such as albumins and gelatins are also used for protective purposes as they preferentially adsorb to surfaces. However, protein based excipients adds another layer of complexity to the system in terms of the complicating analytical methods for protein stability as well as its use from the regulatory point of view. Commonly used amino acids for stabilizing proteins include histidine, arginine and glycine. Amino acids act in the capacity by preferential hydration, as a buffering agent to stabilize pH which potentially causes protein aggregation and as an antioxidant. These additives mentioned above can be added to the protein formulation before subsequent formulation steps.

2.5.3.3. Experimental conditions

Incompatible *in vitro* experimental condition where the release profile of proteins from the carrier is acquired is often overlooked thereby generating misleading

results leading to fallacious conclusions drawn. This can be avoided if experimental parameters compromising protein stability are elucidated allowing protein stabilizing strategies to be formulated. Experimental conditions that have to be considered include the release medium and sampling method.

During *in vitro* release, entrapped proteins are generally susceptible to degradation such as deamidation, peptide bond hydrolysis and aggregation as they are exposed to moisture and elevated temperature [105]. The most common release medium used for *in vitro* studies is phosphate buffer saline (PBS) simulating physiological conditions of pH 7.4 at 37 °C. While some proteins are relatively stable in the above conditions, other researchers have preferred assessing protein release in optimal receptor medium such as acetate, tris-HCl or glycine buffers[86, 106]. Although Jiang et al., had shown good *in vitro in vivo* correlations with pH 2.5 glycine buffers [106], factors such as pH and ionic strength of the receptor buffer frequently alter the release rates of protein with accompanying change in carrier degradation rates and protein stability. Particularly, extreme buffer pH accelerates polymer degradation while incompatible pH will trigger protein aggregation inducing to slower and incomplete release. More often than not, increase buffer ionic strength decreases the release rates [86]. The choice of release medium depends on whether release of bioactive protein or simulation of *in vivo* conditions takes precedence. While physiologically simulating buffer could establish comparable *in vivo* protein release profile if no prior knowledge of protein instability in physiological conditions is known, the cause of protein destabilization becomes convoluted.

Varshochian et al. demonstrated this instability in their *in vitro* release study where the cumulative active and total amount of bevacizumab released reduced over time, as the receptor buffer was not replenished at each time point. However, bevacizumab released from the same particles into *ex vivo* rabbit vitreous could be detected. As their DDS had been optimized to ensure bioactivity, some loss can be attributed to the loss in bevacizumab activity after release due to storage in PBS [21]. Thus the conundrum for the choice of the release medium: between a physiologically simulating buffer or a buffer where the released protein remains stable in, remains an important consideration [88, 107]. While it is important to ensure that the released protein remains quantifiable, great care must be taken

when choosing the release media as components in the release medium had been shown to vastly alter the release patterns [108].

In vitro drug release of proteins is complicated by its fragile nature. Some sampling methods include separation method, dialysis of particulates, and continuous flow [88]. In the separation method, particles and released drugs are separated by centrifugation. However the centrifugal force can affect the particle degradation and consequently alter the protein release profile. To simulate *in vivo* conditions better, the speed of centrifugation and receptor volume have to be optimized. Meanwhile with the membrane diffusion method, particles are contained in a dialysis bag where the products can be released through the pores into the receptor. However, this method requires a large receptor volume, which results in a low concentration of protein collected. Lastly the continuous flow method closely simulates *in vivo* conditions as particles are exposed to receptor buffer that are continuously replaced. Although this method reduces the effect of medium contamination from the byproducts of degraded particles, this method is not popular due to the complexity involved.

2.6. Summary

The impact of AMD, currently the main cause of irreversible blindness in the developed societies, is expected to increase as life expectancy increase. VEGF is the most widely studied factor that modulates CNV by angiogenesis. This culminated in the development of VEGF antagonists such as ranibizumab. Currently, ranibizumab is administered intravitreally monthly. This invasive injection is commonly associated with injection related adverse events, retinal detachment and endophthalmitis. A clear need for a less frequent dosing can be achieved with drug delivery system capable of providing sustained and bioactive ranibizumab release. The concept of sustained VEGF antagonist release has been examined in a number of carriers such as liposomes, hydrogels and polymers. Moreover the translation of these systems from bench to bedside has not been reported. Precedence of PLGA polymer usage for ocular medical devices on the market highlights the translational potential of drug delivery systems made from PLGA polymers. Improvements to the current reported sustained release devices include the need to reduce burst release and to ensure that the released ranibizumab remains bioactive. Thus the techniques to fabricate PLGA particles,

factors affecting drug release and protein stability were reviewed in the literature review. While there are a few fabrication techniques, solvent emulsion evaporation remain the most commonly used, as it is a simple technique that yields particles of different properties by tuning the fabrication parameters. Fabricated drug delivery system for protein in particular frequently suffers from burst release due to pores or drug accumulation on the particle surface. Incomplete release also commonly observed is attributed to instabilities of proteins during fabrication and release. In view of these issues, this dissertation aims to elucidate the instabilities and burst release of ranibizumab released from PLGA carriers, and coming out the strategies to tackle on these problems to achieve sustained ranibizumab release

3. Materials and Methods

3.1. Materials

Poly (dl-lactide-co-glycolide acid) (PLGA- PDLG 5004A and PDLG 5004, both of intrinsic viscosity 0.4 dL/g, Corbion Purac) and Poly (vinyl alcohol) (PVA, Mw 30-70 kDa, Sigma-Aldrich) were used without further purification. Clinical grade ranibizumab (Genentech) were obtained from clinician collaborators. Solvents such as dichloromethane (DCM) and chloroform purchased from Tedia Company Inc were of high performance liquid chromatography (HPLC) grade. Phosphate buffer saline (PBS) tablets and carbonate-bicarbonate buffer capsules were purchased from Sigma-Aldrich and used as instructed. Trehalose, l-histidine hydrochloride, bovine serum albumin (BSA) and Tween 20 were purchased from Sigma-Aldrich. Recombinant human vascular endothelial growth factor-165 (VEGF₁₆₅) was from R&D systems. Immunopure peroxidase conjugated goat anti-human immunoglobulin G secondary antibody, supersignal enzyme linked immunoassay (ELISA) PICO chemiluminescent substrate, bicinchoninic acid protein assay (microBCA), alamarblue and SDS running buffer were purchased from Thermo Fisher. Endothelial cell growth media (EGM-2) bulletkit, trypsin, fetal bovine serum used were purchased from Lonza. Opaque high binding microlite 2+ plates used were from NUNC while transparent 96 well plates were from TPP. Laemmli sample buffer and protein kaleidoscope prestained protein standards were obtained from Bio-rad laboratories.

3.2. Methods

3.2.1. Cell culture

3.2.1.1. Cell maintenance and growth

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza. Cells were grown and maintained using full EGM medium consisting of VEGF, human epidermal growth factor (hEGF), insulin-like growth factor (R³-IGF-I), ascorbic acid, hydrocortisone, human fibroblast growth factor (hFGF- β), heparin, fetal bovine serum (FBS) and gentamicin, amphotericin B. Cells were cultured in an atmosphere of 5% CO₂ in the 37°C humidified incubator. Cells from Passage 5 to 8 were used in this thesis.

3.2.1.2. HUVEC proliferation study

To investigate the effect of VEGF and ranibizumab concentrations on cell proliferation, HUVECs were seeded in 96 well plates using full EGM media at a density of 3000 cells per well 24 hours prior to treatment. For VEGF induced proliferation studies, the full EGM media was removed and a concentration range between 1.56-50 ng/mL VEGF and 2% FBS was added to the wells with two minimum replicates. To investigate of ranibizumab inhibition of VEGF induced proliferation, various concentrations of ranibizumab 1.56-1000 ng/mL was mixed with 12.5 ng/mL VEGF and 2% FBS. The plate was incubated at 5% CO₂ in the 37°C humidified incubator for 3 days.

At the end of the incubation, the metabolic activity of the cells that is representative of the cell proliferation was measured with Alamar Blue dye. Fold change in cell growth were compared with the negative controls- HUVECs incubated with 0 ng/mL VEGF and 2% FBS for the VEGF proliferation study and HUVECs incubated with 0 ng/mL ranibizumab and 2% FBS for the ranibizumab inhibition study respectively.

3.2.2. Protein characterization

To quantify ranibizumab amounts two methods were used in this thesis: ELISA and microBCA. Gel electrophoresis was also used to further determine any changes in the molecular weight of ranibizumab.

3.2.2.1. Ranibizumab ELISA design

ELISA developed and optimized in house, was used to quantify bioactive ranibizumab with the capacity to bind to VEGF. VEGF coated plates were prepared in advance for ranibizumab quantification. VEGF₁₆₅ was immobilized on opaque high binding plates. VEGF diluted to a concentration of 0.1 µg/mL in carbonate buffer (0.05 M, pH 9.6) was added at 100 µL/well. Following incubation for 24 hours at 4 °C, the plates are washed with wash buffer (WB) containing 0.05% Tween 20 in PBS. Blocking buffer made up from 2% (w/v) BSA in PBS was then added for incubation for 24 hours at 4°C. The plates were stored dry at 4 °C or used immediately after washing with WB.

For quantification, samples and standards were diluted and aliquoted in triplicates into the plates prepared above. In each of the plate assayed, a standard curve that

correlates chemiluminescent values to known concentrations of ranibizumab ranging from 3.125 to 50 ng/mL were included. Following incubation with gentle shaking of samples and standards at 25 °C for 2 hours, the plates are washed with WB. Anti-human IgG F(ab')₂ secondary antibody conjugated to horseradish peroxidase was diluted to 40 ng/mL in PBS was aliquoted and incubated with gentle shaking for 45 minutes at room temperature. The plates then were washed with WB. Chemiluminescent signal was triggered by mixing equal parts of SuperSignal ELISA Pico Luminol/Enhancer and SuperSignal ELISA Pico Stable Peroxide Solution to a total volume of 100 µL per well. The plate was agitated for 1 minute before luminescent signal was detected with Infinite200 microplate reader (Tecan Inc).

3.2.2.2. *MicroBCA*

Total protein concentration was determined through microBCA. The linear protein working range had been optimized to be between 0.5- 40 µg/mL. 150 µL of samples or standards were aliquoted into the transparent 96 well plates. Homogenous working reagent in ratios of 25: 24: 1 (Reagent A: B: C) were added into the samples also at a volume of 150 µL. This mixture was left in the shaking incubator at 37°C for 2 hours. The absorbance was detected with a Tecan Reader microplate laminator at wavelength of 562 nm. The absorbance reading was normalized with the background reading from the buffer and concentration is read off from the standard curve.

3.2.2.3. *Gel Electrophoresis (SDS-PAGE)*

All samples were diluted to 0.1 mg/mL and used for SDS-PAGE with 10 % polyacrylamide gel. The study was conducted according to manufacturer's protocol. PowerPac HC High-Current Power Supply electrophoresis system was used. 10 µl of 2X laemmli sample buffer was mixed with 10 µl of samples and heat denatured at 95 °C for 10 minutes. 5 µl of precision plus protein kaleidoscope prestained protein standards and 10 µl of respective protein samples were loaded into each well. Gel electrophoresis was performed at 120 kV for 45 minutes with 1X NuPAGE MES SDS running buffer. The gel was stained with CBB Stain One Ready To Use (Coomassie brilliant blue G-250 staining solution) for 30 minutes and subsequently de-stained with deionized water for 1 hour to visualize protein bands. The gel was imaged with G:Box Chemi XT4 (Syngene, Inc).

3.2.3. Ranibizumab storage study

3.2.3.1. Ranibizumab stability in PBS

Ranibizumab stability upon storage in PBS for 1 day was studied at two concentration ranges. Specifically, 5- 40 µg/mL was studied for the higher microgram concentrations and 6.25- 50 ng/mL for the lower nanogram concentrations. Ranibizumab was diluted to the aforementioned concentrations and stored at 4 °C overnight before measurement by the ELISA assay.

3.2.3.2. Ranibizumab stability in various buffers

Ranibizumab stability in various buffers was also explored across the nanogram concentration range of 6.25- 50 ng/mL. The compositions of the buffers are summarized in the Table 3.1 below.

Table 3.1 Summary of buffer components used in storage study

Buffer	Composition
PBS	Phosphate buffered saline tablet (Sigma-Aldrich)
Intrinsic	10 mM Histidine HCl; 10% (w/v) α - α trehalose dihydrate; 0.01 % (v/v) Tween 20 at pH 5.5
BSA	0.1 % (w/v) BSA in PBS
Serum	0.5 % (w/v) BSA; 0.05% (v/v) Tween 20; 5mM EDTA; 0.35 M NaCl; in PBS at pH 6.35

3.2.3.3. Desorption of ranibizumab

Desorption of ranibizumab from the storage vessels was studied at two concentrations- 50 and 100 ng/mL. The storage vessels were flushed with 1 mL of intrinsic buffer for a period of up to 10 days. At each time point, ranibizumab that had desorbed from the vessels was measured with ELISA.

3.2.4. Particle preparation

Three types of fabrication techniques namely water-in-oil-in-water microparticle (WOW MP), water-in-oil-in-water nanoparticle (WOW NP) and solid-in-oil-in-water microparticle (SOW MP) were investigated to understand the release of the entrapped ranibizumab from PLGA carriers. The different types of PLGA used for the respective techniques are described below in Table 3.2. In all emulsion

techniques, PLGA polymer with 50:50 glycolide:lactide ratio and molecular weight 44 kDa (Intrinsic viscosity 0.4 dL/g) was used. The drug to polymer was kept to a ratio of 1:200. A minimum of three independent batches was fabricated.

Table 3.2 Summary of fabricated PLGA particles

Particles Name	Emulsion technique	Polymer Type	End group	Remarks
WOW NP 0.4A	WOW NP	PDLG 5004A	Acid	Microparticles
WOW MP 0.4A	WOW MP	PDLG 5004A	Acid	Nanoparticles
SOW MP 0.4A also 20% SOW MP	SOW MP	PDLG 5004A	Acid	Microparticles
10% SOW MP	SOW MP	PDLG 5004A	Acid	Microparticles; Smaller size
SOW MP 0.4E	SOW MP	PDLG 5004	Ester	Microparticles

3.2.4.1. Water-in-oil-in-water Microparticle (WOW MP)

20% (w/v) of PLGA polymer (PDLG 5004A) was dissolved in DCM and sonicated (Qsonica, Q125) at 35% amplitude for 2 minutes. Ranibizumab dissolved in PVA aqueous solution 1% (w/v), was subsequently added to the polymer solution at volume ratio of 1:3 and ultra-sonicated for another 2 minutes. The resulting water-in-oil (WO) emulsion was added to 100 mL of 1 % (w/v) PVA solution and stirred at 1000 rpm for 5 hours to evaporate the solvents.

3.2.4.2. Water-in-oil-in-water Nanoparticle (WOW NP)

2.5 % (w/v) of the PLGA polymer (PDLG 5004A) is dissolved in DCM and sonicated at 35% amplitude for 2 minutes. Ranibizumab dissolved in PVA aqueous solution 1 % (w/v) was subsequently added to the polymer solution at volume ratio of 1:1 and ultra-sonicated for another 2 minutes. The resulting WO emulsion was added to 100 ml of 1 % (w/v) PVA solution and stirred at 1000 rpm for 5 hours to evaporate the solvents.

3.2.4.3. Solid-in-oil-in-water Microparticle (SOW MP)

Ranibizumab was lyophilized to its solid form. DCM was added to the crushed ranibizumab powder and the mixture was sonicated at 35% amplitude for 2

minutes to get a solid dispersion. PLGA polymer (PDLG 5004A) dissolved in this dispersion to make up 20 % (w/v) and ultra-sonicated for another 2 minutes. The resulting S/O emulsion was added to 100 ml of 1 % (w/v) PVA solution and stirred at 1000 rpm for 5 hours to evaporate the solvents.

To make smaller SOW particles, the same polymer type (PDLG 5004A) but a lower polymer concentration of 10% (w/v) was used. In the investigation of SOW MP made with PLGA of different hydrophobicity, the polymer concentration was maintained at 20% (w/v) but an ester-terminated PLGA of the same molecular weight (PDLG 5004) was used. The same procedure for SOW MP fabrication described above was used.

3.2.4.4. PLGA microparticles with osmotic pressure in W_2 phase

The effect of osmotic pressure on WOW MP and SOW MP was investigated. To create an osmotic pressure between the internal drug phase and the external continuous phase, NaCl was added to the external continuous phase made up of 1% PVA. The respective primary emulsion including W/O and S/O phases was fabricated with the procedures described in Section 3.2.4.1 and 3.2.4.3. This primary emulsion is added to the PVA-NaCl continuous phase and stirred at 1000 rpm to evaporate the solution for 5 hours. Two concentrations of NaCl including 428 mM and 1000 mM were studied.

Irrespective of the fabrication method, the particles were collected by centrifugation at 8,000 and 10,000 rpm for the microparticles and nanoparticles respectively after stirring. The pelleted particles were washed thrice to remove un-entrapped ranibizumab. After washing these particles were re-suspended in 1 % (w/v) mannitol, frozen and freeze dried. Blank PLGA particles were also prepared using the same procedure excluding the addition of ranibizumab in the PVA phase for the WOW particles and the sonication of ranibizumab for the SOW particles.

3.2.5. Bioactivity studies of ranibizumab after emulsification

The impact of preparation procedure on ranibizumab with different emulsification methods was evaluated by comparing the bioactive ranibizumab and total ranibizumab released as measured by ELISA and microBCA respectively. The respective fabrication procedure was mimicked, but without PLGA to avoid introducing an additional variable of ranibizumab extraction from PLGA polymer.

A further examination of PVA content for the WOW emulsion technique was studied. The PVA concentration added from the internal phase was varied between 0- 2% (w/v) to determine if PVA concentration affects bioactivity. The optimal PVA amount to be used in WOW emulsion was also resolved in this thesis.

3.2.6. Characterization of ranibizumab loaded PLGA particles

3.2.6.1. Encapsulation Efficiency (E.E.)

To determine the total ranibizumab content in the particles, triplicate samples of 10 mg of the microparticles were dissolved in 0.5 mL 1M NaOH. These dissolved samples were measured in terms with total protein with the microBCA assay. The interference of NaOH with the working range of the microBCA assay was removed by diluting the dissolved samples 10 times with PBS before measurement. As NaOH polymer hydrolysis is a destructive method, the percentage encapsulation is measured in terms of total protein content.

$$\% \text{ EE} = \frac{\text{Encapsulated Ranibizumab}}{\text{Initial Ranibizumab Used}} \times 100 \%$$

3.2.6.2. *In vitro* drug release

10 mg of loaded particles (n=3) were placed in 100 kDa dialysis membranes (Spectrum Laboratories) secured with dialysis clips. The dialysis membrane was suspended in 15 mL of PBS. The released ranibizumab was collected, replaced and sampled daily by ELISA. This *in vitro* drug release measures bioactive ranibizumab, which retains its VEGF binding capacity.

3.2.6.3. Percentage bioactivity of the released ranibizumab

Using the same method of measuring E.E. mentioned above, the particles were collected after *in vitro* release, lysed with 1M NaOH and measured for the residual ranibizumab content after release with microBCA. The total (active + non-active) ranibizumab release can first be quantified by taking the difference between the total ranibizumab amounts before and after release. The quantity of non active ranibizumab released can then be calculated from the difference between the total ranibizumab and the active ranibizumab released. An estimation of bioactivity was calculated by normalising the active released ranibizumab measured with ELISA

over the total ranibizumab released as measured by microBCA. The bioactivity of the released ranibizumab from its respective particles was then compared.

$$\begin{aligned} & \text{Total Ranibizumab released} \\ &= \text{Encapsulated Ranibizumab} - \text{Residual Ranibizumab} \end{aligned}$$

$$\begin{aligned} & \text{Non active Ranibizumab released} \\ &= \text{Total Ranibizumab Released} - \text{Active Ranibizumab Released} \end{aligned}$$

$$\begin{aligned} \text{Bioactivity} &= \frac{\text{Active Ranibizumab released measured by ELISA}}{\text{Total Ranibizumab released measured by microBCA}} \\ &\times 100 \% \end{aligned}$$

3.2.6.4. Degradation study

To study the degradation of particles, the *in vitro* released condition was mimicked. 5 mg of the respective particles were dispersed in 7.5 mL of PBS (pH 7.4) (n=3) and incubated at 37 °C with moderate shaking at 50 rpm (Satorius Cartomat). At the designated time points of Day 0, 5, 10, 15 and 30, the particles were centrifuged, rinsed with deionized water, collected and freeze dried for characterization. The molecular weight changes and surface morphology of the particles were studied and detailed below.

3.2.6.5. Size exclusion chromatography

Molecular weight changes of PLGA were determined by size exclusion chromatography (SEC) (Agilent 1100 Series LC System instrument), equipped with a G1262A refractive index detector. All measurements were maintained at 35 °C and carried out using a linear PL gel 5 µm mixed-C column suitable for separating molecular weights between 200 to 2,000,000 Da. Molecular weight of samples was obtained relative to the standard curve constructed using polystyrene standards. HPLC grade chloroform was used as the mobile phase with a flow rate of 1.0 mL/min. Samples were run in triplicate.

3.2.6.6. SEM imaging

The external morphologies and size distributions of the microparticles and nanoparticles are characterized with the scanning electron microscope (SEM; JSM-6360A JEOL, Japan) operating voltage 5kV and field emission scanning electron microscope (FESEM; JSM-6340F JEOL, Japan) operating voltage 3 kV respectively. The nanoparticles were sputter coated with platinum (JFC-1600 JEOL, Japan) for 30 seconds. The microparticles were sputter coated with gold (SPI-Module) for 90 seconds. Particles size and morphologies were found to be similar for the independent batches fabricated. Only one representative SEM micrograph is shown. Through characterizing their external morphologies, particle degradation was monitored over a period of 30 days.

With the same SEM method the internal structure of microparticles is observed. To view the internal morphologies, an additional sample preparation step involves mounting the samples on a metal stub, immersing it in liquid nitrogen followed by cryo fractured with the razor blade.

The size distribution of the particles observed from the SEM images was analyzed with the Image J software. The mean diameter of the particles is taken as the number average diameter of the particles (n= 100).

3.2.6.7. Raman mapping

Raman spectroscopy combined with confocal microscopy provides a label-free and non-destructive method to assess the particles [84]. With this method, the drug distribution within the cross-sectioned particles was investigated in this thesis. The Raman effect is the inelastic scattering of light after interactions with the sample with Stokes scattering mostly observed. Molecular vibrations characteristic of the distinct functional groups from proteins and polymers can be obtained after the scattering of incident photon from the modified oscillating dipole [109].

Raman mapping was used to verify the drug distribution within the microparticles. The cross sectioned microparticles were placed under alpha 300 SR confocal microscope (Witec) employing 785 nm infrared diode laser. Raman point by point mapping was performed on an area of 25 μm X 25 μm with a step size of 0.5 μm in the x and y direction. The backscattered was collected with a 20X objective

lens. Measurement scans were collected with a static 300 groove per mm dispersive grating in a spectral window between 300 to 1800 cm^{-1} with each acquisition time for each spectrum to be around 0.5s.

3.2.7. Statistical analysis

Results are presented as mean $\pm$ standard deviation of at least three independent studies. Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by post hoc test. A p value of < 0.05 is considered significant. Categories with p-value <0.05 are indicated with *, categories with p-value <0.01 are indicated with **, categories with p-value <0.001 are indicated with *** and categories with p-value <0.0001 are indicated with ****.

4. Characterization of ranibizumab

4.1. Introduction

This chapter focuses on the basic characterization of ranibizumab for a better understanding of its properties before proceeding to its encapsulation in the later chapters. With its proteinaceous nature, ranibizumab bioactivity is easily compromised during processing, as a result of formulation choice or even due to experimental conditions [97, 100, 105]. To ensure their pharmaceutical efficacy, several characterizations including enzyme linked immunoassay (ELISA) and bicinchoninic acid protein assay (microBCA) have been conducted to quantitatively evaluate ranibizumab binding ability and further elucidate its stability in different conditions. The potential sources of destabilization were hypothesized and recommendations made in the later sections. Through these understandings, better strategies to handle and incorporate ranibizumab in poly(lactide-co-glycolide acid) (PLGA) carriers could be formulated to retain its bioactivity in treating age-related macular degeneration (AMD).

4.2. Effects of VEGF and ranibizumab on HUVECs proliferation

The efficacy of ranibizumab was evaluated through analyzing its inhibitory effect when co-incubated with media consisting of only vascular endothelial growth factor (VEGF) and fetal bovine serum (FBS). Prior to the study, the proliferative effect of VEGF on human umbilical vein endothelial cells (HUVECs) was firstly determined and it was found that HUVECs grew in a dose dependent manner, as shown in Figure 4.1A. However, the growth ceased and effect plateaued at 12.5 ng/mL. Hence 12.5 ng/mL VEGF was deemed to be the saturation point to induce HUVECs proliferation and would be used to investigate the ranibizumab inhibitory effect.

Various concentrations of ranibizumab, ranging from 1.6 ng/mL to 1000 ng/mL, were pre-incubated with 12.5ng/mL of VEGF media and used for HUVECs treatment. As shown in Figure 4.1.B, ranibizumab demonstrated dose-dependent growth inhibition where 50 ng/mL of ranibizumab was able to achieve 60 % HUVECs growth arrest. It was observed that no further significant inhibition when the ranibizumab concentration increased beyond 50 ng/mL.

Figure 4.1 (A) Effects of VEGF concentration on HUVEC proliferation presented in terms of fold increase of cell number at specified VEGF concentration normalized to cell number at 0 ng/mL VEGF treatment. **(B)** Effects of ranibizumab concentration on HUVEC proliferation presented in terms of fold increase of cell number at specified ranibizumab concentration co-incubated with 12.5 ng/mL VEGF normalized to cell number at 0 ng/mL ranibizumab treatment. Categories with p-value<0.05 are indicated with *, categories with p-value<0.01 are indicated with **, categories with p-value<0.001 are indicated with *** and categories with p-value<0.0001 are indicated with ****.

4.3. Stability of ranibizumab

4.3.1. Ranibizumab stability in PBS

The VEGF binding ability of ranibizumab, quantified with ELISA after PBS storage for 24 hours at 37 °C was normalized to the measured initial concentration to give the percentage recovery after storage. It was found that across the higher concentration range between 5- 40 µg/mL there was no significant total protein loss between ranibizumab stored in PBS and fresh ranibizumab (Figure 4.2A; $p=0.89$). However, significant ranibizumab loss was observed across the lower concentration range of 6.25- 50ng/mL (Figure 4.2B; $p=1.3 \times 10^{-4}$).

Figure 4.2. Percentage ranibizumab recovery in PBS as measured by ELISA after storage at 37 °C for 24 hours. (A) In the microgram concentration range between 5- 40 µg/mL; $P=0.9$. (B) In the nanogram concentration range between 6.25- 50 ng/mL; $P<0.001$. P values indicate significant difference compared to fresh ranibizumab.

4.3.2. Recovery of ranibizumab in various buffers

The effect of the dilution buffer, namely intrinsic, serum and bovine serum albumin (BSA) buffers, on ranibizumab stability after one day of storage at concentrations between 6.25- 50 ng/mL was investigated and shown in Figure 4.3. After one day of storage, ranibizumab in intrinsic (p= 0.6) and serum buffer (p= 0.17) were not significantly different in terms of VEGF binding ability from freshly diluted proteins indicating retention of bioactive ranibizumab after storage. On the other hand, while the amount of bioactive ranibizumab recovered when stored in BSA buffer was significantly different than fresh ranibizumab (p= 0.02), the loss (ie. 20%) was less significant than when stored in PBS (ie. 90%; p= 1.3×10^{-4}).

Figure 4.3 Percentage ranibizumab recovery in various buffers (a) PBS; $P < 0.001$ (b) Intrinsic buffer; $P = 0.6$ (c) Serum buffer; $P = 0.17$ (d) BSA buffer; $P < 0.05$ as measured by ELISA in the concentration range between 6.25- 50 ng/mL when stored at 37°C for 24 hours. P values indicate significant difference compared to fresh ranibizumab.

4.3.3. Desorption of ranibizumab from storage tubes

The buffers used in the previous Section 4.3.2 had the common ability to prevent protein adsorption. To elucidate if adsorption is the mechanism of loss, ranibizumab at two concentrations (i.e. 50 ng/mL and 100 ng/mL) were stored in PBS for one day and the receptor containers were then flushed with intrinsic buffer. Notably, despite no detection of ranibizumab in the PBS storage buffer, there was 25% of bioactive ranibizumab being detected from the flushed solutions in the 10 days of experiment as shown in Figure 4.4.

Figure 4.4 Recovery of ranibizumab loss due to storage in PBS through flushing receptor containers with intrinsic buffer over 10 days. Recovery is measured in terms of bioactive ranibizumab as measured with ELISA.

4.4. Ranibizumab bioactivity during emulsion

4.4.1. Effect of PVA amounts in the internal water phase during WOW NP emulsion

In this thesis, emulsion technique has been considered as the mode of incorporating ranibizumab in the PLGA carriers. Hence, it would be of interest to understand if the use of organic solvent in the fabrication process would be of any effect to the ranibizumab binding affinity during water-in-oil-in-water (WOW) emulsion. Ranibizumab was put through the same fabrication process but in the absence of PLGA polymer. Poly vinyl alcohol (PVA) of various concentrations (0% to 2%) was added in the formulations to check on their protective capability during fabrication. After emulsification, the aqueous and organic solvent content was removed by freeze-drying the mixture. The total and bioactive ranibizumab recovered was quantified by two different techniques. ELISA was used to quantify bioactive ranibizumab as it measures for VEGF binding capable ranibizumab. On the other hand, microBCA was used to measure total protein as it quantifies peptide bonds and certain amino acid groups irrespective of activity of the protein. The difference between the protein amounts measured between both techniques can be considered as loss in activity.

As shown in Figure 4.5, when there was no PVA present in the first internal w_1 phase, only 52% of the protein was recovered of which less than 0.1% of the initial ranibizumab remained active. As the PVA concentration in the w_1 phase is increased to 0.5%, there is no loss of total protein and only 20% loss in activity. This is a significant improvement as compared to when no PVA is used in the w_1 phase for total protein ($p= 0.020$) and active protein ($p= 0.00004$). Significant improvement in the recovery of ranibizumab bioactivity was noted in PVA concentrations of 1% and 2% when compared to that of 0.5% PVA ($p= 0.0015$). However, no further improvement in bioactivity was observed when PVA concentration is increased beyond 1%. These results revealed that PVA concentration in the w_1 phase is critical in preserving ranibizumab activity during emulsification. The optimal PVA concentration, established to be 1% was then used in further WOW nanoparticles (NP) encapsulation studies.

Figure 4.5 Effect of PVA concentration on total and bioactive ranibizumab recovery during the nanoparticle emulsification process in the absence of PLGA polymer. 1% PVA was found to be optimal for total and bioactive recovery of ranibizumab. Categories with p-value<0.05 are indicated with *, categories with p-value<0.01 are indicated with **, categories with p-value<0.001 are indicated with * and categories with p-value<0.0001 are indicated with ****.**

4.4.2. Effect of PVA amounts in the internal water phase during WOW MP emulsion

The same study described above was repeated for WOW microparticles (MP) fabrication (Figure 4.6). Similar trend was observed for 0% of PVA: although 85% of protein was recovered only 13% of the initial ranibizumab remained bioactive. As the PVA concentration increased to 0.5% a 25% loss in bioactivity was detected although there is no loss in total protein content. There is a significant improvement in total ($p= 0.00009$) and active protein ($p= 0.004$) recovery than when no PVA was used. When PVA concentrations of more than 1% was used, no protein loss was detected and total bioactivity of ranibizumab was recovered after the fabrication process. This result corroborate with the previous PVA concentration needed for WOW NP emulsification, that PVA concentration in the w_1 phase is essential in preserving ranibizumab activity during emulsification. The optimal PVA concentration, again shown to be 1% will be used in further WOW MP encapsulation studies.

Figure 4.6 Effect of PVA concentration on total and active ranibizumab recovery during the microparticle emulsification process in the absence of PLGA polymer. 1% PVA was again found to be optimal for total and bioactive recovery of ranibizumab. Categories with p -value <0.05 are indicated with *, categories with p -value <0.01 are indicated with **, categories with p -value <0.001 are indicated with *** and categories with p -value <0.0001 are indicated with ****.

4.4.3. Comparing ranibizumab recovery for three emulsion techniques

Apart from WOW emulsion method, solid-in-oil-in-water (SOW) emulsion is also another method proposed for encapsulating ranibizumab in this thesis. In the SOW fabrication method, ranibizumab particles in the solid state were emulsified with dichloromethane (DCM) solvent. Following emulsification, ranibizumab was recovered by freeze-drying the mixture and its bioactive and total protein content was measured. Ranibizumab retained 94% of its protein bioactivity despite direct exposure to DCM and sonication during particle fabrication and no significant loss of activity ($p=0.07$) was detected (Figure 4.7). For SOW, there is no PVA used in the procedure. This is in contrast to the need for the use of PVA in the other WOW emulsion techniques shown previously.

Figure 4.7 Effect of three emulsion methods: WOW NP; WOW MP; SOW MP on Ranibizumab recovery in the absence of PLGA polymer. WOW NP and WOW MP was stabilized by 1% PVA. No significant loss in bioactivity of the ranibizumab during the respective fabrication methods was observed. Hence, these three methods are suitable for future loading studies.

4.4.4. Effect of structural integrity of ranibizumab after emulsion

In addition to the two protein quantitative methods, the molecular weight of the stressed ranibizumab was evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 4.8). The samples emulsified in its respective conditions were loaded on the gel. These samples included SOW fabrication stressed samples, WOW NP fabrication stressed samples with 0% to

2% PVA concentrations in w_1 phase and WOW NP fabrication stressed samples with 0% to 2% PVA concentrations in w_1 phase. The bands observed confirmed that there were no detectable ranibizumab aggregates or fragments as all samples demonstrated a clear band between 37 kDa and 50 kDa concurring with the pure non-stressed ranibizumab samples shown in lane 13. Although smears were observed in lanes 6-8, it did not affect the protein detected at ranibizumab molecular weight. The smears were attributed to the higher amounts of PVA used in the WOW NP emulsification- proven in lane 5 where no PVA was used and control lane 14 where PVA was mixed with non-stressed ranibizumab.

Lane No	Sample
1-3	SOW fabrication treated ranibizumab
4	Freeze dried and reconstituted ranibizumab
5-8	WOW NP fabrication treated ranibizumab In the order of 0, 0.5, 1, 2 % PVA used in w_1 phase respectively
9-12	WOW MP fabrication treated ranibizumab In the order of 0, 0.5, 1, 2 % PVA used in w_1 phase respectively
13	Control: Non stressed ranibizumab
14	Control: Non stressed ranibizumab mixed with 1% PVA

Figure 4.8 Coomassie-stained SDS-PAGE gel of ranibizumab after various particle fabrication emulsions. No aggregation or fragmentation of the ranibizumab was observed.

4.5. Discussion

In the treatment of AMD, ranibizumab, which has been commonly prescribed, is a recombinant fragment antigen-binding (Fab) that neutralizes all bioactive forms of VEGF and consequently reduces downstream permeability and angiogenesis in the endothelial cells [38, 40]. Its biological activity can be measured in the bioassay where its ability to illicit a biological response is determined. Meanwhile a

potency assay assess the ability of ranibizumab to exert its intended activity such as its binding ability, which is not the biological endpoint of the active ingredient [110]. ELISA is a potency assay used to assess binding and it complements VEGF-induced HUVEC proliferation bioassay to assess the biological responses ranibizumab exerts. In the VEGF induced HUVEC proliferation bioassay, it was demonstrated that HUVEC inhibition plateaus at 50 ng/mL of ranibizumab use. While bioassay is not a reflection of clinical efficacy, this indicates that further increase in ranibizumab concentrations to 1000 ng/mL do not further inhibit HUVEC proliferation or induce a cytotoxic effect. VEGF-ranibizumab binding in the bioassay illicit HUVEC growth inhibition validates the use of ELISA in providing a quantitative measure of VEGF-binding capable ranibizumab as well as the extrapolation of potent ranibizumab measured to inhibit endothelial cell proliferation. The biological activity of bevacizumab, another VEGF antagonist was also assessed through inhibition of VEGF-induced cell proliferation as was shown in this chapter. Wang et al., had also reported bevacizumab to be capable of inhibiting VEGF-induced HUVEC survival, HUVEC permeability, nitric oxide production, HUVEC migration and tissue factor production factors [111].

The stability of clinically obtained ranibizumab was characterized. Storage of ranibizumab in PBS between concentrations of 5- 40 ug/mL indicated that ranibizumab remains stable in PBS, as there was no significant loss in bioactivity. Similarly the stability of ranibizumab at 0.25 mg/mL showed that there was no change in terms VEGF binding ability over a period of 30 days in another report [94]. However in this thesis, significant ranibizumab loss was observed when it was stored under the same conditions but at low concentrations between 6.25- 50 ng/mL. As release studies will be collected at low nanogram concentration range the loss in measurable ranibizumab will be consequential. In our study, the loss of recoverable ranibizumab when stored in PBS had been shown to be due to adsorption loss as 25% of bioactive ranibizumab could be flushed out from the storage containers. As the adsorption loss saturates when the surface is covered, this loss is evident only at lower concentrations and not observed at the higher microgram concentrations. It is speculated that the remaining non-recoverable ranibizumab could either not be desorbed or that surface adsorption of proteins could have led to denaturation or aggregation of proteins [112].

Buffers explored to investigate potential improvements in the recovery of quantifiable ranibizumab at low concentrations include: intrinsic, serum and BSA buffer. These three buffers were able to improve the recovery of ranibizumab as compared to its storage in PBS and they share the characteristic of reducing adsorption. Intrinsic buffer is currently the storage buffer for commercial 10 mg/mL ranibizumab drug formulation which can be used within 2 years when stored between 2-8 °C [49]. The second serum buffer was used by researchers from Genentech as a diluent in measuring ranibizumab concentrations in serum of to determine the pharmacokinetics of ranibizumab [113]. The third BSA buffer is made up of 0.1 % BSA in PBS since albumins have been commonly used as carrier molecules to enhance protein stability by reducing losses to surface with its higher surface activity [100, 114]. The detailed mechanism behind the stabilizing effects of excipients had been explained in the Section 2.5.3.2 [103].

For the intrinsic and serum buffer, the presence of nonionic surfactant- tween 20 likely prevented protein adsorption by preferentially adsorbing to surfaces outcompeting protein for hydrophobic interfaces [103]. Although not significant, there was an average 7% and 11% loss in bioactivity for intrinsic and serum buffer respectively. While tween 20 can prevent most loss due to adsorption, pH of the buffer may also influence ranibizumab stability as it affects the net outer charge of the protein and consequently its tendency to aggregate or unfold. Moreno et al., had also showed that ranibizumab has the highest thermal unfolding temperature at pH 5.5 indicating that ranibizumab is less likely to form non-native aggregates on storage at this pH [94]. This also explains the formulation of ranibizumab in intrinsic buffer of pH 5.5.

Apart from nonionic surfactants, albumins have been used to reduce protein adsorption through modification of either storage vessels or dilution buffer to prevent non-specific adsorption [115], the latter studied in this thesis. Moreover, in our study incomplete ranibizumab recovery was observed despite storage in BSA buffer. One possible explanation could possibly be due to insufficient BSA molecules at the concentration of 0.1% to saturate the receptor chamber surface resulting in some loss of ranibizumab due to adsorption. BSA concentration could be increased to 1% where this concentration had previously shown to improve peptide recovery [116]. However limitations to using BSA as a diluent include the

added complexity in developing analytical methods in quantifying total protein amounts [103]. Additionally, future immunogenicity and regulatory issues might arise with the use of BSA buffer. Hence, intrinsic buffer is a more straightforward choice for future ranibizumab dilutions and storage due to the presence of BSA in serum buffer and its current use in the FDA approved ranibizumab formulation.

To stabilize ranibizumab at concentrations between 6.25- 50 ng/mL, it is worthwhile to use intrinsic buffer in future encapsulation work and release studies. However, use of intrinsic buffer in release experiments is not without its limitations. The slightly acidic pH of a sink medium for release can potentially affect the release rates of protein from the particles particularly since particle degradation can be accelerated [86]. Additionally, release of ranibizumab from PLGA particles in intrinsic buffer may not exactly simulate the physiological release profile as factors such as ionic strength and pH may affect release. The effect of intrinsic buffer on ranibizumab release kinetics will be explored in the next chapter.

Since proteins and peptides generally have short half-lives *in vivo*, it is necessary to encapsulate them in carriers such as PLGA particles, and if the burst release is minimized then sustained delivery is feasible [24, 88]. A number of key challenges exist for the use of polymeric carriers including: stability and recovery of therapeutic agent, its encapsulation efficiency and loading, as well as *in vitro* and *in vivo* release kinetics. With this in mind we have explored different stabilization and encapsulation approaches with the aim of improved and sustained delivery of ranibizumab for the treatment of AMD.

The impact of the preparation method on ranibizumab bioactivity will first be discussed. Among the techniques used for preparing PLGA particles, emulsion techniques have been the most widely explored partly due to simplicity [23, 24]. Generally proteins that are denatured during the fabrication step remain so after emulsification. Hence when designing a DDS for therapeutic proteins, protein bioactivity and recovery after fabrication should be maximized. In view of this, ranibizumab bioactivity after different fabrication methods was evaluated in the present work. In WOW emulsions, a trend of significant loss in total and active ranibizumab was observed when ranibizumab was emulsified with DCM.

However both total and bioactive protein recovery was significantly improved in the presence of PVA, whereby recovery was PVA concentration dependent and the optimal concentration identified at 1% PVA (Figure 4.5 and Figure 4.6). Physical loss of protein and loss in its bioactivity could be attributed to the emulsification process, during which proteins reconfigure, unfold or even precipitate at the water-oil interface consequently resulting in activity loss [66, 101]. This explains why total protein loss was more obvious in the WOW NP emulsion than the WOW MP emulsion- as there is a higher DCM to ranibizumab ratio and exposure during nanoparticle fabrication. In order to reduce irreversible denaturation, various techniques explored include the use of amphiphilic agents such as sacrificial carrier proteins and polymers to improve the bioactivity of the model proteins such as lysozyme [117] ovalbumin [118] and α -chymotrypsin [119]. Amphiphilic agents including PVA, compete with ranibizumab for the water-organic interface reducing its exposure and potential loss in bioactivity at such interface [117]. Similar improvements were observed in the work of Varshochian et al. where they revealed that PVA could preserve 48% of Bevacizumab bioactivity in WOW emulsions whereas only less than 3% of activity remained when no PVA was used. Bioactivity can be improved by increasing PVA concentration used in the internal water phase such that sufficient PVA is present to reduce exposure of ranibizumab to the water-organic interface as shown in this work.

Apart from using PVA to stabilize ranibizumab, a non-aqueous approach without the use of amphiphilic agents, utilizing a suspension of solid ranibizumab in organic solvent (the SOW emulsion method) has emerged [66, 70]. With very little conformational mobility in the solid state, proteins in general will not unfold and lose their activity when emulsified in this fashion. Hence, direct contact of solid state ranibizumab with DCM in the absence of surfactants did not cause significant loss in bioactivity (Figure 4.7). Notably, this is the first in depth study demonstrating that the bioactivity of ranibizumab could be fully preserved either by varying PVA concentration in WOW emulsion or by using solid proteins in the SOW emulsion.

4.6. Summary

In this chapter we validate the use of our in-house ELISA for the quantification of VEGF binding capable ranibizumab and for the measurable ranibizumab to be translated into HUVEC inhibition. This lays the foundation for the use of ELISA for future studies in quantifying bioactive ranibizumab. The clinical ranibizumab obtained was characterized on its stability in physiological buffer-PBS. Ranibizumab was found to be stable in PBS, but recovery was minimal at low nanogram concentrations as loss due to adsorption was prominent at such low concentrations. This loss can be prevented with the use of buffers formulated to prevent adsorption. Following the basic characterization of ranibizumab, the effect of fabrication process on the stability of ranibizumab was investigated. The oil-water interface was the main factor destabilizing ranibizumab and the use of sufficient PVA surfactant can prevent its exposure to such interface. Additionally, the use of solid ranibizumab was also found to improve the recovery of bioactive ranibizumab. With such understanding, 1% PVA in WOW fabrication and solid protein particles in SOW will be strategically used for incorporating ranibizumab in the PLGA carriers, where their loading properties would be further explored in the next chapter.

5. Incorporation of ranibizumab into PLGA particles

5.1. Introduction

In this chapter we set out to identify an optimal fabrication method to achieve sustained release of ranibizumab from PLGA carriers that remains bioactive. Generally, the fabrication process parameters affect the particle properties and eventually its release kinetics. For example, the speed of polymer solidification is affected by factors such as solubility of polymer in organic solvent, and the polymer concentration and water-to-oil ratio determine the encapsulation efficiency (E.E.). In this thesis, the focus was placed on the effects of 1) polyvinyl alcohol (PVA) concentration in the internal aqueous phase (W_1) phase and 2) poly(lactide-co-glycolide acid) (PLGA) concentration on the resulting particles properties, on particle size, size distribution as well as surface morphology. Building on the process parameters, the most suitable fabrication technique was assessed from its E.E., length of sustained release and bioactivity of released ranibizumab. To evaluate the applicability of the optimal fabrication method, a more detailed study such as study of smaller size particles and particles fabricated with a different polymer were also studied. The correlation between the release profile with degradation kinetics of various PLGA particles and evaluation for the loss of bioactivity was also established, paving the way for future improvements of the particles to achieve a more sustained release.

5.2. Fabrication of PLGA particles

PLGA particles were fabricated according to Section 3.2.4. For water-in-oil-in-water (WOW) emulsions, PVA concentration in the W_1 was varied. As PVA concentration was identified to not be crucial in preserving ranibizumab when solid proteins were used in solid-in-oil-in-water (SOW) emulsions (in Section 4.4.3), only the effect of PLGA concentration was investigated in SOW emulsions. The resulting particles were evaluated under the SEM for its size, size distribution and surface morphology.

5.2.1. Effect of PVA concentration on the size of PLGA NPs

The PVA concentration was varied from 0 to 2 w/v% with the other experimental conditions were kept constant. Shown in Figure 5.1 the size of the resulting nanoparticles (NP) generally decreases with PVA concentration from 329.7 ± 137.7 nm to 169.8 ± 36.7 nm for 0 to 2 % of PVA used. When 0 % PVA was used in the internal aqueous phase, a large size distribution was observed.

A

Figure 5.1 The effect of PVA concentration in the internal aqueous phase on the size of WOW NP. (A) As visualized under SEM. (B) Size distribution of the particles in terms of diameter. Categories with p-value<0.05 are indicated with *, categories with p-value<0.01 are indicated with **, categories with p-value<0.001 are indicated with *** and categories with p-value<0.0001 are indicated with ****.

5.2.2. Effect of PVA concentrations on the size of PLGA MPs

The effect of PVA concentration used in the internal aqueous phase during WOW microparticles (MP) emulsion was also investigated. Similarly, the PVA concentration was varied from 0 to 2 w/v% while the other experimental conditions were kept constant. Between 0.5 to 2% of PVA used, there is no significant difference in the size of the MP varying from $133.7 \pm 36.8 \mu\text{m}$ to $147.4 \pm 35.8 \mu\text{m}$. When 0 % PVA was used in the internal aqueous phase, a smaller sized particle with $119.9 \pm 38.4 \mu\text{m}$ diameter was observed ($p= 0.0009$).

Taking into consideration the maintenance of ranibizumab stability only when concentrations of more than 1 % PVA was used during the emulsion as shown in Section 4.4 and that 1% PVA also yield homogenous particles with homogenous size distribution, 1 % PVA was used for encapsulating ranibizumab in WOW NP and WOW MP and further discussed in Section 5.3.

Figure 5.2 The effect of PVA concentration in the internal aqueous phase on the size and morphology of WOW MP. (A) As visualized under SEM. (B) Size distribution of the particles in terms of diameter.

5.2.3. Effect of PLGA concentration on the size of SOW MPs

Generally polymer concentration would have an effect on particle size, where the latter poses as an important contribution in affecting release kinetics of encapsulated therapeutic agents [72, 73]. Hence, in this thesis, polymer concentration was varied from 5- 40 w/v% in SOW emulsions and their effect on size was evaluated, as shown in Figure 5.3. The size of the MP increases with

PLGA concentration from $29.7 \pm 7.4 \mu\text{m}$ to $142.3 \pm 42.3 \mu\text{m}$ for 5- 40 % of PLGA used. The significant difference of $p < 0.001$ between each polymer concentration increment investigated indicates the impact of polymer concentration on size. It should be noted that in current medical practice, needle with an internal diameter of $160 \mu\text{m}$ is generally used for intravitreal injections of ranibizumab for AMD treatment. Judging from the particles size as shown in Figure 5.3A, 40 w/v% PLGA generated too large particles with broad size distribution ($142 \pm 42 \mu\text{m}$) and smaller size particles were identified with low drug encapsulation capability (will be discussed in Section 5.4.1.), 20w/v% PLGA concentration was deemed to be the suitable concentration in SOW emulsions for further investigations. For consistency in comparisons to evaluate effect of particle fabrication, 20 w/v % PLGA concentration was also used to fabricate WOW MP.

A

Figure 5.3 The effect of polymer concentration on the size of SOW MP. (A) As visualized under SEM. (B) Size distribution of the particles in terms of diameter.

5.3. Ranibizumab loading into PLGA particles

5.3.1. Characterizing ranibizumab loaded PLGA particles

Calculations were based on the release of ranibizumab from PLGA particles after 15 days for WOW NP, WOW MP and SOW MP. Total bioactive ranibizumab released was calculated from the daily ranibizumab release amounts, measured by ELISA. At the end of release particles were collected, lysed with NaOH and analyzed for total residual ranibizumab via microBCA. Non bioactive ranibizumab released can then be calculated by taking the difference between the total ranibizumab encapsulated with bioactive ranibizumab released and residual ranibizumab.

It was previously identified that 1 % PVA in WOW emulsions and 20 % PLGA concentration in SOW emulsions was suitable to fabricate ranibizumab-loaded particles using PLGA 0.4A polymers. The effect of these identified processing parameters on some key properties of ranibizumab loading in particles using PLGA 0.4A polymer is shown in Figure 5.4 and Table 5.1. For the WOW NP, a diameter of approximately 208.5 ± 24.3 nm was obtained. The WOW MP fabricated was significantly larger than the SOW MP. E.E. obtained in terms of total protein was higher in both types of WOW emulsion methods and lowest in the SOW emulsion method. Comparing between WOW emulsions, higher encapsulation was observed in the microparticles, compared to the nanoparticles.

Maintenance of bioactivity for the released ranibizumab is crucial for evaluating the therapeutic application of the particles. In Section 4.3, pure ranibizumab was

shown to be stable during all fabrication techniques. To evaluate any bioactivity loss due to release, bioactivity of released samples was calculated in terms of percentage active ranibizumab released (■) normalized by total ranibizumab released (■ + ▨) at the end of the release. The released ranibizumab as calculated at the end of *in vitro* release was not fully bioactive. The released ranibizumab from WOW NP and SOW MP retained the highest bioactivity of more than 80%. However, less than 60% of the released ranibizumab from WOW MP remained bioactive. The breakdown for the released active ranibizumab, released non-active ranibizumab and residual ranibizumab in terms of initial drug used is presented in Figure 5.4.

Figure 5.4 Summary of ranibizumab after release, as a percentage of the drug used during encapsulation- including percentage active ranibizumab released, calculated percentage of non active ranibizumab released and percentage of residual ranibizumab. These three characteristic sums up to give the encapslation efficiency of each system.

Table 5.1 Characteristics of respective fabrication techniques examined include: Size of fabricated particles, E.E., and bioactivity calculated at the end of the release.

Fabrication Method	Size	Encapsulation Efficiency (%)	Bioactivity of Total Released Samples (%)
WOW MP 0.4A	112.3 ± 6.8 μm	83 ± 6.6 %	56.8 ± 7.7 %
WOW NP 0.4A	208.5 ± 24.3 nm	41 ± 2.0 %	81.87 ± 7.9 %
SOW MP 0.4A	69.9 ± 19.4 μm	22 ± 0.9 %	81.74 ± 6.3 %

5.3.2. In vitro release of ranibizumab from three emulsion methods

The cumulative percentage release and daily release amounts of bioactive ranibizumab as measured by ELISA under *in vitro* sink conditions are shown in Figure 5.5. The release profile for both the WOW MP and WOW NP exhibited a huge burst release of 35% and 66% respectively. Thereafter, very little ranibizumab was released from WOW MP (~4%) and WOW NP (~9%) over the next 7 days. In contrast, SOW MP displayed a lower burst release of $23 \pm 2\%$ followed by a subsequent sustained release of $25 \pm 2.5\%$ over a period of 15 days.

Figure 5.5 (A) In vitro cumulative percentage release profile of bioactive ranibizumab from WOW NP, WOW MP and SOW MP over a period of at least 15 days as measured by ELISA. WOW NP and WOW MP demonstrated a burst release followed by a plateau. With SOW MP a sustained release of 15 days was observed. **(B)** In vitro daily release amounts of ranibizumab from WOW NP, WOW MP and SOW MP over a period of at least 15 days. WOW NP and WOW MP demonstrated a burst release followed decreased release amounts for 7 days. With SOW MP a burst release was observed followed by a sustained release of 15 days.

5.4. Modification of SOW MP

5.4.1. Effect of particle size on release kinetics of SOW MP

To check that the sustained release trend of the SOW MP is not just an effect of size, the same fabrication technique was used to make smaller particles. Keeping all other parameters constant, a lower concentration of polymer- 10 w/v % was used during fabrication to obtain smaller SOW particles of diameter $44.8 \pm 12.0 \mu\text{m}$. This 10% SOW MP is significantly smaller than the 20% SOW MP (also known as SOW MP 0.4A) particles of diameter $69.9 \pm 19.4 \mu\text{m}$ presented in Section 5.3. ($p= 5*10^{-12}$). The E.E. was also expectedly significantly lower at $7 \pm 0.8 \%$. The bioactivity of $81.6 \pm 3.4 \%$ was obtained for the ranibizumab released from 10% SOW MP. With the SOW MP fabrication method a very consistent value of at least 80% bioactivity was recovered irrespective of size. 10% SOW MP experienced a 20% burst release and a subsequent release of another 10% over the next 20 days.

Table 5.2 Effect of different polymer concentration on the size, E.E. and bioactivity of fabricated SOW MP with reference to 20% SOW MP.

Polymer Conc/ Fabrication Method	Size	Encapsulation Efficiency (%)	Bioactivity of Released Samples (%)
10 % SOW MP	$44.8 \pm 12.0 \mu\text{m}$	$7 \pm 0.8 \%$	$81.6 \pm 3.4 \%$
20 % SOW MP also SOW MP 0.4A	$69.9 \pm 19.4 \mu\text{m}$	$22 \pm 0.9 \%$	$81.74 \pm 6.3\%$

Figure 5.6 In vitro cumulative percentage release profile of ranibizumab from smaller sized SOW MP over a period of at least 15 days.

5.4.2. Effect of polymer nature on the release kinetics of SOW MP

With SOW identified as the optimal fabrication method for sustaining release, the effect of end-capped ester group (0.4E) or uncapped carboxylic acid terminus group (0.4A) with the same molecular weight of 44 kDa on the release kinetics was also investigated. The end-capped group confers a more hydrophobic property to the overall polymer [62]. The size of SOW MP 0.4E fabricated with 0.4E polymer has similar diameter of $75.9 \pm 4.3 \mu\text{m}$ than SOW MP 0.4A ($p = 0.45$). The E.E. obtained was similar to the SOW MP 0.4A at $24 \pm 3.2\%$. The SOW MP 0.4E showed a suppressed burst release of 12% followed by a release of 13.3% over the next 24 days.

Table 5.3 Effect of polymer hydrophobicity on the size and E.E. of fabricated SOW MP with reference to SOW 0.4A.

Fabrication Method	Polymer Type Mw/ End group	Size	Encapsulation Efficiency (%)	Bioactivity of Released Samples (%)
SOW MP 0.4A	44 kDa/ Acid	69.9 ± 19.4 µm	22 ± 0.9 %	81.74 ± 6.3%
SOW MP 0.4E	44 kDa/ Ester	75.9 ± 4.3 µm	24 ± 3.2 %	83.81 ± 6.1%

A

B

Figure 5.7 The hydrophobicity of the polymer used to fabricate SOW MP was examined. (A) In vitro cumulative percentage release profile of ranibizumab from SOW MP made with different hydrophobicity over a period of at least 15 days. (B) Size and surface morphology difference was visualized under SEM at 100X for SOW 0.4A and SOW 0.4E.

5.5. *In vitro* degradation of ranibizumab encapsulated particles

5.5.1. Comparisons of the three fabrication methods

To explain the differences in the release profiles, the degradation of PLGA polymer was studied. The release profile and kinetics of the ranibizumab loaded polymeric particles may be understood from the hydrolytic degradation of these particles. Molecular weight of the polymer as measured by size exclusion chromatography (SEC), mass loss and the surface morphology of the particles were observed under SEM were used to track physical changes to the particles. The mass loss of WOW NP was not recorded due to limitations in recovering the nanoparticles as it degrades.

As shown in Figure 5.8A, the WOW NP exhibited less than 15% loss in molecular weight over the 30 days. From the SEM images, it can also be seen that these nanoparticles retained their spherical shape through the whole period. Pores were observed from these particles from day 10 and they became increasingly apparent from Day 15.

For the WOW MP there was a moderate loss in molecular weight till day 15; thereafter, the rate of molecular weight change was retarded. This molecular weight loss was only translated to mass loss in the particles after day 15. From the SEM images, WOW MP is a very porous particle and there was no apparent change in this porosity throughout the release.

In the SOW MP, there was a rapid decrease in the molecular weight of the polymer over the first 15 days due to hydrolysis. Such loss in molecular weight contributed to a large mass loss after day 15 with sufficient degradation. The SEM images showed a smooth and non-porous surface at Day 0. Dimples and pits could be observed on the surface from Day 5. The most drastic change was seen on day 10 where the particles collapsed losing its round shape.

Figure 5.8 In vitro degradation of ranibizumab loaded PLGA particles fabricated with different techniques characterized by (A) Molecular weight change and (B) Gravimetric measurements of particles weight loss over time mass.

A

B

Figure 5.9 SEM micrographs chronicling the degradation of (A) WOW NP, (B) WOW MP, (C) SOW MP at 15,000 X, and 500 X magnification following in vitro degradation up to a period of 30 days.

5.5.2. Comparisons of the effect of size

The degradation of SOW MP made from concentration of 10 w/v% is compared to that made from 20 w/v% PLGA polymer. SOW MP made from the 2 concentrations followed a rapid decrease in the molecular weight of the polymer over the first 15 days. However, the molecular weight loss for 10 % PLGA SOW MP occurred at faster rate where $p = 0.005$ on Day 5 and $p = 0.03$ on Day 10. This faster molecular weight loss was also translate to the faster mass loss and SEM images where the collapse in the particle shape is observed at Day 5 rather than Day 10 as observed in the 20 wt% SOW MP.

C

Figure 5.10 In vitro degradation of ranibizumab loaded 10 wt% SOW MP up to a period of 30 days. (A) Characterized by molecular weight change of PLGA polymer. (B) Gravimetric measurements of particles weight loss over time mass. (C) SEM micrographs chronicling particle degradation at 500 X.

5.5.3. Comparisons of the effect of polymer physiochemical properties

The degradation of SOW MP made from end-capped ester group polymer is compared to that made from acid terminated polymer of the same molecular weight. Loss of molecular weight in the SOW MP made from the end-capped ester group occurred much slower than the SOW MP made from acid terminated group. This slower molecular weight loss is also reflected in the mass loss, with less than 30% of the PLGA polymer was eroded by Day 30. SEM images where no change in the particle shape was observed even at Day 15 rather than the collapse observed at Day 10 in the 20 wt% SOW MP.

C

Figure 5.11 In vitro degradation of ranibizumab loaded SOW MP fabricated with end-capped ester group up to a period of 30 days. (A) Characterized by molecular weight change of PLGA polymer. (B) Gravimetric measurements of particles weight loss over time mass. (C) SEM micrographs chronicling SOW MP 0.4E degradation at 500 X.

5.6. Ranibizumab distribution within SOW MP and WOW MP

Confocal raman spectroscopy was to visualize the ranibizumab distribution within the particles. Raman spectral signatures of PLGA and ranibizumab is shown in Figure 5.12. PLGA exhibits highest peak intensities at the C-COO stretch vibration at 875 cm^{-1} band [120] while ranibizumab at the tryptophan doublet at $1360/1340\text{ cm}^{-1}$ band [121]. Figure 5.12 shows the associated score image of SOW MP. PLGA is detected in the whole area with ranibizumab particulates heterogeneously dispersed within the particle. In Figure 5.12B PLGA was also detected in the scanned area, whereas no ranibizumab was detected. Combining the knowledge of the high E.E. in WOW MP with the lack of ranibizumab spectral signatures detected in WOW MP, it is postulated that the encapsulated ranibizumab is homogeneously dispersed throughout the particle resulting in weak signal undetected.

Figure 5.12 Pure component raman spectra estimates and their associated score images obtained from: (A) Ranibizumab loaded SOW MP. (B) Ranibizumab loaded WOW MP.

5.7. Release of SOW MP in different buffers

Pure ranibizumab loss on storage was shown to be due to adsorption in Section 4.2. To elucidate if the approximate 20% bioactivity loss for SOW MP quantified at the end of release was due to adsorption during *in vitro* release, the same batch of particle was released in various buffers one of which is intrinsic buffer (IB) where losses due to adsorption can be prevented as shown in Section 4.3. However, little release as shown in Figure 5.13 was observed in this buffer after the initial burst. The same trend was also observed when the particles were

released in PBS of pH 5.5. A faster release trend was observed when PBS of pH 7.4 was used. When the release buffer pH increases to a slightly more alkaline range of pH 8.5, the release profile follows a trend similar to pH 7.4 but at a faster rate.

Figure 5.13 In vitro cumulative percentage release profile of ranibizumab from various buffers including IB at pH 5.5, PBS at pH 5.5, PBS at pH 7.4, PBS at pH 8.5.

As identified previously that some of the loss of ranibizumab could be due to adsorption on the receptor chambers, hence it was postulated that the loss might be prevented by coating the receptor chambers with BSA proteins to block the surface. However, when the same set of particles as shown in Figure 5.14 was released in BSA and non BSA coated receptor, there was a similar *in vitro* release trend was observed.

Figure 5.14 In vitro cumulative percentage release profile of ranibizumab into BSA coated and non BSA coated receptor chambers.

5.8. Discussion

Prior to encapsulating ranibizumab the influence of process parameters including PVA and PLGA concentration on the PLGA particles size and morphology was studied. PVA concentration, which had been shown to be an important parameter in maintaining ranibizumab bioactivity, was studied for its effect on the size and size distribution for WOW NP and WOW MP. Notably, the effect of PVA concentration in the internal phase has not been widely explored. Most studies focused on the influence of PVA in the external water phase on the size of the microspheres. PVA stabilizes the emulsion droplets from coalescence and consequently higher PVA concentrations yield smaller size microparticles [122]. Similarly, the PVA stabilization effect in the secondary emulsion can be extrapolated to the stabilization of the primary emulsion. In the WOW NP, the micron sized particles observed from the SEM images when 0% PVA was used indicates the coalescence of the emulsion droplets during solvent evaporation. On the other hand, the effect of varying PVA concentration on the size of the WOW MP was not as pronounced as that observed in the WOW NP system as small differences will be magnified when comparing at the nano scale. Additionally, the PVA concentration used in the internal aqueous phase for the WOW MP did not cause any visible changes on the morphology of the PLGA particle and the particle remained porous. 1% PVA for both WOW NP and WOW MP was shown to achieve a reasonable particle size and size distribution. Meanwhile in the second parameter studied, we showed that PLGA concentration is a key factor

influencing microparticle size and this can be varied to achieve the desired particle size. This phenomenon has also been confirmed in other studies whereby increasing polymer concentration increases the viscosity of the organic phase leading to larger sized particles formed [62, 85]. With these understanding, we identified the most optimal processing parameters (i.e. 1% PVA in WOW emulsions and 20% of PLGA concentrations) and proceeded to incorporate ranibizumab for further characterizations.

A system's potential for sustained release of therapeutically bioactive proteins was evaluated by firstly measuring E.E., secondly the associated *in vitro* release profiles and lastly the bioactivity of the ranibizumab released. To examine our systems, the first factor, E.E. was studied. E.E. can generally be improved by reducing drug loss during emulsification as the drug partitions from the internal water phase to the external continuous phase. Greater drug loss is expected to occur in smaller particles as the diffusion distance for drug to the surrounding environment decreases [123]. This explains the lower E.E. of ranibizumab in WOW NP compared to WOW MP (Table 1). In general, a suspension of solid drug or protein will yield a lower E.E. in the SOW process, as the larger solid protein volume may not co-localize with the carrier polymer as much and result in higher loss as it dissolves into the aqueous continuous phase. This difference has also been reported elsewhere [64, 65].

The second factor studied- *in vitro* drug release showed that ranibizumab WOW MP and NP suffered from a burst release on the first day followed by very little release (Figure 5.5A). The WOW emulsion in this study produced microparticles with significant amount of surface pores (Figure 5.12B- Day 0) leading to ease of drug release. Additionally, burst release could also be due to significant amount of ranibizumab segregated on the surface, most likely near the pores leading to burst release. This is consistent with the spatial analysis of WOW MP encapsulating lysozyme by Rafati et al., where surface segregation of protein is mostly limited to the areas around the pores [84]. In one WOW MP system where ranibizumab was loaded, a burst release of 15% in the initial stage was also experienced followed by no significant release for more than 120 days preceding a second burst [12]. In another PLGA microparticles system encapsulating ranibizumab, PLGA microparticles fabricated by the WOW emulsion based methods suffered from a

50% burst release with minimal release of less than 10% beyond the first week [26] similar to what was observed in our study.

It should be noted that significant burst release of proteins from WOW nanoparticles is the rule rather than the exception. Pandit et al. had also showed that bare WOW NP encapsulating bevacizumab suffer from a burst release and only managed to prolong the release for 48 more hours by coating chitosan on the PLGA NPs [11]. In another report, Song et al. had also shown that both hydrophilic and hydrophobic drug experienced burst release from PLGA nanocarriers [74]. The surface area to volume ratio of nanoparticles is higher allowing for more drug to escape from the surface [72].

Notably, the third emulsion system- SOW MP displayed a relatively lower burst release at 20% and then sustained release for 15 days. A similar trend was reported for interleukin-18 *vitro* release from SOW MP and WOW MP [65]. The lower burst release could be due to the reduced pore-associated surface segregation (Figure 5.12C) since this method yields less porous particles. Following the initial burst, SOW particles displayed a region of diffusion-controlled release of ranibizumab as catastrophic mass loss only started after day 15. Release can then be accelerated by PLGA degradation as a result of hydrolysis of the ester bonds into shorter oligomers resulting in a decrease in molecular weight. Upon sufficient degradation, at a reported molecular weight of 1100 Da, oligomers are sufficiently water soluble and hence able to diffuse out of the microspheres [90], carrying soluble drug with them.

Particle degradation was investigated to explain the different release kinetics. Acidic byproducts of PLGA polymer produced during hydrolysis reduce the pH within the particles creating a pH gradient between the particles and the surrounding release media [93, 124]. The removal of the acidic byproducts or the ingress of surrounding medium to neutralize this generated acidic microenvironment is very much dependent on the properties of these particles such as size and porosity. Klose et al., had reported that autocatalytic effect to be more prominent in less porous particles [125]. As seen from its SEM micrographs on Day 0 (Figure 5.12C), it is likely that relatively lower porosity of the SOW particles facilitated acidity build up in the particle core, leading to catalytic acceleration of degradation where molecular weight loss was occurred faster than

in WOW MP (Figure 5.11A) [91]. Nevertheless release from the WOW and SOW MP before Day 15 was mainly due to diffusion, as bulk degradation that is characterized by catastrophic particle mass loss was only observed between Day 15 to Day 30.

Bioactivity of the released ranibizumab at the end of the study is the last factor studied for evaluating sustained release potential of the three systems presented in this chapter. Maintaining the bioactivity of released ranibizumab remains crucial for therapeutic application of the particles. While WOW NP and SOW MP retained a high percentage of bioactivity at more than 80%, a significant 40% loss in bioactivity was observed in WOW MP. Most likely, this loss in bioactivity is due to an interaction between polymer molecules and the protein molecules when exposed to moisture and elevated temperature [105].

We propose the following mechanism for the observed additional loss in bioactivity observed for WOW MP: Comparing WOW MP and SOW MP fabrication, a more homogenous distribution of protein molecules is seen from the raman spectra and its associated score images within the WOW MP while ranibizumab distribution in SOW MP is less dispersed hence “more concentrated”. For the SOW MP, the more concentrated ranibizumab present indicate bigger protein “clumps” present, where proteins in the interior of the protein clump is shielded by the exterior sacrificial proteins from contact with the polymer, reducing its propensity to chemical degradation [101]. Additionally, interaction between the polymer and ranibizumab limits the ranibizumab stability and complete release due to adsorption and is aggravated in the highly porous WOW MP due to the larger surface area available for polymer-drug interaction as reviewed by Giteau et al. and van de Weert et al. [88, 126]. Hence, the use of solid ranibizumab proteins in the SOW emulsion might have reduced denaturation at the water-oil interface as well as inside the particles.

On the other hand, approximately 20% loss in bioactivity was observed in SOW MP. One potential source for the loss could be adsorption during release as ranibizumab was released into PBS and ranibizumab recovery on storage in PBS was shown to be poor in Section 4.2. To elucidate if there was any potential loss in bioactivity due to protein adsorption during release, the use of intrinsic buffer (IB)

where ranibizumab was fully recovered on storage, as a release medium was investigated. However, no further release from SOW MP in IB was detected beyond the burst release indicating its incompatibility as a release buffer. Further investigation yielded similar trend when the SOW MP was released in PBS of pH 5.5. The lack in release was attributed to the interaction between the negatively charged PLGA particles [126] and positively charged ranibizumab [94]. Ranibizumab with a theoretical isoelectric point of 8.1 is more positively charged at a lower pH leading to stronger electrostatic interaction with the PLGA particle. Consequently at a more alkaline pH of 8.5, ranibizumab is relatively less positive charged and its electrostatic interaction with the negatively charged end capped groups is reduced leading to a faster diffusion based release. Apart from the use of stabilizing buffer, potential adsorption effect could also be circumvented with release in BSA coated bottles where the surface available for adsorption is blocked with BSA [115]. However, *in vitro* release showed that there was no significant difference between release of the particles in BSA and non BSA coated receptor. Hence loss of bioactivity due to adsorption was not significant and the release amount is not altered by experimental conditions. It is believed that this loss was not significant as the burst release of ranibizumab in the first day was sufficient to block and prevent further loss in the subsequent days. Hence, after ruling out the possibility of adsorption loss, the 20% loss in bioactivity in SOW MP could be attributed to the denaturation due to polymer-protein interactions as discussed in paragraph above.

Following the identification of SOW to provide a sustained release of therapeutically bioactive ranibizumab, various factors including particle size and polymer hydrophobicity was further investigated to challenge the applicability of SOW emulsion under different conditions. To determine the effect of particle size on release, smaller size SOW MP of 45 μm has been fabricated compared to the previous 70 μm SOW particles. The lower E.E. was recorded in smaller SOW MP is attributed to the lesser volume available for encapsulating the solid protein in each of the smaller oil droplet formed with lower viscosity and polymer concentration [123]. Conversely, higher E.E. in SOW MP can also be achieved by reducing the initial size of solid protein powder [64, 66-70]. Moreover, for both sizes of SOW MP similar ranibizumab bioactivity on release and burst release was observed (Table 5.2 and Figure 5.6). The faster release rate of ranibizumab from

the bigger particles does not adhere to Fick's diffusion law where diffusion pathway increase with increased particles size should lead to slower release rates [127]. Hereby, the counterintuitive faster release from the larger 20 % SOW MP was proposed: the larger particles with higher loading could have leave behind more voids as drug is released resulting in increased drug diffusivity. This results in faster water ingress and consequently higher drug release rates [123, 128]. However, the low loading achieved for 10 % and 20 % SOW MP at 0.11% and 0.035% respectively could have impeded the observation of this phenomenon under the SEM. In spite of its reduced size, 10% SOW MP followed the similar burst, diffusion-controlled release and degradation-controlled release over 15 days indicating the applicability of this fabrication method for sustaining release.

To achieve a longer sustained release, a more hydrophobic ester terminated polymer of the same molecular weight was used to fabricate SOW MP. With the more hydrophobic PLGA polymer release was extended for a longer period of time (Figure 5.7) from SOW MP 0.4E, and the burst release was suppressed as well. In comparison, the SOW MP 0.4A fabricated with the hydrophilic uncapped polymer, experienced a higher burst release as the particles are more readily hydrated facilitating the diffusion of drugs out to the release media [129-131]. This slower release could also be attributed to the slower degradation of ester end-capped polymer extending the polymer's diffusion release phase beyond 15 days.

5.9. Summary

In this chapter the process parameters including PVA concentration and PLGA concentration on the size, size distribution and morphology of the fabricated particles was investigated. Building on the process parameters, the effect of fabrication techniques to achieve a bioactive and sustained release of ranibizumab was compared. In these studies, we have demonstrated that SOW emulsion offers the most potential for a sustained and bioactive release of incorporated ranibizumab for at least 15 days. We attribute the higher bioactivity to reduced interactions of the solid protein with either solvent or biodegradable polymer, when compared to the WOW emulsion method. To challenge the applicability of SOW emulsion, a more detailed study including fabrication of smaller SOW MP and SOW MP with a more hydrophobic polymer was studied and sustained release can be achieved with both particles. The correlation of the release profile and

degradation kinetics of various PLGA particles was also established, paving the way for future improvements of the particles to achieve a more sustained release. The sustained release observed from SOW MP is mainly from diffusion-controlled release of ranibizumab. Interestingly, despite the higher burst release and undesirable release pattern in WOW MPs, these particles possessed higher E.E. compared to SOW particles. Strategies in reducing the burst release have been proposed through the observation of WOW particle porous morphology in this chapter, and will be further discussed in Chapter 6.

6. Modulating ranibizumab release from WOW MP with sodium chloride

6.1. Introduction

In the Chapter 5, we showed that the water-in-oil-in-water (WOW) emulsion provides a system with high encapsulation efficiency although it did not translate into a sustained release delivery system. On the contrary although solid-in-oil-in-water (SOW) emulsion yielded low encapsulation efficiency, sustained release of bioactive ranibizumab for more than 15 days was achieved. The lack of sustained release in the WOW MP was hypothesized to be due to the high porosity of the particles. Some formulation strategies identified to suppress the burst release include the use of high molecular weight polymers, high polymer concentration, more hydrophobic polymer and lower drug loading [62]. In this chapter, we explore the usage of sodium chloride (NaCl) as an osmotic agent in the external aqueous phase (W_2) during the WOW emulsion to attain particles of different surface porosity. Notably, this is the first in depth study on suppressing ranibizumab release through the use of osmotic gradient to produce non-porous WOW particles. The encapsulation efficiency, *in vitro* release and bioactivity of the ranibizumab are key parameters studied to characterize the system. The effect of NaCl concentration on the particles morphology was studied prior to loading ranibizumab into the particles. Release from WOW MP made with various NaCl concentrations in the W_2 was then compared to identify the most suitable drug delivery system where sustained and bioactive ranibizumab release can be achieved. As ranibizumab maintained high bioactivity in SOW MP, it was used as a benchmark to identify if the usage of osmotic agent is the cause of any loss in bioactivity during SOW emulsion with NaCl in the W_2 phase.

6.2. Effect of NaCl concentration on morphology of PLGA particles

In Chapter 5, it was shown that despite the higher burst release and undesirable release profile of WOW MPs these particles possess higher encapsulation efficiency compared to SOW system. In order to provide long term therapeutic efficacy, an ideal PLGA delivery system is capable of high encapsulation efficiency as well as sustained release with low burst release. From the observations in Chapter 5 Figure 5.5A, it is postulated that the porous morphology on WOW MP has contributed to the burst release of the entrapped ranibizumab. In this chapter, we propose that the burst release issues in WOW emulsion could be resolved through producing particles with non-porous morphology. One such method is by introducing osmotic pressure across the oil phase through the addition of osmotic agents (NaCl in this thesis) in the external water phase (W_2) thereby maintaining the rest of the fabrication conditions [87]. This method allows for gradual control of the particle morphology depending on the osmotic agent concentration.

Figure 6.1 shows the SEM micrographs of the WOW MP formed with the introduction of NaCl in the W_2 phase. With increasing concentrations of NaCl from a concentration of 0 to 1000 mM, a trend of smaller microparticles, reduced surface porosity and more compact (denser) microparticle was observed. The surface and internal morphology of WOW MP formed with 1000 mM NaCl concentration appears more comparable to SOW MP.

		Surface Morphology	Cross Section
WOW MP	No NaCl 0 mM
		$112.3 \pm 25.2 \mu\text{m}$	
	Mid NaCl 500 mM
		$92.7 \pm 20.5 \mu\text{m}$	
	High NaCl 1000 mM
		$78.0 \pm 20.3 \mu\text{m}$	
SOW MP	No NaCl
		$69.9 \pm 19.4 \mu\text{m}$	

Figure 6.1 SEM micrographs of PLGA microparticles prepared with WOW and SOW emulsion methods showing the surface morphology and cross section at 5000X and 1000X magnification respectively. In the WOW emulsion method, NaCl concentration used in the W₂ phase was varied from 0 to 1000 mM. SOW MP is also used as a reference.

6.3. Ranibizumab loading into WOW MP with various NaCl concentrations

6.3.1. Characterizing ranibizumab loaded WOW MPs

Calculations were based on the release of ranibizumab from WOW MP, where release was stopped at Day 15 for WOW MP no NaCl and Day 40 for WOW MP mid and high NaCl. Total bioactive ranibizumab released was calculated from the daily ranibizumab release amounts, measured by ELISA. At the end of release particles were collected, lysed with NaOH and analyzed for total residual ranibizumab via microBCA. Non bioactive ranibizumab released can then be calculated by taking the difference between the total ranibizumab encapsulated with bioactive ranibizumab released and residual ranibizumab.

The effect of two NaCl concentrations- mid (500 mM) and high (1000 mM) were compared to WOW MP fabricated without NaCl in the W₂ phase, on ranibizumab loading and release from PLGA particles is shown in Table 6.1. The breakdown of active, total and residual ranibizumab after release is presented in Figure 6.2. The encapsulation efficiencies of all WOW MP fabricated with various NaCl concentrations in the W₂ phase is similar within the range of 80% a significant improvement to the encapsulation efficiency obtained in the SOW MP (i.e. 22%).

The bioactivity for the released ranibizumab is crucial for evaluating the therapeutic application of the particles. To evaluate any bioactivity loss due to release, bioactivity of released samples was calculated in terms of percentage active ranibizumab released (■) normalized by total ranibizumab released (■ + ▨) at the end of the release. It was observed that the bioactivity of ranibizumab released reduces with increasing concentration of NaCl used in the W₂ phase. The bioactivity of the released ranibizumab dropped from 48% to 21% when the NaCl concentration is increased from 500 mM to 1000 mM.

Figure 6.2 Summary of ranibizumab after release from WOW MP fabricated with mid and high NaCl concentrations, as a percentage of the drug used during encapsulation- including percentage active ranibizumab released, calculated percentage of non active ranibizumab released and percentage of residual ranibizumab as calculated at the end of release. These three characteristic sums up to give the encapsulation efficiency of each system.

Table 6.1 Characteristics of respective fabrication techniques examined include: Size of fabricated particles, E.E., and bioactivity calculated at the end of the release.

Fabrication Method	Size	Encapsulation Efficiency (%)	Bioactivity of Total Released Samples (%)
WOW MP No NaCl	112.3 ± 6.8 μm	83 ± 6.6 %	56.8 ± 7.7 %
WOW MP Mid NaCl	92.7 ± 20.5 μm	82 ± 2.3 %	48.2 ± 2.8 %
WOW MP High NaCl	78.0 ± 20.3 μm	82 ± 2.0 %	21.0 ± 1.5 %

6.3.2. In vitro release of ranibizumab from WOW MPs

The cumulative percentage of bioactive ranibizumab released and daily bioactive ranibizumab release amounts measured with ELISA under *in vitro* sink condition is shown in Figure 6.3 and Figure 6.4 respectively. Unlike the WOW MP, WOW MP fabricated with mid and high concentrations of NaCl suppressed the burst release by more than 30%. While there was no subsequent release for the WOW MP made without NaCl in the W₂ phase, two release rates were observed for both WOW MP made with mid and high concentrations. For both formulations, a

sustained release of 40 days was achieved where a slow release was first observed for approximately 20 days followed by a period of faster release. Moreover, only 13.6% and 8.2% of the encapsulated ranibizumab was released and bioactive for the mid and high NaCl WOW MP respectively.

WOW MP mid NaCl achieved the longest sustained release and the released ranibizumab retained the highest bioactivity. The first 15 days of release from WOW MP mid NaCl was fitted to zero-order, first-order, Higuchi model [132] and Korsmeyer-Peppas equation [133]. The release is best fitted to Higuchi model ($R^2 = 0.944$) where release is proportional to square root of time.

Figure 6.3 In vitro cumulative percentage release profile of bioactive ranibizumab from WOW MP fabricated with mid and high concentrations of NaCl over a period 40 days as measured by ELISA (Secondary y axis). WOW MP fabricated without NaCl was used as a reference (Primary y axis). WOW MP fabricated with NaCl in the W₂ phase followed a trend of minimal burst, slow release followed by second burst after 20 days.

Table 6.2 Fit of release from SOW MP Mid NaCl to various release models. Rate of release (k) and correlation function (R^2) was calculated.

Release Model	Equation	Rate (k)	R^2
Zero order	$M_t = k_0 t$	0.0773	0.903
First order	$M_t = 100 \times (1 - e^{-k_1 t})$	0.000340	0.904
Higuchi	$M_t = k_H t^{0.5}$	0.402	0.944
Korsmeyer-Peppas	$M_t = k t^n$	1.42	0.940

Figure 6.4 In vitro daily release amounts of ranibizumab from WOW MP fabricated with mid and high concentrations of NaCl over a period 40 days. WOW MP fabricated with NaCl in the W₂ phase showed minimal burst release with release of at least 35 days.

6.4. *In vitro* degradation of WOW particles emulsified with various NaCl concentrations

Degradation of PLGA particles was studied in terms of molecular weight change, mass loss and surface morphology changes to the particles. WOW MP No NaCl and SOW MP were included as a reference. There was no significant difference in the molecular weight loss between mid and high NaCl WOW MPs over the 30 days period. Additionally, both WOW MP fabricated with mid and high concentrations of NaCl in the W₂ phase followed the same trend of the molecular weight loss of SOW MP and a faster molecular weight loss than WOW MP fabricated without NaCl in W₂. For all particles, this molecular weight loss contributed to the rapid mass loss representing bulk degradation of the particles after Day 15.

The same rapid molecular weight loss was reflected in the PLGA particle morphology, which presented a relatively smooth and non porous morphology on Day 0. Pores were observed to have formed on the PLGA particles by Day 5 and loss of its round morphology by Day 15. Meanwhile the morphology of SOW MP remained round to Day 15 and loss of round morphology was observed on the Day 30 timepoint.

C
Mid NaCl

High NaCl

Figure 6.5 In vitro degradation of ranibizumab loaded WOW MP fabricated with mid and high concentrations of NaCl up to a period of 30 days. (A) Characterized by molecular weight change of PLGA polymer. (B) Mass loss of PLGA particles (C) SEM micrographs chronicling particle degradation at magnification of 500 X.

6.5. Fabrication of SOW MP NaCl

6.5.1. Characterizing ranibizumab loaded SOW MP NaCl

WOW MP fabricated with NaCl in the W_2 phase has been shown to yield smaller particles, high encapsulation efficiency and longer sustained release achieve. However, the bioactivity of ranibizumab was compromised with higher NaCl concentrations from 57% to 21% when NaCl concentration is increased from 0 mM to 1000 mM (Table 6.1). To isolate the effect of NaCl on ranibizumab release from PLGA particles, properties of SOW MP was compared with SOW MP fabricated with NaCl in the W_2 phase. Mid (500 mM) NaCl was used in this study for encapsulating solid protein particles as WOW MP mid NaCl particles showed the most ideal release. The internal morphology of SOW MP and SOW MP NaCl is shown in Figure 6.6. Visually, there is no significant difference in the internal porosity and density between both particles.

Figure 6.6 SEM micrographs showing the cross section of (a) SOW MP (b) SOW MP fabricated with NaCl in W₂.

The SOW MP NaCl particulate and *in vitro* release properties are presented in Table 6.3 and Figure 6.7 respectively. The addition of NaCl in to the W₂ phase for SOW MP results in a particle of similar size ($P= 0.1$) with higher encapsulation efficiency ($P= 0.01$). However, the bioactivity of ranibizumab released from SOW MP NaCl was also significantly reduced similar to the bioactivity loss for WOW MP.

Figure 6.7 Summary of ranibizumab after release from SOW MP fabricated with mid NaCl concentrations, as a percentage of the drug used during encapsulation- including percentage active ranibizumab released, calculated percentage of non active ranibizumab released and percentage of residual ranibizumab. These three characteristic sums up to give the encapsulation efficiency of each system.

Table 6.3 Characteristics of respective fabrication techniques examined include: Size of fabricated particles, E.E., and bioactivity calculated at the end of the release.

Fabrication Method	Size	Encapsulation Efficiency (%)	Bioactivity of Total Released Samples (%)
SOW MP NaCl	65.2 ± 15.7 µm	30.6 ± 1.5 %	56.0 ± 1.9 %
SOW MP	69.9 ± 19.4 µm	22 ± 0.9 %	81.7 ± 6.3 %

6.5.2. *In vitro* release of ranibizumab from SOW MP NaCl

The cumulative percentage release and daily amounts of bioactive ranibizumab from SOW MP and SOW MP NaCl measured with ELISA under *in vitro* sink conditions are shown in Figure 6.8 and Figure 6.9. For both SOW MP NaCl and SOW MP 18% and 23% was released on the first day respectively and there was no significant difference ($p= 0.053$) in burst release. Moreover, SOW MP NaCl showed a slower rate of release beyond 20 days, extending beyond the SOW MP release of 15 days. Despite the different rates of release, SOW MP NaCl followed the same release profile of SOW MP.

Figure 6.8 In vitro cumulative percentage release profile of bioactive ranibizumab from SOW MP fabricated with NaCl over a period 20 days as measured by ELISA. SOW MP fabricated without NaCl was used as a reference. SOW MP fabricated with NaCl in the W₂ phase followed the same trend of release as SOW MP.

Figure 6.9 In vitro daily release release amounts of ranibizumab from SOW MP fabricated with NaCl over a period 20 days. SOW MP fabricated with NaCl in the W₂ phase showed a more prolonged release.

6.6. *In vitro* degradation of ranibizumab encapsulated SOW MP NaCl

SOW MP made with NaCl in the W₂ phase presented a similar trend to SOW MP for the loss in molecular weight. This degradation is also reflected in the SEM morphology of SOW MP NaCl, where the particles nearly lost its round morphology by Day 15. Similarly, pitting observed on the SOW MP was also observed in SOW MP NaCl.

Figure 6.10 In vitro degradation of ranibizumab loaded SOW MP fabricated with NaCl up to a period of 30 days. (A) Characterized by molecular weight change of PLGA polymer. (B) Mass loss of PLGA particles. (C) SEM micrographs chronicling particle degradation at magnification of 500 X.

6.7. Effect of ranibizumab VEGF binding ability on incubation with NaCl

Bioactivity loss of ranibizumab post release from PLGA WOW MP NaCl and SOW MP NaCl needs to be accounted for. Ranibizumab released from SOW MP NaCl suffer from an additional 25% loss in bioactivity than SOW MP and the presence of NaCl in W₂ is the only variable during fabrication. To deduce if NaCl-ranibizumab contact during fabrication affects ranibizumab stability, two NaCl fabrication concentrations 500 mM, 1000 mM and a lower NaCl concentration

simulating PBS salt concentration at 137 mM was investigated. To simulate the fabrication, ranibizumab was stored in these NaCl solutions for the same period of time used for solvent extraction/ particle solidification. Storage of ranibizumab was compared against storage at 137 mM of NaCl. While nearly 40% of ranibizumab bioactivity was lost when stored at 1000 mM ($p=0.04$), no significant difference in bioactivity was ranibizumab was stored at 500 mM.

Figure 6.11 Recovery of ranibizumab measured by ELISA on storage of ranibizumab in NaCl buffer after 4 hours to simulate potential contact during PLGA particle fabrication.

6.8. Discussion

Building on Chapter 5, we hypothesized that the lack of sustained release in the WOW MP was to be due to the high porosity of the particles. Moreover, the high encapsulation efficiency of this emulsion method is beneficial for the practical application of this sustained release system due to lower drug wastage. There are a few methods to suppressed burst mostly through the adjustment of the formulation parameters through the use of high molecular weight polymers, high polymer concentration, more hydrophobic polymer and lower drug loading [62]. However, osmotic pressure in the WOW emulsion offers the advantage for easy control in particle pore size by merely adjusting the salt concentrations.

The presence of NaCl in W_2 alters the aqueous phase and organic solvent extraction of WOW emulsion [87]. Hence, PLGA particles with different internal and surface morphology are formed through varying NaCl concentration shown in Figure 6.1. In our study, higher osmotic pressures yield smaller microparticles with

reduced surface porosity and denser internal structure. This trend of less porous and denser particles formed as a result of the osmotic pressure created was also shown in the fabrication of WOW MP encapsulating BSA protein by Jiang et al. and Pistel et al. [87, 134]. The three transitional states of particle solidification suggested by Li et al. [135] include: PLGA-solvent in the solution state transiting into an intermediate gel state and eventually into the glassy state as the solvent is removed to from PLGA particles. Based on this polymer transition during solidification, Jiang et al. had proposed the mechanism of polymeric solidification in the presence of osmotic agents in W_2 [87].

During polymer solidification to form particles, the polymer in the form of an intermediate gel state separates W_1 and W_2 as illustrated in Figure 6.12. PLGA particle is formed when the most of the solvent is extracted into the continuous aqueous phase- W_2 , which is limited by the DCM solubility in water at 2%. Meanwhile water from W_1 can also partition into DCM-polymer oil phase and subsequently into the W_2 phase. Presence of an osmotic pressure gradient between W_1 and W_2 promote this outward water exchange, whereas water exchange is at equilibrium when there is a lack of osmotic pressure gradient. This presence of osmotic agent, specifically NaCl in W_2 encourages unidirectional diffusion of water resulting in a smaller W_1 volume and consequently lesser pores in the final particle morphology. This corresponds to the less porous and smaller particle formed due to reduced W_1 volume. Osmotic pressure gradient increases with increasing NaCl concentration used in W_2 phase. Despite the outward water exchange, encapsulation efficiency of WOW MP as shown in Table 6.3 was not affected with different osmotic pressure, generated with various NaCl concentrations in W_2 .

Figure 6.12 Schematic diagram depicting the mechanism for solidification of WOW MP with and without NaCl in the external aqueous phase W2 [87].

Apart from water exchange, the presence of NaCl as an osmotic agent increases the polarity of the continuous aqueous phase W_2 . This increase in polarity lowers DCM solubility in the aqueous phase resulting in the slower DCM extraction from the polymer phase. Hence polymer remains in the intermediate gel state longer allowing more time for W_1 to diffuse out into W_2 and the formation of a more densely packed particle with a smaller diameter as was observed in our study. Our results show that WOW MP made with more than 500 mM of NaCl forms a relatively less porous particle with the surface morphology similar to SOW MP.

The particle solidification mechanism explained above is applied for the fabrication of SOW MP with osmotic agent in the external water phase. Due to the absence of the internal water phase, the morphology of the particle was not affected. Although the particle solidification rate may be affected as the NaCl increases the polarity of the external aqueous phase, there was no significant difference between the size of SOW MP NaCl and SOW MP. Since the surface morphology and size of SOW MP is not altered in the fabrication process, SOW MP NaCl will be a useful reference to investigate the effect of NaCl on the ranibizumab release from PLGA particles as well as the overall bioactivity of ranibizumab released.

Similar to Chapter 5, the WOW MP prepared with NaCl as an osmotic agent in the W₂ phase was evaluated in terms of encapsulation efficiency, *in vitro* release profile and bioactivity of released ranibizumab. Adaptation of WOW MP fabrication with NaCl in the external aqueous phase was found to not significantly affect the encapsulation efficiency. This indicates that the slower solidification rate due to the presence of osmotic agents did not affect the encapsulation efficiencies with this set of fabrication parameters.

The second factor evaluated- *in vitro* release of ranibizumab from both 500 mM and 1000 mM NaCl followed the same trend with minimal burst release (less than 1%), slow release followed by a faster release after 15 days. It was suggested in Chapter 5 that burst release from WOW MP could be attributed to the significant amount of ranibizumab segregated near the pores leaving poor control over ranibizumab diffusion from pores, enhancing burst release. Notably, this postulation is supported by the suppressed burst release observed from the WOW MP NaCl where there is negligible pores observed under SEM (Figure 6.1C and 6.1G). Similarly, Lee et al. had shown the effectiveness of pores in modulating drug release and burst release from PLGA WOW MP [136]. In the systematic study, the pore size on the particle was controlled through varying BSA porogen concentration in the internal water phase. Porous PLGA encapsulating either insulin or VEGF demonstrated a faster release with increasing pore size.

Following the burst release, a period of diffusional release from WOW MP NaCl in initial 15 days, fitted the Higuchi model where release is proportional to square root time [132]. Diffusion of ranibizumab from PLGA is facilitated by the formation of pores seen from the SEM micrographs on Day 5. The second quicker release occurred after 20 days was likely due to the bulk degradation of the PLGA particles characterized by a catastrophic mass loss of PLGA particles after Day 15. On the contrary, ranibizumab release from SOW MP is mainly controlled by diffusion through the water filled pores, which plateaus at Day 15 despite the onset of bulk degradation. This could be accounted for by the heterogeneous drug distribution within the SOW MP resulting in reduced drug-polymer interaction and a quicker release explained in Chapter 5 [83].

The final factor evaluating the potential of a sustained release system is the bioactivity of the ranibizumab released. Although both PLGA particles fabricated with mid and high NaCl in W₂ followed the same trend of release, different release rates and eventually release amounts of bioactive ranibizumab were observed. Back calculating from the residual ranibizumab, the total amount of ranibizumab released from WOW MP mid and high NaCl is similar at 24 % and 32% respectively. Hence, the difference in release rates was attributed to the loss of ranibizumab bioactivity during release. The bioactivity of ranibizumab released was reduced with increasing concentrations of NaCl used during fabrication. 57%, 48% and 21% of ranibizumab remained bioactive after released from WOW MP made from 0 mM, 500 mM and 1000 mM NaCl in the W₂ phase respectively. Direct incubation of ranibizumab with NaCl maintained its VEGF binding ability below concentrations of 500 mM while 40% loss in bioactivity was detected with 1000 mM NaCl incubation. It had been reported that high ionic strength can lead to aggregation of proteins such as interferon-gamma eventually altering its release from the microparticles [137]. The aggregation mechanism of immunoglobulin G (IgG) proposed that the presence of salt shield the initially charged IgG from electrostatic repulsion. This allows intermolecular hydrophobic interactions eventually increase the propensity for aggregation [138]. However loss in bioactivity due to NaCl-ranibizumab contact is unlikely as diffusion of polar NaCl salt through the non-polar DCM solvent is limited [87] and vice versa ranibizumab diffusion through the intermediate gel state is restricted due to its size and hydrophilic properties [83]. This was confirmed when no NaCl was detected in the polymer matrix despite the use of 1700 mM of NaCl in the W₂ phase by Péan et al. [139].

Encapsulation of ranibizumab in the solid protein state into SOW MP NaCl particles also elicited 25% more reduction of bioactivity for the released ranibizumab despite the use of only mid NaCl concentration in W₂. Similar to the WOW MP, the reduced release amounts of ranibizumab from SOW MP NaCl could also be attributed to the loss in bioactivity. Should the released ranibizumab from SOW MP NaCl retain 80% bioactivity, cumulative released amounts similar to SOW MP will be obtained. Similarly, Péan et al. reported that 93% of the released nerve growth factor (NGF) from WOW MP NaCl lost its bioactivity

during release. They had proposed the loss to be due to the increased outflow of water from the internal aqueous phase aggravating NGF exposure to the oil-water interface leading to denaturation [139]. As the SOW MP NaCl also suffered the loss in bioactivity, exposure of ranibizumab to oil-water interface is unlikely the cause of bioactivity loss. Instead we suggest that interaction between NaCl, PLGA polymer and ranibizumab could have led to a loss in bioactivity during release although the mechanism remains unknown. Maintenance of ranibizumab bioactivity after release is crucial for the success of the drug delivery system. For encapsulation of sensitive proteins, sugars can be used to create the osmotic gradient. However due to the lack of polarity of the sugar molecules, sugar molecules may diffuse through the polymeric barrier reducing the osmotic pressure gradient [87]. Use of sugar may not prolong release as efficiently as use of NaCl as the osmotic agent.

Similar degradation rate was obtained between WOW MP NaCl and SOW MP as opposed to WOW MP, where a continuous molecular weight loss contributed to the catastrophic mass loss after 15 days. This indicates that the morphology of the particles rather than the fabrication technique was more critical on the degradation kinetics. In this case, the less porous particles experienced an accelerated molecular weight loss due to the autocatalytic effect as explained in Chapter 5. It was also observed that SOW MP NaCl underwent similar loss in molecular weight as compared to its counterpart when fabricated without NaCl in the W₂ phase. This translated to similar bulk degradation after Day 15 and had no obvious impact on the release kinetics of ranibizumab.

6.9. Summary

In this chapter we report the use of an osmotic gradient in the WOW emulsion between the internal and external aqueous phase to create PLGA particles of varying pore sizes. The WOW particles fabricated with this method achieved high encapsulation efficiency with a non porous morphology. The morphology of the PLGA particle was shown to be critical in sustaining the release of ranibizumab from PLGA particles. While the use of NaCl markedly reduce the burst release allowing for a sustained release, a balance with released bioactivity need to be reached through the use of optimal NaCl concentration. Mid NaCl concentration

(500 mM) was found to be optimal for sustaining release of ranibizumab up to 40 days. Although it was found that WOW emulsion suffered from the loss in bioactivity, the high encapsulation efficiency obtained ensured that the overall amount released was similar that from SOW MP. The sustained release observed is a combination of diffusion-controlled and degradation-controlled release of ranibizumab. The pragmatic use of this drug delivery system *in vivo* will be discussed in the future work section, where the polymer amounts needed to achieve the dosage for suppressing the disease will be discussed.

7. Conclusions

The impact of AMD, currently the main cause of irreversible blindness in the developed societies, is expected to increase as life expectancy increase. For the treatment for AMD, ranibizumab one of the VEGF antagonists, has been commonly prescribed for patients to inhibit the progression of CNV by angiogenesis. However, ranibizumab administered monthly via intravitreal injections, is commonly associated with injection associated adverse events. This highlights the need for a sustained ranibizumab delivery. In view of this, this thesis aims to deliver ranibizumab sustainably in a PLGA based particle in order to address the issues associated with intravitreal injections. To achieve sustained release, this work was divided into three objectives. The first objective of the project is to study and understand the basic properties of ranibizumab. VEGF binding ability of ranibizumab was crucial for assessing its bioactivity and an in house ELISA kit was developed for its quantification. Usage of this ELISA kit for quantification of bioactive ranibizumab was validated through the translational ability of ranibizumab in inhibiting HUVEC proliferation. This lays the foundation for the use of ELISA in quantifying ranibizumab for future studies.

As release studies will be conducted in physiologically simulating buffer- PBS, its stability on storage in PBS was studied. Ranibizumab was found to be stable in PBS, but recovery was found to be minimal at low nanogram concentrations. Low recovery was attributed to adsorption loss as ranibizumab can be recovered on these storage tubes become especially prominent at low concentrations. Such adsorption loss can be prevented with the use of appropriate buffers through leveraging on the excipients such as surfactants, proteins and pH of the buffer.

Apart from the stability of pure ranibizumab, the effect of the three main fabrication techniques on the stability of ranibizumab was also investigated. The oil-water interface was main cause of loss of protein instability and this was avoided with use of sufficient PVA concentration. For both WOW MP and WOW NP, no loss in ranibizumab bioactivity was found at 1% PVA concentration. This would be an important consideration for encapsulation of ranibizumab into PLGA

particles. Oil-water interface can also be prevented when ranibizumab was encapsulated in the solid form. No loss of ranibizumab was found when encapsulated as such without PVA. Characterization of the basic properties of ranibizumab would be important for subsequent encapsulation of ranibizumab into PLGA particles.

The second objective of this project is to develop an optimal fabrication method for bioactive and sustained release ranibizumab release. Prior to encapsulating ranibizumab into PLGA particles some fabrication parameters were studied. For the WOW emulsions to fabricate both micron and nano sized particles, the effect of PVA concentrations was studied. The effect of PVA concentrations on the morphology and porosity was not pronounced as observed from the SEM micrographs. Hence taking into account the effect of PVA on the stability of ranibizumab after fabrication, 1% PVA will be used for encapsulation studies. The second parameter studied was the effect of polymer concentration. Polymer concentration was shown to be an important factor influencing particle size. This factor can be varied in future studies investigating the effect of particle size on sustaining ranibizumab release.

Three fabrication methods studied include: (i) WOW NP; (ii) WOW MP; (iii) SOW MP. Various characteristics were used to evaluate the potential of the sustained delivery device specifically encapsulation efficiency, its associated in vitro release profile and the bioactivity of the released ranibizumab. In these studies, SOW MP showed the most potential for a sustained release of bioactive ranibizumab. SOW MP displayed a relatively lower burst release and sustained release for 15 days while WOW MP and WOW NP suffered from a burst release with no further release. The high burst release was believed to be due to either the pores present on the particles or the protein segregation on the surface near the pores. Meanwhile, retention of its high bioactivity was attributed to the reduced interaction between protein and polymer due less homogenous drug distribution in the SOW MP. This less dispersed protein distribution contributed to the shielding effect where proteins in the interior of the protein particle are shielded by the exterior sacrificial protein and less prone to degradation when exposed to moisture and elevated temperature. Release of ranibizumab from SOW MP in the 15 days

was mainly diffusion controlled as bulk degradation occurred only after 15 day as indicated by the mass loss.

The sustained release and high bioactivity of ranibizumab released from SOW emulsion were also replicable in the fabrication of smaller SOW MP and SOW MP of a more hydrophobic polymer. Smaller SOW MP was fabricated with lower polymer concentration to yield 45 μm particles as compared to the 70 μm particles. Lower encapsulation efficiency obtained was due to the smaller volume available for encapsulating solid protein. Moreover, similar bioactivity of the released ranibizumab and release profile was achieved. This indicated the applicability of this fabrication method as sustained release was not limited to bigger sized SOW MP. Release can be prolonged with SOW MP fabricated using a more hydrophobic polymer of the same molecular weight. Burst release of ranibizumab was also suppressed with the use of this hydrophobic polymer. This slower release was attributed to the slower hydration of polymer retarding the diffusion of ranibizumab from the particle.

To address if the loss of bioactivity is due to adsorption of released ranibizumab on storage in Chapter 4, release from PLGA in intrinsic buffer (IB) was studied. However, no further release was detected in IB beyond the burst release. This lack of release was attributed to the interaction between the negatively charged PLGA particles and positively charged ranibizumab. Hence use of IB buffer to prevent adsorption loss in release studies was not a viable option as release kinetics will not be an accurate reflection of release in physiological conditions. Moreover, the same release study was carried out in BSA coated bottles where adsorption phenomenon would be circumvented. No significant difference was detected between the release in BSA coated and non-BSA coated bottles. Hence bioactivity loss due to adsorption was not significant. In this chapter, we found that SOW MP is the optimal fabrication method for sustained release of bioactive ranibizumab and this method can be extended to fabricating particles of different size and polymer properties. The bioactivity loss was not due to adsorption of ranibizumab during release.

The last objective is to elucidate the mechanism of release such that appropriate strategies can be applied to prolong the sustained release effect. In chapter 5 we

showed that the WOW emulsion provides a system with high encapsulation efficiency although poor sustained release profile. This lack of sustained release in WOW MP was attributed to the presence of pores or surface associated proteins. On the contrary SOW emulsion provides sustained release of bioactive ranibizumab for more than 15 days but the low encapsulation efficiency limits the translational potential of this delivery system. A non-porous WOW MP would be a promising solution for achieving high encapsulation efficiency and yet a sustained release.

We successfully varied the porosity of WOW MP with NaCl as an osmotic agent in the external aqueous phase (W_2). Higher osmotic pressure was achieved with higher NaCl concentration and yielded smaller microparticles with reduced surface porosity and denser internal structure. The presence of NaCl as an osmotic agent in the W_2 phase was due to the increased outward water exchange and consequently smaller W_1 volume. Additionally, presence of NaCl in the W_2 phase increases the polarity of the W_2 phase. This slows down DCM extraction from the polymer phase, providing more time for the water exchange between W_1 and W_2 resulting in a more densely packed particle

Similarly these particles encapsulating ranibizumab fabricated with various NaCl concentrations in W_2 were evaluated in terms of encapsulation efficiency, in vitro release profile and bioactivity of the released ranibizumab. The WOW MP fabricated with various NaCl concentrations retained similar encapsulation efficiencies. In vitro release of ranibizumab from these particles followed the trend of minimal burst release, slow release and faster release after 15 days. The minimal burst release indicates that the burst release observed in WOW MP was due to the presence of surface pores because cumulation of proteins on the surface would also occur in the presence as the same emulsion technique was used. The period of slow release was attributed to the diffusional release while the second faster release was due to bulk degradation that corroborates with the particle mass loss. In this case, the less porous WOW MP NaCl experienced an accelerated molecular weight loss due to autocatalytic effect, similar to that observed that SOW MP fabricated without NaCl in the W_2 phase.

Despite the improved encapsulation efficiency and sustained release with this NaCl particle, the bioactivity of the released ranibizumab was not improved. This was expected as the particles were fabricated with the WOW emulsion and the ranibizumab would be homogenously distributed within the particles. Moreover, the bioactivity of ranibizumab was reduced with increasing NaCl concentration used in the W₂ phase. As direct incubation of ranibizumab with NaCl solution did not affect ranibizumab activity, the loss of bioactivity was not due to ranibizumab contact with NaCl. The same bioactivity loss was also observed when SOW MP was fabricated with NaCl in the W₂ phase. Hence we suggest the lost in bioactivity to be due to interaction between NaCl, polymer and ranibizumab.

In view of the difficulties in obtaining sustained release formulations of proteins in general, and antibodies in particular, this work highlights the use of a biodegradable polymer to incorporate and release bioactive ranibizumab over a period of 40 days. This is a significant achievement, and points the way to particulate or implantable systems that can deliver ranibizumab against various conditions, for example AMD and diabetic retinopathy.

8. Recommendations

8.1. Dosing perspective

The practical implications of the drug delivery system need to be addressed. The dosage needed from a population pharmacokinetic study of ranibizumab using a one compartmental model with first order absorption and first order elimination from the systemic circulation study is calculated [140]. With this model, drug transportation through the tissues to the diseased site at the choroid is assumed to be negligible.

$$Dosage = C_{ss} \times CL$$

$$CL = \frac{V_d \times \ln 2}{t_{0.5}}$$

The parameters extracted from the study for monthly 0.5 mg dosage include:

- Median steady state serum minimum concentration estimated C_{ss} at 0.22 ng/mL
- CL is clearance
- Systematic to vitreous exposure at ratio of 1:90,000
- Vitreous half-life $t_{0.5}$ for 9 days
- V_d volume of distribution also the vitreous volume of 4 mL

6.1 μg of ranibizumab is needed in the vitreous on a daily basis an amount equivalent to a dosage of 183 μg on a monthly basis. Currently, the most sustained WOW MP NaCl released approximately 6.5 μg / 10 mg of particles after 40 days. With the current released amounts, 300 mg of PLGA particle need to be injected into the vitreous to meet the dosage needs. However, this amount translates to a volume too large for injection. To provide some perspective onto how this system can be made more practical the following suggestions for future work is suggested:

8.2. In vivo testing for the suppression of AMD with sustained release

Current injection of VEGF antagonist into the vitreous is able suppress VEGF for a mean period of 38 days [141]. The vitreous provides a depot effect, similar to a sustained release system, evidenced by the significantly shorter serum half-life when VEGF antagonist is injected intravenously as compared to intravitreally.

While all release studies in this thesis were conducted in vitro, meaningful data can be obtained from in vivo studies. Firstly, although current studies took into account the bioactivity of released ranibizumab in terms of binding ability to VEGF, the effect of released ranibizumab in regulating growth of blood vessels in AMD in vivo needs to be studied. Additionally, the dosage needed in a sustained release system may be less than that needed from a one-time bolus (calculated above) injection due to the increased half-life of the drug in the system. Lastly, release of ranibizumab from PLGA is controlled by the hydrolysis of the ester bonds in the polymer. Due to the various systems present in vivo, the degradation of PLGA and consequently drug release kinetics might change in vivo. Factors such as foreign body reaction and systemic circulation will change the rate of release.

A simplified design of the vivo study can be classified into three sections: pharmacokinetic, pharmacodynamics and toxicity study. In the pharmacokinetic study, the effect of the sustained release can be studied in terms of drug concentrations in the serum and tissues of interest. This would be compared to control- a bolus drug injections to determine if the drug concentrations can be maintained for a longer period time with the use of the drug delivery systems. Pharmacodynamics is an indicator for the regression of the disease. Disease regression can be evaluated in terms of reduction in size of experimentally induced choroidal neovascularization (CNV) through visualizing and quantifying fluorescein isothiocyanate dextran labeled CNV [57]. Electroretinography (ERG) is standard approach to investigate ocular toxicity in the retina providing information towards the outer and inner retinal function before and after injections [56]. The presence of in vivo cytotoxicity is also detectable with histological analysis by hematoxylin and eosin staining verifying any retinal necrosis, morphological changes in the eye and infiltration of inflammatory cells after treatment.

8.3. Further improvements to the drug delivery system

8.3.1. Increase bioactivity of the released ranibizumab from PLGA MP NaCl

Calculations from the dosing studies indicate that higher doses of ranibizumab need to be released from the drug delivery system. This can be achieved by improving the bioactivity of ranibizumab released.

Loss of bioactivity from WOW MP NaCl was attributed to the presence of NaCl during fabrication. To reduce loss of bioactivity due to the suspected ranibizumab-PLGA-NaCl interaction, sugars such as sucrose, mannitol or trehalose can be used in the W_2 phase instead. In fact sugars are commonly used excipients used to optimize protein stability due to changes in water content [66]. Osmotic gradient is created between the W_1 and W_2 phase. There is some differences in the use of this two osmotic agent. Due to the lack of polarity of the sugar molecules, sugar molecules may diffuse through the polymeric barrier during particle solidification reducing the osmotic pressure gradient [87]. Hence particles fabricated with sugar in the W_2 may not be as densely packed and release may not be as efficiently prolonged than with the use of NaCl. However, this lowered encapsulation efficiency might be sufficiently compensated for by the improved bioactivity which makes this worth exploring.

Incomplete protein release that was observed in our formulations had commonly been attributed to protein denaturation. PLGA acidic degradation products resulting in a drop in pH is one contributing factor for non covalent aggregation of proteins which are not released [126]. Basic salts such as $Mg(OH)_2$ have been incorporated to neutralized the degradation byproducts [142]. One possible drawback from this could be that the basic salts could generate osmotic gradient from W_2 into W_1 during release encouraging a faster release due to faster water uptake. Moreover, considering that more than 50% of ranibizumab remained unreleased, the faster release might be compensated by higher overall amount of ranibizumab.

8.3.2. Further suppression in the burst release of ranibizumab

Release of ranibizumab from SOW MP achieved a sustained release over 15 days. However, a burst release of 20% was observed on the first day. While burst release could be important for the initial suppression of the disease, such burst release can be achieved by mixing free drug and the particles. Ideally a longer period of sustained release can be obtained with minimal burst release.

Zhang et al., and Hu et al., had demonstrated the effectiveness of lipid coating on PLGA nanoparticles in suppressing hydrophobic- docetaxel [143] and hydrophilic protein- BSA release [144] respectively. We propose that the burst release of ranibizumab can be suppressed with a lipid layer on a SOW PLGA MP.

Two fabrication methods for hybrid-PLGA particles have been reported. Hybrid-PLGA particles can be fabricated through a two-step fabrication method where PLGA nanoparticles were first fabricated followed by the encapsulation of PLGA nanoparticles into the liposomes [144-146]. However this method provide poor control over the physicochemical structure of the particle making it difficult for clinical translation. A one step fabrication method is preferred such as the one reported by Zhang et al., where the PLGA polymeric solution can be precipitated on addition to a lipid-ethanol mixture to form PLGA particles [143]. In a preliminary study, we demonstrated a, a one-step fabrication of the lipid-SOW MP particle by dissolving lipid, PLGA and solid ranibizumab protein in DCM organic solvent phase. 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) lipid used in this study was added at a weight ratio of 30% to the PLGA polymer. Upon the addition of this mixture to the external aqueous phase, it is expected that the hybrid PLGA-lipid coated SOW MP is form through self-assembly.

A pilot study on the release kinetics of ranibizumab from hybrid DPPC-PLGA particles is shown in Figure 8.1. The burst release of ranibizumab from hybrid DPPC-PLGA was significantly suppressed which the release only starting after Day 10. Suppression of this burst release probably contributed the prolonged release for at least 20 days. However, more work needs to be done to characterize lipid-PLGA hybrid particle. Some parameters to be optimized include lysis of the particle and characterization of the lipid bilayer on the particle. Currently the

NaOH method used to lyse the PLGA particle cannot be used on the hybrid particle as the lipid undergoes saponification on contact with NaOH and a gel like substance is formed [147]. Hence the encapsulated ranibizumab cannot be released for quantification. Further characterization of the hybrid particles need to be studied to ensure that the lipid is coated on the PLGA particle. Current reported characterization involves the use of transmission electron microscopy (TEM) to indicate the spatial location of the lipid in the hybrid nanoparticles [143, 144]. However, this method is may not be viable for the microparticles as the micron size particles fabricated in our studies are too large for the grid that is commonly used in TEM. Another possible method would be to image the particles using confocal microscopy where the lipids and polymer used during fabrication can be tagged with different fluorescence to indicate their spatial location.

Figure 8.1 In vitro cumulative release profile of ranibizumab from SOW MP and hybrid DPPC_SOW MP.

The additional benefits of the lipid layer on the PLGA particle include ease of surface modification to confer properties of the lipid bilayer to the PLGA particle as well as an additional physical layer to impede ranibizumab release. Such beneficial effect would be most prominent should the lipid bilayer be able to suppress the burst that was observed in the WOW nanoparticles. The benefits of nano size include: optical clarity in the vitreous, size range capable of particulate transport and reduced settling velocity consequently reduced particle clearance [19]. However, challenges also stem from its small size such as lower encapsulation efficiency due to increased surface area to volume ratio leading to

burst release. We propose that sustained release of ranibizumab can be achieved through appropriate fabrication of lipid layer on the PLGA particle to yield a hybrid lipid-PLGA particle.

8.3.3. Increase loading of ranibizumab in a bulk implant

Insufficient drug loading can be improved by loading ranibizumab into a bulk implant to achieve longer sustained release of ranibizumab. There are currently some biodegradable and nonbiodegradable implants used in the market. This includes Ozurdex® (length: 6.5 mm by diameter 0.45 mm) which delivers dexamethansone, to treat posterior uveitis and macular edema for a period of up to 6 months, Retisert® (tablet; diameter 1.5 mm) and Iluvien® (length: 3.5 mm by diameter 0.37 mm) for delivering fluocinolone to treat posterior segment uveitis for a period of up to 3 years [148]. Devices in the market mentioned can be used as a reference for encapsulation of ranibizumab into such devices. While microparticles provide more potential administration locations such as the subconjunctival space, suprachoroidal space and the vitreous due to its smaller size, bulk implants are typically limited to administration into the larger vitreous volume. Even though there is limitation in the implantation site, the smaller surface area to volume ratio of the bulk implant implies the potential for a longer sustained release. Furthermore, through employing the approach of using solid-state proteins to better preserve the bioactivity during release as reported in this thesis, the resulting bulk implant might further enhance the therapeutic opportunities for treating AMD by prolonging the release of ranibizumab.

9. Reference

- [1] A. Chappelow and P. Kaiser, "Neovascular Age-Related Macular Degeneration," (in English), *Drugs*, vol. 68, no. 8, pp. 1029-1036, 2008/06/01 2008.
- [2] D. Pauleikhoff, "Neovascular Age-Related Macular Degeneration: Natural History and Treatment Outcomes," *RETINA*, vol. 25, no. 8, pp. 1065-1084, 2005.
- [3] N. Congdon *et al.*, "Causes and prevalence of visual impairment among adults in the United States," *Archives of Ophthalmology*, vol. 122, no. 4, pp. 477-485, Apr 2004.
- [4] P. T. V. M. de Jong, "Age-Related Macular Degeneration," *New England Journal of Medicine*, vol. 355, no. 14, pp. 1474-1485, 2006.
- [5] "Risk factors for choroidal neovascularization in the second eye of patients with juxtafoveal or subfoveal choroidal neovascularization secondary to age-related macular degeneration. Macular Photocoagulation Study Group," *Arch Ophthalmol*, vol. 115, no. 6, pp. 741-7, 1997.
- [6] "Surgery for subfoveal choroidal neovascularization in age-related macular degeneration: Ophthalmic findings* *A list of the members of the Group who contributed data for the 2 SST clinical trials for patients with age-related macular degeneration appears in "Appendix 1." The members of the Writing Committee for this report who take responsibility for its content on behalf of the SST Research Group are included at the end of that list. Documentation of approval of the manuscript submitted for publication by all individuals listed is on file at the SST Coordinating Center, Baltimore, Maryland.: SST report no. 11," *Ophthalmology*, vol. 111, no. 11, pp. 1967-1980, 2004.
- [7] A. P. Adamis, "CHAPTER 4 - Ocular angiogenesis: vascular endothelial growth factor and other factors," in *Retinal Pharmacotherapy*, Q. D. Nguyen, E. B. Rodrigues, M. E. Farah, and W. F. Mieler, Eds. Edinburgh: W.B. Saunders, 2010, pp. 23-36.
- [8] M. S. Blumenkranz, L.-S. Leung, D. F. Martin, P. J. Rosenfeld, and M. A. Zarbin, "Chapter 67 - Pharmacotherapy of Age-Related Macular Degeneration," in *Retina (Fifth Edition)* London: W.B. Saunders, 2013, pp. 1213-1255.
- [9] H. F. Edelhauser *et al.*, "Ophthalmic drug delivery systems for the treatment of retinal diseases: basic research to clinical applications," *Investigative Ophthalmology & Visual Science*, vol. 51, no. 11, pp. 5403-5420, 2010.
- [10] I. D. Ladas, D. A. Karagiannis, A. A. Rouvas, A. I. Kotsolis, A. Liotsou, and I. Vergados, "Safety of repeat intravitreal injections of bevacizumab versus ranibizumab: our experience after 2,000 injections," *RETINA*, vol. 29, no. 3, pp. 313-318, 2009.
- [11] J. Pandit, Y. Sultana, and M. Aqil, "Chitosan-coated PLGA nanoparticles of bevacizumab as novel drug delivery to target retina: optimization, characterization, and in vitro toxicity evaluation," *Artif Cells Nanomed Biotechnol*, pp. 1-11, Nov 18 2016.

- [12] N. Elsaid, T. L. Jackson, Z. Elsaid, A. Alqathama, and S. Somavarapu, "PLGA Microparticles Entrapping Chitosan-Based Nanoparticles for the Ocular Delivery of Ranibizumab," *Mol Pharm*, vol. 13, no. 9, pp. 2923-40, Sep 06 2016.
- [13] S. K. Yandrapu, A. K. Upadhyay, J. M. Petrash, and U. B. Kompella, "Nanoparticles in porous microparticles prepared by supercritical infusion and pressure quench technology for sustained delivery of bevacizumab," *Mol Pharm*, vol. 10, no. 12, pp. 4676-86, Dec 02 2013.
- [14] M. Abrishami, S. Zarei-Ghanavati, D. Soroush, M. Rouhbakhsh, M. R. Jaafari, and B. Malaekhe-Nikouei, "Preparation, characterization, and in vivo evaluation of nanoliposomes-encapsulated bevacizumab (avastin) for intravitreal administration," *Retina*, vol. 29, no. 5, pp. 699-703, May 2009.
- [15] C. C. Hu, J. R. Chaw, C. F. Chen, and H. W. Liu, "Controlled release bevacizumab in thermoresponsive hydrogel found to inhibit angiogenesis," *Biomed Mater Eng*, vol. 24, no. 6, pp. 1941-50, 2014.
- [16] D. P. Chang, V. K. Garripelli, J. Rea, R. Kelley, and K. Rajagopal, "Investigation of Fragment Antibody Stability and Its Release Mechanism from Poly(Lactide-co-Glycolide)-Triacetin Depots for Sustained-Release Applications," *J Pharm Sci*, vol. 104, no. 10, pp. 3404-17, Oct 2015.
- [17] G. E. Visscher, R. L. Robison, H. V. Maulding, J. W. Fong, J. E. Pearson, and G. J. Argentieri, "Biodegradation of and tissue reaction to 50:50 poly(DL-lactide-co-glycolide) microcapsules," *J Biomed Mater Res*, vol. 19, no. 3, pp. 349-65, Mar 1985.
- [18] E. Fournier, C. Passirani, C. N. Montero-Menei, and J. P. Benoit, "Biocompatibility of implantable synthetic polymeric drug carriers: focus on brain biocompatibility," *Biomaterials*, vol. 24, no. 19, pp. 3311-31, Aug 2003.
- [19] U. B. Kompella, A. C. Amrite, R. Pacha Ravi, and S. A. Durazo, "Nanomedicines for back of the eye drug delivery, gene delivery, and imaging," *Progress in Retinal and Eye Research*, vol. 36, pp. 172-198, 2013.
- [20] R. Varshochian *et al.*, "Albuminated PLGA nanoparticles containing bevacizumab intended for ocular neovascularization treatment," *J Biomed Mater Res A*, vol. 103, no. 10, pp. 3148-56, Oct 2015.
- [21] R. Varshochian *et al.*, "The protective effect of albumin on bevacizumab activity and stability in PLGA nanoparticles intended for retinal and choroidal neovascularization treatments," *Eur J Pharm Sci*, vol. 50, no. 3-4, pp. 341-52, Nov 20 2013.
- [22] S. Mohammadi-Samani and B. Taghipour, "PLGA micro and nanoparticles in delivery of peptides and proteins; problems and approaches," *Pharmaceutical development and technology*, no. 0, pp. 1-9, 2014.
- [23] Y. Y. Yang, T. S. Chung, and N. P. Ng, "Morphology, drug distribution, and in vitro release profiles of biodegradable polymeric microspheres containing protein fabricated by double-emulsion solvent extraction/evaporation method," *Biomaterials*, vol. 22, no. 3, pp. 231-41, Feb 2001.
- [24] M. Ye, S. Kim, and K. Park, "Issues in long-term protein delivery using biodegradable microparticles," *J Control Release*, vol. 146, no. 2, pp. 241-60, Sep 01 2010.

- [25] F. Li, B. Hurley, Y. Liu, B. Leonard, and M. Griffith, "Controlled release of bevacizumab through nanospheres for extended treatment of age-related macular degeneration," *Open Ophthalmol J*, vol. 6, pp. 54-8, 2012.
- [26] C. R. Osswald and J. J. Kang-Mieler, "Controlled and Extended In Vitro Release of Bioactive Anti-Vascular Endothelial Growth Factors from a Microsphere-Hydrogel Drug Delivery System," *Curr Eye Res*, vol. 41, no. 9, pp. 1216-22, Sep 2016.
- [27] R. Steinbrook, "The Price of Sight — Ranibizumab, Bevacizumab, and the Treatment of Macular Degeneration," *New England Journal of Medicine*, vol. 355, no. 14, pp. 1409-1412, 2006.
- [28] R. B. Bhisitkul, "Vascular endothelial growth factor biology: clinical implications for ocular treatments," *British Journal of Ophthalmology*, vol. 90, no. 12, pp. 1542-1547, December 1, 2006 2006.
- [29] N. Papadopoulos *et al.*, "Binding and neutralization of vascular endothelial growth factor (VEGF) and related ligands by VEGF Trap, ranibizumab and bevacizumab," (in English), *Angiogenesis*, vol. 15, no. 2, pp. 171-185, 2012/06/01 2012.
- [30] E. W. M. Ng, D. T. Shima, P. Calias, E. T. Cunningham, D. R. Guyer, and A. P. Adamis, "Pegaptanib, a targeted anti-VEGF aptamer for ocular vascular disease," *Nat Rev Drug Discov*, 10.1038/nrd1955 vol. 5, no. 2, pp. 123-132, 2006.
- [31] A. V. Chappelow and A. P. Schachat, "CHAPTER 18 - Neovascular age-related macular degeneration," in *Retinal Pharmacotherapy* Edinburgh: W.B. Saunders, 2010, pp. 128-132.
- [32] R. R. White, B. A. Sullenger, and C. P. Rusconi, "Developing aptamers into therapeutics," *The Journal of Clinical Investigation*, vol. 106, no. 8, pp. 929-934, 2000.
- [33] E. B. Rodrigues *et al.*, "Therapeutic monoclonal antibodies in ophthalmology," *Progress in Retinal and Eye Research*, vol. 28, no. 2, pp. 117-144, 3// 2009.
- [34] F. Penha and P. Rosenfeld, "Management of Neovascular AMD," in *Age-related Macular Degeneration Diagnosis and Treatment*, A. C. Ho and C. D. Regillo, Eds.: Springer New York, 2011, pp. 79-98.
- [35] P. J. Rosenfeld *et al.*, "Ranibizumab for Neovascular Age-Related Macular Degeneration," *New England Journal of Medicine*, vol. 355, no. 14, pp. 1419-1431, 2006.
- [36] R. Keizer, A. R. Huitema, J. M. Schellens, and J. Beijnen, "Clinical Pharmacokinetics of Therapeutic Monoclonal Antibodies," (in English), *Clinical Pharmacokinetics*, vol. 49, no. 8, pp. 493-507, 2010/08/01 2010.
- [37] V. Ghetie and E. S. Ward, "Multiple roles for the major histocompatibility complex class I-related receptor FcRn," *Annual Review of Immunology*, vol. 18, pp. 739-766, 2000 2000.
- [38] N. Ferrara, L. Damico, N. Shams, H. Lowman, and R. Kim, "Development of ranibizumab, an anti-vascular endothelial growth factor antigen binding fragment, as therapy for neovascular age-related macular degeneration," *Retina-the Journal of Retinal and Vitreous Diseases*, vol. 26, no. 8, pp. 859-870, Oct 2006.
- [39] J. Mordenti *et al.*, "Comparisons of the Intraocular Tissue Distribution, Pharmacokinetics, and Safety of 125I-Labeled Full-Length and Fab Antibodies in Rhesus Monkeys Following Intravitreal Administration,"

- Toxicologic Pathology*, vol. 27, no. 5, pp. 536-544, September 1, 1999
- [40] Y. Chen *et al.*, "Selection and analysis of an optimized anti-VEGF antibody: crystal structure of an affinity-matured fab in complex with antigen," *Journal of Molecular Biology*, vol. 293, no. 4, pp. 865-881, 1999.
 - [41] J. Holash *et al.*, "VEGF-Trap: A VEGF blocker with potent antitumor effects," *Proceedings of the National Academy of Sciences*, vol. 99, no. 17, pp. 11393-11398, August 20, 2002
 - [42] N. Ferrara, L. Damico, N. Shams, H. Lowman, and R. Kim, "Development of ranibizumab, an anti-vascular endothelial growth factor antigen binding fragment, as therapy for neovascular age-related macular degeneration," *Retina*, vol. 26, no. 8, pp. 859-70, Oct 2006.
 - [43] R. L. Avery, "What is the evidence for systemic effects of intravitreal anti-VEGF agents, and should we be concerned?," *Br J Ophthalmol*, vol. 98 Suppl 1, pp. i7-10, Jun 2014.
 - [44] P. M. Hughes, O. Olejnik, J.-E. Chang-Lin, and C. G. Wilson, "Topical and systemic drug delivery to the posterior segments," *Advanced Drug Delivery Reviews*, vol. 57, no. 14, pp. 2010-2032, 2005.
 - [45] D. M. Maurice, "Drug delivery to the posterior segment from drops," *Survey of ophthalmology*, vol. 47, pp. S41-S52, 2002.
 - [46] S. Raghava, M. Hammond, and U. B. Kompella, "Periocular routes for retinal drug delivery," *Expert Opinion on Drug Delivery*, vol. 1, no. 1, pp. 99-114, 2004.
 - [47] D. H. Geroski and H. F. Edelhauser, "Drug delivery for posterior segment eye disease," *Investigative Ophthalmology & Visual Science*, vol. 41, no. 5, pp. 961-964, 2000.
 - [48] T. W. Olsen, "CHAPTER 11 - Routes for drug delivery: topical, transscleral, suprachoroidal, and intravitreal gas-phase nanoparticles A2 - Nguyen, Quan Dong," in *Retinal Pharmacotherapy*, E. B. Rodrigues, M. E. Farah, and W. F. Mieler, Eds. Edinburgh: W.B. Saunders, 2010, pp. 74-80.
 - [49] (21 July). *Lucentis (ranibizumab injection) (US prescribing information ed.)*. Available: http://www.gene.com/download/pdf/lucentis_prescribing.pdf
 - [50] (21 July). *European public assessment report (EPAR) for Lucentis*. Available: http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/000715/WC500043546.pdf
 - [51] U. B. Kompella, R. S. Kadam, and V. H. Lee, "Recent advances in ophthalmic drug delivery," *Ther Deliv*, vol. 1, no. 3, pp. 435-56, Sep 2010.
 - [52] J. L. Bourges *et al.*, "Intraocular implants for extended drug delivery: Therapeutic applications," *Advanced Drug Delivery Reviews*, vol. 58, no. 11, pp. 1182-1202, 11/15/ 2006.
 - [53] G. J. Jaffe, D. Martin, D. Callanan, P. A. Pearson, B. Levy, and T. Comstock, "Fluocinolone Acetonide Implant (Retisert) for Noninfectious Posterior Uveitis," *Ophthalmology*, vol. 113, no. 6, pp. 1020-1027, 2006.
 - [54] F. E. Kane, J. Burdan, A. Cutino, and K. E. Green, "Iluvien: a new sustained delivery technology for posterior eye disease," *Expert Opin Drug Deliv*, vol. 5, no. 9, pp. 1039-46, Sep 2008.
 - [55] J. A. Haller *et al.*, "Dexamethasone Intravitreal Implant in Patients with Macular Edema Related to Branch or Central Retinal Vein Occlusion:

- Twelve-Month Study Results," *Ophthalmology*, vol. 118, no. 12, pp. 2453-2460, 12// 2011.
- [56] C. H. Wang, Y. S. Hwang, P. R. Chiang, C. R. Shen, W. H. Hong, and G. H. Hsiue, "Extended release of bevacizumab by thermosensitive biodegradable and biocompatible hydrogel," *Biomacromolecules*, vol. 13, no. 1, pp. 40-8, Jan 09 2012.
 - [57] C. K. Pan *et al.*, "Comparison of long-acting bevacizumab formulations in the treatment of choroidal neovascularization in a rat model," *J Ocul Pharmacol Ther*, vol. 27, no. 3, pp. 219-24, Jun 2011.
 - [58] M. S. Shive and J. M. Anderson, "Biodegradation and biocompatibility of PLA and PLGA microspheres," *Adv Drug Deliv Rev*, vol. 28, no. 1, pp. 5-24, Oct 13 1997.
 - [59] J. E. Chang-Lin *et al.*, "Pharmacokinetics and pharmacodynamics of a sustained-release dexamethasone intravitreal implant," *Invest Ophthalmol Vis Sci*, vol. 52, no. 1, pp. 80-6, Jan 05 2011.
 - [60] Y. Yeo, N. Baek, and K. Park, "Microencapsulation methods for delivery of protein drugs," *Biotechnology and Bioprocess Engineering*, vol. 6, no. 4, pp. 213-230, 2001.
 - [61] J. V. Natarajan, C. Nugraha, X. W. Ng, and S. Venkatraman, "Sustained-release from nanocarriers: a review," *Journal of Controlled Release*, no. 0.
 - [62] Y. Yeo and K. Park, "Control of encapsulation efficiency and initial burst in polymeric microparticle systems," *Archives of Pharmacal Research*, vol. 27, no. 1, p. 1, 2004// 2004.
 - [63] R. C. Mehta, B. C. Thanoo, and P. P. Deluca, "Peptide containing microspheres from low molecular weight and hydrophilic poly(d,l-lactide-co-glycolide)," *Journal of Controlled Release*, vol. 41, no. 3, pp. 249-257, 1996/09/01 1996.
 - [64] J. Wang, K. M. Chua, and C. H. Wang, "Stabilization and encapsulation of human immunoglobulin G into biodegradable microspheres," *J Colloid Interface Sci*, vol. 271, no. 1, pp. 92-101, Mar 01 2004.
 - [65] F. Lagarce *et al.*, "Development and characterization of interleukin-18-loaded biodegradable microspheres," *Int J Pharm*, vol. 314, no. 2, pp. 179-88, May 18 2006.
 - [66] C. Perez, I. J. Castellanos, H. R. Costantino, W. Al-Azzam, and K. Griebenow, "Recent trends in stabilizing protein structure upon encapsulation and release from bioerodible polymers," *J Pharm Pharmacol*, vol. 54, no. 3, pp. 301-13, Mar 2002.
 - [67] T. Morita, Y. Sakamura, Y. Horikiri, T. Suzuki, and H. Yoshino, "Protein encapsulation into biodegradable microspheres by a novel S/O/W emulsion method using poly(ethylene glycol) as a protein micronization adjuvant," *J Control Release*, vol. 69, no. 3, pp. 435-44, Dec 03 2000.
 - [68] T. Morita, Y. Horikiri, H. Yamahara, T. Suzuki, and H. Yoshino, "Formation and isolation of spherical fine protein microparticles through lyophilization of protein-poly(ethylene glycol) aqueous mixture," *Pharm Res*, vol. 17, no. 11, pp. 1367-73, Nov 2000.
 - [69] H. R. Costantino *et al.*, "Protein Spray-Freeze Drying. Effect of Atomization Conditions on Particle Size and Stability," *Pharmaceutical Research*, journal article vol. 17, no. 11, pp. 1374-1382, 2000.

- [70] A. Giteau *et al.*, "Reversible protein precipitation to ensure stability during encapsulation within PLGA microspheres," *Eur J Pharm Biopharm*, vol. 70, no. 1, pp. 127-36, Sep 2008.
- [71] H. Wen, J. Hao, and S. K. Li, "Characterization of human sclera barrier properties for transscleral delivery of bevacizumab and ranibizumab," *Journal of pharmaceutical sciences*, vol. 102, no. 3, pp. 892-903, 2013.
- [72] J. Panyam *et al.*, "Polymer degradation and in vitro release of a model protein from poly(D,L-lactide-co-glycolide) nano- and microparticles," *J Control Release*, vol. 92, no. 1-2, pp. 173-87, Sep 19 2003.
- [73] X. Huang and C. S. Brazel, "On the importance and mechanisms of burst release in matrix-controlled drug delivery systems," *Journal of Controlled Release*, vol. 73, no. 2-3, pp. 121-136, 2001.
- [74] X. Song *et al.*, "Dual agents loaded PLGA nanoparticles: Systematic study of particle size and drug entrapment efficiency," *European Journal of Pharmaceutics and Biopharmaceutics*, vol. 69, no. 2, pp. 445-453, 6// 2008.
- [75] R. A. Jain, "The manufacturing techniques of various drug loaded biodegradable poly(lactide-co-glycolide) (PLGA) devices," *Biomaterials*, vol. 21, no. 23, pp. 2475-2490, 12/1/ 2000.
- [76] C. Thomasin, H. P. Merkle, and B. Gander, "Drug Microencapsulation by PLA/PLGA Coacervation in the Light of Thermodynamics. 2. Parameters Determining Microsphere Formation," *Journal of Pharmaceutical Sciences*, vol. 87, no. 3, pp. 269-275.
- [77] K. Cal and K. Sollohub, "Spray drying technique. I: Hardware and process parameters," *Journal of Pharmaceutical Sciences*, vol. 99, no. 2, pp. 575-586, 2010.
- [78] P. Johansen, H. P. Merkle, and B. Gander, "Technological considerations related to the up-scaling of protein microencapsulation by spray-drying," *European Journal of Pharmaceutics and Biopharmaceutics*, vol. 50, no. 3, pp. 413-417, 11// 2000.
- [79] S. Freitas, H. P. Merkle, and B. Gander, "Microencapsulation by solvent extraction/evaporation: reviewing the state of the art of microsphere preparation process technology," *Journal of Controlled Release*, vol. 102, no. 2, pp. 313-332, 2/2/ 2005.
- [80] K. Koushik and U. B. Kompella, "Preparation of Large Porous Deslorelin-PLGA Microparticles with Reduced Residual Solvent and Cellular Uptake Using a Supercritical Carbon Dioxide Process," *Pharmaceutical Research*, vol. 21, no. 3, pp. 524-535, 2004// 2004.
- [81] M. A. McHugh and V. J. Krukonis, "Supercritical Fluid Extraction (2nd Edition)," ed: Elsevier.
- [82] J. Kluge, F. Fusaro, N. Casas, M. Mazzotti, and G. Muhrer, "Production of PLGA micro- and nanocomposites by supercritical fluid extraction of emulsions: I. Encapsulation of lysozyme," *The Journal of Supercritical Fluids*, vol. 50, no. 3, pp. 327-335, 10// 2009.
- [83] S. Fredenberg, M. Wahlgren, M. Reslow, and A. Axelsson, "The mechanisms of drug release in poly(lactic-co-glycolic acid)-based drug delivery systems--a review," *Int J Pharm*, vol. 415, no. 1-2, pp. 34-52, Aug 30 2011.

- [84] A. Rafati *et al.*, "Chemical and spatial analysis of protein loaded PLGA microspheres for drug delivery applications," *J Control Release*, vol. 162, no. 2, pp. 321-9, Sep 10 2012.
- [85] S. Freiberg and X. X. Zhu, "Polymer microspheres for controlled drug release," *Int J Pharm*, vol. 282, no. 1-2, pp. 1-18, Sep 10 2004.
- [86] A. Giteau, M.-C. Venier-Julienne, A. Aubert-Pouëssel, and J.-P. Benoit, "How to achieve sustained and complete protein release from PLGA-based microparticles?," *International Journal of Pharmaceutics*, vol. 350, no. 1, pp. 14-26, 2008.
- [87] G. Jiang, B. C. Thanoo, and P. P. DeLuca, "Effect of osmotic pressure in the solvent extraction phase on BSA release profile from PLGA microspheres," *Pharm Dev Technol*, vol. 7, no. 4, pp. 391-9, Nov 2002.
- [88] A. Giteau, M. C. Venier-Julienne, A. Aubert-Pouessel, and J. P. Benoit, "How to achieve sustained and complete protein release from PLGA-based microparticles?," *Int J Pharm*, vol. 350, no. 1-2, pp. 14-26, Feb 28 2008.
- [89] T. G. Park, H. Yong Lee, and Y. Sung Nam, "A new preparation method for protein loaded poly(D, L-lactic-co-glycolic acid) microspheres and protein release mechanism study," *J Control Release*, vol. 55, no. 2-3, pp. 181-91, Nov 13 1998.
- [90] T. G. Park, "Degradation of Poly(D,L-Lactic Acid) Microspheres - Effect of Molecular-Weight," *Journal of Controlled Release*, vol. 30, no. 2, pp. 161-173, May 1994.
- [91] T. G. Park, "Degradation of poly(lactic-co-glycolic acid) microspheres: effect of copolymer composition," *Biomaterials*, vol. 16, no. 15, pp. 1123-30, Oct 1995.
- [92] F. v. Burkersroda, L. Schedl, and A. Göpferich, "Why degradable polymers undergo surface erosion or bulk erosion," *Biomaterials*, vol. 23, no. 21, pp. 4221-4231, 11// 2002.
- [93] L. Li and S. P. Schwendeman, "Mapping neutral microclimate pH in PLGA microspheres," *J Control Release*, vol. 101, no. 1-3, pp. 163-73, Jan 03 2005.
- [94] M. R. Moreno *et al.*, "Study of stability and biophysical characterization of ranibizumab and aflibercept," *Eur J Pharm Biopharm*, vol. 108, pp. 156-167, Nov 2016.
- [95] M. van de Weert, J. Hoehstetter, W. E. Hennink, and D. J. Crommelin, "The effect of a water/organic solvent interface on the structural stability of lysozyme," *Journal of Controlled Release*, vol. 68, no. 3, pp. 351-359, 2000.
- [96] F. Wu and T. Jin, "Polymer-based sustained-release dosage forms for protein drugs, challenges, and recent advances," *Aaps Pharmscitech*, vol. 9, no. 4, pp. 1218-1229, 2008.
- [97] K. Griebenow and A. M. Klibanov, "On Protein Denaturation in Aqueous–Organic Mixtures but Not in Pure Organic Solvents," *Journal of the American Chemical Society*, vol. 118, no. 47, pp. 11695-11700, 1996/01/01 1996.
- [98] U. Bilati, E. Allémann, and E. Doelker, "Strategic approaches for overcoming peptide and protein instability within biodegradable nano-and microparticles," *European Journal of Pharmaceutics and Biopharmaceutics*, vol. 59, no. 3, pp. 375-388, 2005.

- [99] S. H. Jeong, "Analytical methods and formulation factors to enhance protein stability in solution," *Archives of pharmacal research*, vol. 35, no. 11, pp. 1871-1886, 2012.
- [100] A. L. Daugherty and R. J. Mersny, "Formulation and delivery issues for monoclonal antibody therapeutics," *Advanced Drug Delivery Reviews*, vol. 58, no. 5, pp. 686-706, 2006.
- [101] U. Bilati, E. Allemann, and E. Doelker, "Strategic approaches for overcoming peptide and protein instability within biodegradable nano- and microparticles," *Eur J Pharm Biopharm*, vol. 59, no. 3, pp. 375-88, Apr 2005.
- [102] W. Jiang and S. P. Schwendeman, "Stabilization and controlled release of bovine serum albumin encapsulated in poly (D, L-lactide) and poly (ethylene glycol) microsphere blends," *Pharmaceutical Research*, vol. 18, no. 6, pp. 878-885, 2001.
- [103] T. J. Kamerzell, R. Esfandiary, S. B. Joshi, C. R. Middaugh, and D. B. Volkin, "Protein-excipient interactions: mechanisms and biophysical characterization applied to protein formulation development," *Adv Drug Deliv Rev*, vol. 63, no. 13, pp. 1118-59, Oct 2011.
- [104] N. K. Jain and I. Roy, "Effect of trehalose on protein structure," *Protein Science*, vol. 18, no. 1, pp. 24-36, 2009.
- [105] K. Fu, A. M. Klibanov, and R. Langer, "Protein stability in controlled-release systems," *Nat Biotechnol*, vol. 18, no. 1, pp. 24-5, Jan 2000.
- [106] G. Jiang, B. H. Woo, F. Kang, J. Singh, and P. P. DeLuca, "Assessment of protein release kinetics, stability and protein polymer interaction of lysozyme encapsulated poly (D, L-lactide-co-glycolide) microspheres," *Journal of Controlled Release*, vol. 79, no. 1, pp. 137-145, 2002.
- [107] A. Aubert-Pouessel *et al.*, "In vitro study of GDNF release from biodegradable PLGA microspheres," *J Control Release*, vol. 95, no. 3, pp. 463-75, Mar 24 2004.
- [108] N. Faisant, J. Akiki, F. Siepmann, J. P. Benoit, and J. Siepmann, "Effects of the type of release medium on drug release from PLGA-based microparticles: experiment and theory," *Int J Pharm*, vol. 314, no. 2, pp. 189-97, May 18 2006.
- [109] K. C. Gordon and C. M. McGoverin, "Raman mapping of pharmaceuticals," *Int J Pharm*, vol. 417, no. 1-2, pp. 151-62, Sep 30 2011.
- [110] A. R. Mire-Sluis, "Progress in the Use of Biological Assays During the Development of Biotechnology Products," *Pharmaceutical Research*, journal article vol. 18, no. 9, pp. 1239-1246, September 01 2001.
- [111] Y. Wang, D. Fei, M. Vanderlaan, and A. Song, "Biological activity of bevacizumab, a humanized anti-VEGF antibody in vitro," *Angiogenesis*, vol. 7, no. 4, pp. 335-45, 2004.
- [112] L. Jorgensen, S. Hostrup, E. H. Moeller, and H. Grohgan, "Recent trends in stabilising peptides and proteins in pharmaceutical formulation - considerations in the choice of excipients," *Expert Opin Drug Deliv*, vol. 6, no. 11, pp. 1219-30, Nov 2009.
- [113] J. Lowe *et al.*, "Development of a novel homogenous electrochemiluminescence assay for quantitation of ranibizumab in human serum," *Journal of pharmaceutical and biomedical analysis*, vol. 52, no. 5, pp. 680-686, 2010.

- [114] S. J. Bakri, M. R. Snyder, J. M. Reid, J. S. Pulido, and R. J. Singh, "Pharmacokinetics of intravitreal bevacizumab (Avastin)," *Ophthalmology*, vol. 114, no. 5, pp. 855-859, 2007.
- [115] C. H. Suelter and M. DeLuca, "How to prevent losses of protein by adsorption to glass and plastic," *Analytical Biochemistry*, vol. 135, no. 1, pp. 112-119, 1983/11/01 1983.
- [116] M. Goebel-Stengel, A. Stengel, Y. Taché, and J. R. Reeve Jr, "The importance of using the optimal plasticware and glassware in studies involving peptides," *Analytical Biochemistry*, vol. 414, no. 1, pp. 38-46, 7/1/ 2011.
- [117] M. van de Weert, J. Hoehstetter, W. E. Hennink, and D. J. A. Crommelin, "The effect of a water/organic solvent interface on the structural stability of lysozyme," *Journal of Controlled Release*, vol. 68, no. 3, pp. 351-359, 9/3/ 2000.
- [118] H. Sah, "Stabilization of proteins against methylene chloride/water interface-induced denaturation and aggregation," *J Control Release*, vol. 58, no. 2, pp. 143-51, Mar 29 1999.
- [119] C. Perez-Rodriguez, N. Montano, K. Gonzalez, and K. Griebenow, "Stabilization of alpha-chymotrypsin at the CH₂Cl₂/water interface and upon water-in-oil-in-water encapsulation in PLGA microspheres," *J Control Release*, vol. 89, no. 1, pp. 71-85, Apr 14 2003.
- [120] A. A. van Apeldoorn, H. J. van Manen, J. M. Bezemer, J. D. de Bruijn, C. A. van Blitterswijk, and C. Otto, "Raman imaging of PLGA microsphere degradation inside macrophages," *J Am Chem Soc*, vol. 126, no. 41, pp. 13226-7, Oct 20 2004.
- [121] A. Rygula, K. Majzner, K. M. Marzec, A. Kaczor, M. Pilarczyk, and M. Baranska, "Raman spectroscopy of proteins: a review," *Journal of Raman Spectroscopy*, vol. 44, no. 8, pp. 1061-1076, 2013.
- [122] S. Mao, J. Xu, C. Cai, O. Germershaus, A. Schaper, and T. Kissel, "Effect of WOW process parameters on morphology and burst release of FITC-dextran loaded PLGA microspheres," *Int J Pharm*, vol. 334, no. 1-2, pp. 137-48, Apr 04 2007.
- [123] J. Siepmann, N. Faisant, J. Akiki, J. Richard, and J. P. Benoit, "Effect of the size of biodegradable microparticles on drug release: experiment and theory," *J Control Release*, vol. 96, no. 1, pp. 123-34, Apr 16 2004.
- [124] K. Fu, D. W. Pack, A. M. Klibanov, and R. Langer, "Visual evidence of acidic environment within degrading poly(lactic-co-glycolic acid) (PLGA) microspheres," *Pharm Res*, vol. 17, no. 1, pp. 100-6, Jan 2000.
- [125] D. Klose, F. Siepmann, K. Elkharraz, S. Krenzlin, and J. Siepmann, "How porosity and size affect the drug release mechanisms from PLGA-based microparticles," *Int J Pharm*, vol. 314, no. 2, pp. 198-206, May 18 2006.
- [126] M. van de Weert, W. E. Hennink, and W. Jiskoot, "Protein Instability in Poly(Lactic-co-Glycolic Acid) Microparticles," *Pharmaceutical Research*, journal article vol. 17, no. 10, pp. 1159-1167, 2000.
- [127] L. Fan and S. K. Singh, *Controlled Release: A Quantitative Treatment*. Springer-Verlag, 1989.
- [128] W. Chen, A. Palazzo, W. E. Hennink, and R. J. Kok, "The effect of particle size on drug loading and release kinetics of gefitinib-loaded PLGA microspheres," *Molecular Pharmaceutics*, 2016/12/14 2016.

- [129] E. Walter *et al.*, "Hydrophilic poly(DL-lactide-co-glycolide) microspheres for the delivery of DNA to human-derived macrophages and dendritic cells," *J Control Release*, vol. 76, no. 1-2, pp. 149-68, Sep 11 2001.
- [130] X. M. Lam, E. T. Duenas, A. L. Daugherty, N. Levin, and J. L. Cleland, "Sustained release of recombinant human insulin-like growth factor-I for treatment of diabetes," *J Control Release*, vol. 67, no. 2-3, pp. 281-92, Jul 03 2000.
- [131] M. J. Blanco-Prieto, M. A. Campanero, K. Besseghir, F. Heimgatner, and B. Gander, "Importance of single or blended polymer types for controlled in vitro release and plasma levels of a somatostatin analogue entrapped in PLA/PLGA microspheres," *J Control Release*, vol. 96, no. 3, pp. 437-48, May 18 2004.
- [132] J. Siepmann and N. A. Peppas, "Higuchi equation: derivation, applications, use and misuse," *Int J Pharm*, vol. 418, no. 1, pp. 6-12, Oct 10 2011.
- [133] R. W. Korsmeyer, R. Gurny, E. Doelker, P. Buri, and N. A. Peppas, "Mechanisms of solute release from porous hydrophilic polymers," *International Journal of Pharmaceutics*, vol. 15, no. 1, pp. 25-35, 1983/05/01/ 1983.
- [134] T. K. K. F. Pistel, "Effects of salt addition on the microencapsulation of proteins using W/O/W double emulsion technique," *Journal of Microencapsulation*, vol. 17, no. 4, pp. 467-483, 2000/01/01 2000.
- [135] W.-I. Li, K. W. Anderson, and P. P. Deluca, "Kinetic and thermodynamic modeling of the formation of polymeric microspheres using solvent extraction/evaporation method," *Journal of Controlled Release*, vol. 37, no. 3, pp. 187-198, 1995/12/01/ 1995.
- [136] J. Lee, Y. J. Oh, S. K. Lee, and K. Y. Lee, "Facile control of porous structures of polymer microspheres using an osmotic agent for pulmonary delivery," *Journal of Controlled Release*, vol. 146, no. 1, pp. 61-67, 2010/08/17/ 2010.
- [137] J. Yang and J. L. Cleland, "Factors Affecting the in Vitro Release of Recombinant Human Interferon- γ (rhIFN- γ) from PLGA Microspheres," *Journal of Pharmaceutical Sciences*, vol. 86, no. 8, pp. 908-914.
- [138] F. Bickel, E. M. Herold, A. Signes, S. Romeijn, W. Jiskoot, and H. Kiefer, "Reversible NaCl-induced aggregation of a monoclonal antibody at low pH: Characterization of aggregates and factors affecting aggregation," *European Journal of Pharmaceutics and Biopharmaceutics*, vol. 107, pp. 310-320, 2016/10/01/ 2016.
- [139] J. M. Pean, M. C. Venier-Julienne, F. Boury, P. Menei, B. Denizot, and J. P. Benoit, "NGF release from poly(D,L-lactide-co-glycolide) microspheres. Effect of some formulation parameters on encapsulated NGF stability," *J Control Release*, vol. 56, no. 1-3, pp. 175-87, Dec 04 1998.
- [140] L. Xu *et al.*, "Pharmacokinetics of ranibizumab in patients with neovascular age-related macular degeneration: a population approach," *Invest Ophthalmol Vis Sci*, vol. 54, no. 3, pp. 1616-24, Mar 05 2013.
- [141] P. S. Muether, M. M. Hermann, U. Viebahn, B. Kirchhof, and S. Fauser, "Vascular endothelial growth factor in patients with exudative age-related macular degeneration treated with ranibizumab," *Ophthalmology*, vol. 119, no. 10, pp. 2082-6, Oct 2012.

- [142] G. Zhu, S. R. Mallery, and S. P. Schwendeman, "Stabilization of proteins encapsulated in injectable poly (lactide- co-glycolide)," *Nat Biotech*, 10.1038/71916 vol. 18, no. 1, pp. 52-57, 01/print 2000.
- [143] L. Zhang *et al.*, "Self-assembled lipid--polymer hybrid nanoparticles: a robust drug delivery platform," *ACS Nano*, vol. 2, no. 8, pp. 1696-702, Aug 2008.
- [144] Y. Hu, R. Hoerle, M. Ehrich, and C. Zhang, "Engineering the lipid layer of lipid-PLGA hybrid nanoparticles for enhanced in vitro cellular uptake and improved stability," *Acta Biomater*, vol. 28, pp. 149-59, Dec 2015.
- [145] H. L. Wong, R. Bendayan, A. M. Rauth, and X. Y. Wu, "Simultaneous delivery of doxorubicin and GG918 (Elacridar) by new polymer-lipid hybrid nanoparticles (PLN) for enhanced treatment of multidrug-resistant breast cancer," *J Control Release*, vol. 116, no. 3, pp. 275-84, Dec 01 2006.
- [146] J. Thevenot, A. L. Troutier, L. David, T. Delair, and C. Ladaviere, "Steric stabilization of lipid/polymer particle assemblies by poly(ethylene glycol)-lipids," *Biomacromolecules*, vol. 8, no. 11, pp. 3651-60, Nov 2007.
- [147] Y. Li and B. A. Watkins, "Analysis of Fatty Acids in Food Lipids," in *Current Protocols in Food Analytical Chemistry*: John Wiley & Sons, Inc., 2001.
- [148] S. G. Schwartz, I. U. Scott, H. W. Flynn, Jr., and M. W. Stewart, "Drug delivery techniques for treating age-related macular degeneration," *Expert Opin Drug Deliv*, vol. 11, no. 1, pp. 61-8, Jan 2014.