Fundamental study of nanoliposomes for ocular therapy

Chaw, Su Yin

2020

Chaw, S. Y. (2020). Fundamental study of nanoliposomes for ocular therapy. Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/144959>

<https://doi.org/10.32657/10356/144959>

This work is licensed under a Creative Commons Attribution-NonCommercial 4.0 International License (CC BY-NC 4.0).

Downloaded on 26 Apr 2023 21:48:32 SGT

**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

**FUNDAMENTAL STUDY OF NANOLIPOSOMES FOR
OCULAR THERAPY**

CHAW SU YIN

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

2020

FUNDAMENTAL STUDY OF NANOLIPOSOMES FOR OCULAR THERAPY

CHAW SU YIN

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

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

2020

Statement of Originality

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

26/11//2020

.....
Date

.....
Chaw Su Yin

Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

26/11/2020

.....
Date

.....
Prof. Subbu Venkatraman

Authorship Attribution Statement

This thesis contains material from 1 paper published in the following peer-reviewed journal(s) / from papers accepted at conferences in which I am listed as an author.

Chapter 5, 6 and 7 published as Su Yin Chaw; Wisna Novera; Ann-Marie Chacko; Tina Tzee Ling Wong; Subbu Venkatraman. *In Vivo* Fate of Liposomes after Subconjunctival Ocular Delivery. *Journal of Controlled Release* (Accepted on Nov 2020)

The contributions of the co-authors are as follows:

- Prof Subbu Venkatraman provided the project direction, supervised the project and reviewed the manuscript.
- Prof Tina Tzee Ling Wong supervised the project and reviewed the manuscript.
- Asst. Prof Ann-Marie Chacko (Duke-NUS Medical School) supervised the project, revised and edited the manuscript.
- I conducted all experiments, analyzed the data and prepared the manuscript drafts.
- Wisna Novera edited the manuscript.
- All non-radioactive materials preparations were done in School of Materials Science and Engineering, NTU. All radioactive material preparations and animal studies were done in Laboratory for Translational and Molecular Imaging (LTMI), Duke-NUS Medical School

26/11/2020

.....
Date

.....
Chaw Su Yin

Abstract

Delivering ocular drugs via subconjunctival administration has garnered much interest due to its ability to utilize the trans-scleral route. Sustained therapeutic effects of drugs such as Latanoprost and Brimonidine Tartrate have been achieved through subconjunctival administration. Although it has been proposed that sub-conjunctivally administered particles either 1) enter the intraocular tissues through the sclera or through intraocular circulation or 2) are retained at the site of administration to slowly release the drug until the drug delivery system (DDS) degrades, the exact distribution or fate of nano-carriers is still largely unknown. In order to achieve and maintain therapeutic drug concentrations in the eye, it is important to know the *in vivo* fate of the DDS in the eye after administration.

This research aims to study the *in vivo* fate of nano-carriers after sub-conjunctivally injecting them into the eyes. The distribution of the nano-carriers in the eyes after administration would greatly impact the way we design DDS to the eye. By focusing on liposomes, factors that affect the *in vivo* ocular distribution and disposition of the liposomes was studied. The factors include liposomal charge, size, incorporation of cholesterol and chain saturation.

The first part of this research involved optimization of the protocol for imaging the injected liposomes in the eye. By employing the use of CellVizio® Dual Band, a real time *in vivo* confocal laser imaging system, ocular distribution of up to a few microns depth can be detected. To distinguish ocular features along with visualization of injected liposomes, the staining of vasculature (using Evans Blue Dye, EVD) and cell nuclei (DRAQ5) were optimized. A pilot study was conducted to investigate the feasibility of the CellVizio® Dual Band for longitudinal ocular distribution study by following the distribution of 100 nm POPC liposomes over 28 days. It was concluded that CellVizio® Dual Band was indeed a suitable imaging system for longitudinal study of distribution of liposomes in the eye and 2% Evans Blue is suitable for use as a vasculature stain. This was also the first time CellVizio® Dual Band has been used for longitudinal ocular imaging study.

The effect of liposomal charge, size, cholesterol and chain saturation on the distribution in the eye was studied using the CellVizio® Dual Band system by improving on the protocol used for the pilot study. The effect of the charge and size on the distribution of liposomes in the eye was evident 3 days after injection. The liposomes were observed to be distributed to both the sclera and cornea on Day 1, probably due to clearance through blood circulation and convective fluid flow. The mobility for micron sized liposomes was much lesser as they were too big to move away from the injection site. At Day 3, most of the negatively charged liposomes have moved away from the injection site, while neutral and positively charged liposomes can still be seen in the sclera region. By day 5, the liposomes have already migrated to their preferred locations. Neutral liposome and negatively charged liposomes settled at the limbus zone (near the corneal periphery) while positively charged liposomes remained at the sclera. Both neutral and negatively charged liposomes could have left the injection site due to the repulsive interactions between the negatively-charged sclera which drove the liposomes away from the sclera. Positively charged liposomes naturally remained at the sclera due to attractive interactions with the negatively charged environment of the sclera. Liposomes with cholesterol and DPPC liposomes were distributed in a similar manner to POPC. Liposomes with cholesterol resided for a longer time in the eye while DPPC liposomes appeared to be cleared much faster than POPC liposomes.

The studies done on radiolabeled liposomes to quantify the ocular distribution of the liposomes complemented the findings from the imaging study. The radioactivity detected was highest in the eye for POPC/DOTAP and POPC MLVs while the amount detected for POPC liposomes and DPPC liposomes were similar. The DPPC liposomes could have gone deeper into the eye or further posterior beyond the detection limits of the probe.

It can be concluded from both the qualitative and quantitative studies that the main factors that affect distribution of the liposomes in the eye were size and charge.

Lay Summary

Subconjunctival injection involves injecting into the space between the conjunctiva and sclera of the eye. Drugs like Latanoprost and Brimonidine Tartrate have been shown to be slowly released into the eye from their drug delivery systems (DDS) when injected into the subconjunctival space. However, not much is known about where the DDS goes after injection. As this mode of injection allows the drug formulation to be placed right on top of the sclera, the DDS could either enter the eye through the sclera or through blood circulation in the eye, while protecting the drug from breaking down. They could also stay at the site of injection to slowly release the drug until the DDS fully breaks down. What happens to the DDS after injection is still largely unknown. In order to achieve and maintain therapeutic drug concentrations in the eye, it is important to know fate of the DDS in the eye after administration. This research aims to study where the DDS are distributed to after sub-conjunctivally injecting them into the eyes

Drug delivery systems can be in the form of particles in which the drug is protected by a shell of material that is compatible with the body. The system that would be focused in this work would be liposomes, in which the protective shell is made up of lipids. By focusing on liposomes, factors that affect the distribution of the liposomes was studied. The factors include liposomal charge, size, incorporation of cholesterol and chain saturation.

This research involves using imaging to study the distribution of the liposomes in the eye, followed by a more in-depth study of the quantity of liposomes found in each individual part of the eye. From the studies, it was concluded that the charge of the liposomes and the size of the liposomes affected the distribution of the liposomes in the eye. Positively charged liposomes and micron-sized liposomes tend to stay in the eye for a longer time compared to neutral and smaller liposomes.

Acknowledgements

I would like to thank my supervisor, Professor Subbu Venkatraman and co-supervisor Associate Professor Tina Wong for their constant support throughout this project so far. I'm especially grateful for Professor Subbu for his financial support towards my PhD studies.

I would like to thank my collaborators at DUKE-NUS. Assistant Professor Ann-Marie Chacko has provided me with much guidance on the imaging studies done using CellVizio Dual Band and radioactive studies. I am also very grateful for her team at Laboratory for Translational and Molecular Imaging (LTMI), consisting of Dr Keira, Dr Raymond, Ms. Marie, Ms. Joanne and Mr. Jing Yang. They have been very kind in facilitating the animal orders and protocols and have given me freedom to use their equipment and vivarium whenever I needed. I am also exceptionally grateful to Dr Satoru Watanabe for assisting me with the intravenous injections of the dye.

I would like to thank Dr Seet Li Fong, Ms. Toh Li Zhen and Ms. Stephanie Chu from SERI for imparting some of their animal handling and histology skills that are much needed for this project. I would also like to thank Ms. Gan Pei from SERI for facilitating the submission of animal protocols.

I would like to thank my friends in OTEC, SERI and the rest of Professor Subbu's group for their support and any help rendered. I'm especially thankful to Mr. Tan Yang Fei, who is both my close friend and fellow group member, for brain-storming with me whenever I encountered problems.

Table of Contents

Abstract.....	i
Lay Summary.....	iii
Acknowledgements	v
Table of Contents	vii
Table Captions	xi
Figure Captions.....	xiii
Abbreviations	xix
 Chapter 1 Introduction.....	 1
1.1 Background	2
1.2 Hypothesis/Problem Statement	3
1.3 Objectives and Scope	3
1.4 Dissertation Overview	4
1.5 Findings and Outcomes/Originality	5
References.....	6
 Chapter 2 Literature Review	 9
2.1 Eye.....	10
2.2 Eye Diseases.....	12
2.3 Routes of drug administration	13
2.3.1 Topical	13
2.3.2 Intravitreal.....	14
2.3.3 Periocular	14
2.4 Barriers to ocular delivery	15
2.4.1 Static barriers	15
2.4.2 Dynamic barriers.....	17
2.5 Distribution in the eye after subconjunctival injection	19

2.6 Liposomes for Ocular Drug Delivery	25
2.7 Factors affecting clearance of liposomes	26
References	30
Chapter 3 Experiment Methods	39
3.1 Materials	40
3.2 Methods	41
3.2 Liposomal Synthesis.....	42
3.2.1 Preparation of Liposomes	42
3.2.2 Zeta Size and Zeta Potential Measurements	44
3.3 <i>In vivo</i> Study.....	45
3.3.1 Animals	45
3.3.2 Subconjunctival administration	45
3.4 Qualitative Imaging Studies	45
3.4.1 Histological assessment	45
3.4.2 <i>In vivo</i> Confocal Imaging.....	46
3.4.3 Pilot study: Feasibility of CellVizio® for eye imaging	49
3.4.4 Distribution study using CellVizio®	51
3.5 Quantitative Radioactive Studies	52
3.5.1 Preparation of radiolabeled liposomes.....	52
3.5.2 Biodistribution Studies.....	52
3.5.3 Tissue Solubilization Protocol	53
3.5.4 Determination of radioactivity	54
3.6 Statistical Analysis	55
References	55
Chapter 4 Using Cellvizio® to study fate of liposomes in the eye	57
4.1 Characterization of liposomes	58
4.2 Feasibility of the CellVizio dual band for ocular distribution studies	58
4.2.1 Choice of Dye used for CellVizio Imaging	58

4.2.2 Pilot study	64
4.3 Conclusion.....	68
Chapter 5 Qualitative studies on effect of liposomal properties on ocular distribution	69
5.1 Effect of charge of liposome	70
5.2 Effect of size of liposome.....	78
5.3 Effect of incorporation of cholesterol	83
5.4 Effect of chain saturation	86
5.5 Natural Lipid versus synthetic lipid	88
5.6 Conclusion.....	91
References.....	92
Chapter 6 Quantitative study on fate of liposomes in the eye.....	93
6.1 Neutral POPC liposomes.....	94
6.2 Positively charged liposomes (POPC/DOTAP).....	95
6.3 Neutral POPC MLVs.....	96
6.4 DPPC liposomes.....	97
6.5 EggPC liposomes	98
6.6 Effect of liposomal properties on the distribution.....	99
6.7 Conclusion.....	103
Chapter 7 Discussion, conclusion and future work.....	105
7.1 Discussion and conclusion	106
7.2 Future work	110
7.2.1 Study the integrity of liposomes <i>in vivo</i>	111
7.2.2 Study of fate of liposomes and drugs.....	111
References.....	113

Table Captions

Table 2.5: Compilation of studies done on fate of sub-conjunctivally injected molecules.

Table 2.7: Summary of effect of liposomal properties on protein interaction and *in vivo* systemic clearance.

Table 3.2.1.1: No. of moles of lipids used for each type of liposome.

Table 3.2.1.2: Molecular structure of radiolabeled lipids provided by ARC and Perkin Elmer respectively.

Table 3.4.2.2: Description of dyes tested for use with CellVizio® system.

Table 3.4.4: Liposomal properties studied using CellVizio®.

Table 3.5.1: Liposomes for quantitative studies.

Table 3.5.3: Solubilization protocol for tissues.

Table 4.1: Size of liposomes used.

Table 5.1: Zeta size and zeta potential of the liposomes.

Table 5.2: Size of the liposomes

Table 5.3: Size of the liposomes

Table 5.4: Size of the liposomes.

Table 5.51: List of main components of EggPC.

Table 5.52: Size of the liposomes prepared.

Figure Captions

Figure 2.1: Structure of human eye.

Figure 2.3: Ocular drug delivery routes.

Figure 2.3.3: Subconjunctival Injection [30].

Figure 2.4.1: Static barriers.

Figure 2.4.2.1: Clearance pathways.

Figure 3.2: Overview of experiments done.

Figure 3.4.2: a) Cellvizio® Dual Band Imaging System [1] and b) Image of Cellvizio® probe on mouse eye.

Figure 3.4.2.1: Eye map for CellVizio Scans. Image of eye retrieved from *Dorland's Medical Dictionary for Health Consumers*. (2007).

Figure 3.4.3: Flow chart highlighting main procedures in the CellVizio imaging protocol.

Figure 3.5.4: Overview of Scintillation Counting Process.

Figure 4.2.1.1.1: CellVizio images of mice eyes taken 5 days after i.v. injection of 1% and 2% EVD. Evans Blue was not injected in the control mouse. (3S: superior limbus, 3N: Nasal limbus, 3I: Inferior limbus, 3T: Temporal limbus)

Figure 4.2.1.1.2: CellVizio images of mice eyes taken 7 days after i.v. injection of 1% and 2% Evans Blue. Evans Blue was not injected in the control mouse. (3S: superior limbus, 3N: Nasal limbus, 3I: Inferior limbus, 3T: Temporal limbus)

Figure 4.2.1.2.1: Effect of DRAQ5 concentration cell nuclei staining. Compilation of CellVizio images of mice eyes taken 25 minutes after instillation of 10 μ M, 20 μ M and 40 μ M DRAQ5. Images were taken at the cornea (1A: Cornea/Pupil, 2S: Superior Cornea/Iris, 2N: Nasal Cornea/Iris, 2I: Inferior Cornea/Iris, 2T: Temporal Cornea/Iris). DRAQ5 was not administered in the control mouse.

Figure 4.2.1.2.2: Effect of DRAQ5 concentration on cell nuclei staining. Compilation of CellVizio images of mice eyes taken 90 minutes after instillation of 10 μ M, 20 μ M and 40 μ M DRAQ5. Images were taken at the cornea (1A: Cornea/Pupil, 2S: Superior Cornea/Iris, 2N: Nasal Cornea/Iris, 2I: Inferior Cornea/Iris, 2T: Temporal Cornea/Iris). DRAQ5 was not administered in the control mouse.

Figure 4.2.1.2.3: Effect of time on DRAQ5 staining. Compilation of CellVizio images of a mouse eye taken at 25 minutes, 90 minutes, 180 minutes after instillation of 40 μ M

DRAQ5. Images were taken at the cornea (1A: Cornea/Pupil, 2S: Superior Cornea/Iris, 2N: Nasal Cornea/Iris, 2I: Inferior Cornea/Iris, 2T: Temporal Cornea/Iris) of the same mouse.

Figure 4.2.1.2.4: Effect of DRAQ5 on the weight of mice. 10 μ L of 40 μ M of DRAQ5 was directly instilled on the eye on Day 0, 1, 2, 5 and 6. Weight was taken on Day 1, 2, 5, 6 and 7.

Figure 4.2.2.1: Photographs of mice eyes after subconjunctival injection of fluorescent liposomes. The arrows indicate the bulge in the subconjunctival space which contains the injected liposomes.

Figure 4.2.2.2: Compilation of the distribution of the FL-POPC liposomes in the limbus region of the eye over 35 days (3S: Superior limbus, 3T: Temporal limbus). Images were only taken from the 488 channel.

Figure 4.2.2.3: Compilation of the distribution of the FL-POPC liposomes in the cornea, limbus and sclera region of the eye over 28 days with both EBD vasculature staining and DRAQ5 cell nuclei staining for one representative mouse. S represents the sclera and C represents the cornea side of the limbus when identifiable.

Figure 4.2.2.4: Histology images of the mouse eye 1 day post injection. Arrows point to the cornea where most of the liposomes can be observed at this time point.

Figure 4.2.2.5: Effect of Cellvizio® imaging protocol on the weight of mice. Weight was taken on all the days the CellVizio imaging was done.

Figure 5.11: Photographs of mouse taken right after subconjunctival injection of 10 μ L of fluorescent liposomes: neutral, POPC (A), positively charged, POPC-DOTAP (B), negatively charged, POPC-POPG (C), and Fluorescein (D). Arrows indicate the injected liposomes/fluorescein.

Figure 5.12: Distribution of neutral liposomes at the limbus (3S: Superior, 3N: Nasal, 3I: Inferior, 3T: Temporal) and sclera (4S: Superior, 4N: Nasal, 4I: Inferior, 4T: Temporal) region of 1 representative mouse over 14 days. C: Cornea, S: Sclera.

Figure 5.13: Average Fluorescence intensity of neutral POPC liposomes at the limbus and sclera region over 7 days. * and ^ represents significant difference $p < 0.05$.

Figure 5.14: Distribution of positively charged liposomes at the limbus (3S: Superior, 3N: Nasal, 3I: Inferior, 3T: Temporal) and sclera (4S: Superior, 4N: Nasal, 4I: Inferior, 4T: Temporal) region of 1 representative mouse over 14 days. C: Cornea, S: Sclera.

Figure 5.15: Average Fluorescence intensity of positively charged liposomes at the

limbus and sclera region over 7 days. * and ^ represents significant difference $p<0.05$.

Figure 5.16: Distribution of negatively charged liposomes at the limbus (3S: Superior, 3N: Nasal, 3I: Inferior, 3T: Temporal) and sclera (4S: Superior, 4N: Nasal, 4I: Inferior, 4T: Temporal) region of 1 representative mouse over 14 days. C: Cornea, S: Sclera.

Figure 5.17: Average Fluorescence intensity of negatively charged liposomes at the limbus and sclera region over 7 days. * and ^ represents significant difference $p<0.05$.

Figure 5.18: CellVizio images showing disposition of neutral, positively charged, negatively charged liposomes and fluorescein dye at limbus (3S: Superior, 3N: Nasal, 3I: Inferior, 3T: Temporal) and sclera (4S: Superior, 4N: Nasal, 4I: Inferior, 4T: Temporal) region at day 7 of one representative mouse from each group. C: Cornea, S: Sclera.

Figure 5.19: Comparison of Fluorescence intensity of liposomes of different charges at the limbus and sclera region over 7 days. * and ^ represents significant difference $p<0.05$.

Figure 5.21: Distribution of 250 nm POPC neutral liposomes at the limbus (3S: Superior, 3N: Nasal, 3I: Inferior, 3T: Temporal) and sclera (4S: Superior, 4N: Nasal, 4I: Inferior, 4T: Temporal) region of 1 representative mouse over 7 days. C: Cornea, S: Sclera.

Figure 5.22: Average Fluorescence intensity of positively charged liposomes at the limbus and sclera region over 7 days. * and ^ represents significant difference $p<0.05$.

Figure 5.23: Distribution of MLV POPC neutral liposomes at the limbus (3S: Superior, 3N: Nasal, 3I: Inferior, 3T: Temporal) and sclera (4S: Superior, 4N: Nasal, 4I: Inferior, 4T: Temporal) region of 1 representative mouse over 7 days. C: Cornea, S: Sclera.

Figure 5.24: Average Fluorescence intensity of positively charged liposomes at the limbus and sclera region over 7 days. * and ^ represents significant difference $p<0.05$.

Figure 5.26: Comparison of Fluorescence intensity of liposomes of different sizes at the limbus region and sclera region over 7 days. * and ^ represents significant difference $p<0.05$.

Figure 5.31: Distribution of POPC/Chol (60:40) liposomes (100 nm) at the limbus (3S: Superior, 3N: Nasal, 3I: Inferior, 3T: Temporal) and sclera (4S: Superior, 4N: Nasal, 4I: Inferior, 4T: Temporal) region of 1 representative mouse over 7 days. C: Cornea, S: Sclera.

Figure 5.32: Average Fluorescence intensity of POPC/Cholesterol liposomes at the

limbus and sclera region over 7 days. * and ^ represents significant difference $p < 0.05$.

Figure 5.33: Comparison of Fluorescence intensity of liposomes with and without cholesterol at the limbus region and sclera region over 7 days. * and ^ represents significant difference $p < 0.05$.

Figure 5.34: CellVizio images showing disposition of POPC and POPC/Chol (60:40) liposomes at limbus (3S: Superior, 3N: Nasal, 3I: Inferior, 3T: Temporal) and sclera (4S: Superior, 4N: Nasal, 4I: Inferior, 4T: Temporal) region at day 7 of one representative mouse from each group. C: Cornea, S: Sclera. All the liposomes are 100 nm in size.

Figure 5.41: Distribution of DPPC liposomes (100 nm) at the limbus (3S: Superior, 3N: Nasal, 3I: Inferior, 3T: Temporal) and sclera (4S: Superior, 4N: Nasal, 4I: Inferior, 4T: Temporal) region of 1 representative mouse over 7 days. C: Cornea, S: Sclera.

Figure 5.42: CellVizio images showing disposition of DPPC the limbus (3S: Superior, 3T: Temporal) and sclera (4S: Superior, 4T: Temporal) region at over time for 5 mice. All the liposomes are 100 nm in size.

Figure 5.43: CellVizio images showing disposition of POPC and DPPC at the limbus (3S: Superior, 3N: Nasal, 3I: Inferior, 3T: Temporal) and sclera (4S: Superior, 4N: Nasal, 4I: Inferior, 4T: Temporal) region at day 7 of one representative mouse from each group. C: Cornea, S: Sclera. All the liposomes are 100 nm in size.

Figure 5.51: Distribution of EggPC liposomes (100 nm) at the limbus (3S: Superior, 3T: Temporal) and sclera (4S: Superior, 4T: Temporal) region of 1 representative mouse over 14 days. C: Cornea, S: Sclera.

Figure 5.52: CellVizio images showing disposition of EggPC liposomes and its main constituents, POPC, PLPC and SLPC liposomes at the limbus (3S: Superior, 3T: Temporal) and sclera (4S: Superior, 4T: Temporal) region at day 7 of one representative mouse from each group. C: Cornea, S: Sclera. All the liposomes are neutral and 100 nm in size.

Figure 6.1: Distribution of 100 nm POPC liposomes after subconjunctival injection into the mice eye. (* $p < 0.05$ in comparison to previous timepoint).

Figure 6.2: Distribution of 100 nm POPC/DOTAP (positively charged) liposomes after subconjunctival injection into the mice eye. (* $p < 0.05$ in comparison to previous timepoint).

Figure 6.3: Distribution of POPC MLVs (micron size) after subconjunctival injection into the mice eye. (* $p < 0.05$ in comparison to previous timepoint).

Figure 6.4: Distribution of 100 nm DPPC liposomes after subconjunctival injection into the mice eye. (* $p < 0.05$ in comparison to previous timepoint).

Figure 6.5.1: Comparison of quantity of liposomes detected in ocular tissues for 100 nm POPC, 100 nm POPC/DOTAP, POPC MLV and 100 nm DPPC liposomes after subconjunctival injection into the mice eye. (* $p < 0.05$ in comparison to POPC liposomes).

Figure 6.5.2: Comparison of quantity of liposomes detected in lymphatic system for 100 nm POPC, 100 nm POPC/DOTAP, POPC MLV and 100 nm DPPC liposomes after subconjunctival injection into the mice eye. (* $p < 0.05$ in comparison to POPC liposomes).

Figure 6.6.3: Comparison of quantity of liposomes detected in the blood and excretory organs for 100 nm POPC, 100 nm POPC/DOTAP, POPC MLV and 100 nm DPPC liposomes after subconjunctival injection into the mice eye. (* $p < 0.05$ in comparison to POPC liposomes).

Abbreviations

AMD	Age-related Macular Degeneration
ARVO	Association for Research in Vision and Ophthalmology
BRB	Blood retinal barrier
CF	Carboxyfluorescein
CGN	Collagen-Glycosaminoglycan Network
Ci	Curie
CPM	Counts Per Minute
DDS	Drug Delivery System
DMPC	1,2-dimyristoyl-sn-glycero-3-phosphocholine
DNA	Deoxyribonucleic acid
DOTAP	1,2-dioleoyl-3-trimethylammonium-propane (chloride salt)
DPM	Disintegrations Per Minute
DPPC	1,2-dipalmitoyl-sn-glycero-3-phosphocholine
DTPA	Diethylenetriamine Pentaacetic Acid
EBD	Evans Blue Dye
Fl-DHPE	(N-(Fluorescein-5-Thiocarbamoyl)-1,2-Dihexadecanoyl-sn-Glycero-3-Phosphoethanolamine, Triethylammonium Salt)
I.v.	Intravenous
IACUC	Institutional animal care and use committee
IOP	Intraocular pressure
IP	Intra- peritoneal
LUV	Large, Unilamellar Vesicles
LWMH	Low-molecular-weight Heparin
MLV	Multilamellar Vesicles
MRI	Magnetic resonance imaging
OCT	Optimal cutting temperature
PBS	Phosphate Buffered Solution
PC	Phosphocholine
PEG	Polyethylene Glycol
PI	Phosphatidylinositol
PLA	Polylactic Acid

PLPC	1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine
POPC	1-palmitoyl-2-oleoyl-glycero-3-phosphocholine
pRNA	Promoter-associated ribonucleic acid
PS	Phosphatidyl serine
PVDF	Polyvinylidene fluoride
RES	Reticuloendothelial System
RPE	Retinal Pigment Epithelium
SD	Standard deviation
SLPC	1-stearoyl-2-linoleoyl-sn-glycero-3-phosphatidylcholine
VEGF	Vascular Endothelial Growth Factor

Chapter 1

Introduction

This chapter briefly introduces the limitations of ocular drug delivery and the advantages sub-conjunctival injection has over other routes of ocular drug administration. The importance of knowing the fate of drug delivery systems was discussed. In summary, this chapter introduces the background, problem statement, hypothesis, objective and scope of this thesis. A dissertation overview and findings are also summarized in this chapter.

1.1 Background

Many people worldwide are affected by ocular diseases that have direct impact on the patient's vision and quality of life [1]. Due to the presence of ocular barriers, there is little efficiency in systemic delivery of drugs to the eyes, making it a challenge to deliver therapeutic agents to the eye [2]. Therapeutic agents must be delivered across several protective barriers regardless of the administration route (E.g. topical, subconjunctival or intravitreal injection) [3].

Eye-drops often eliminate rapidly after administration and only 1–3% of the active drug actually reaches the intraocular tissue [3]. One major disadvantage of eye drops is poor treatment compliance, especially with elderly people who have a tendency to forget their routine medications. Approximately 20% of glaucoma patients tend to miss out on their eye drops which in turn resulted in an increased severity of their condition [4]. Intravitreal administration is an invasive procedure that requires penetration of all layers of the ocular globe. While it is able to deliver the drugs directly into the vitreous cavity of the eye, complications such as retinal detachment and intraocular hemorrhage can occur [1, 3].

Due to its ability to utilize the trans-scleral route, there has been much interest in administration of ocular drugs through sub-conjunctival injection. Being less invasive than intravitreal injection, sustained therapeutic effect for glaucoma medications such as Latanoprost [4-8] and Brimonidine Tartrate [9] have been achieved through sub-conjunctival administration. However, the fate of the drug delivery systems after sub-conjunctival injection is not clearly known. To have a therapeutic effect, sub-conjunctivally administered particles could have either entered the intraocular tissues through the sclera or through intraocular circulation or been retained at the site of administration to slowly release the drug until the drug delivery system (DDS) degrades [10, 11]. In order to achieve and maintain therapeutic drug concentrations in the eye, it is important to know the *in vivo* fate of the DDS in the eye after administration.

Among various colloidal carriers, liposomes have been most extensively investigated [12] and have been widely studied for their use as an ocular delivery system both as ophthalmic applications and injectable formulations. Liposomes are phospholipid

vesicles with discrete aqueous space enclosed by one or more concentric lipid bilayers [13]. This gives liposomal systems the ability to entrap both lipophilic (in the bilayer membrane) and hydrophilic (in the aqueous center) compounds, allowing encapsulation of a diverse range of drugs [12, 14-18]. Liposomes offer several advantages such as biocompatibility and the freedom to control their characteristics by modifying their physicochemical and biophysical properties [12, 14-19]. By tuning the size and the charge of liposomes, liposomes can be used to deliver drugs with different release profiles. Positively charged liposomes also allow delivery of negatively charged macromolecules while incorporation of cholesterol can adjust the fluidity of the liposomes and corresponding permeability of the membrane to water soluble molecules. [20] When compared with topical application of drug solution, Fluconazole loaded liposomal formulations were found to show better improvements in rabbits infected with *Candida* keratitis [21]. Liposomal formulations of Bevacizumab [22], Cyclosporine [23] and Ganciclovir [24] have also shown longer half-life and prolonged effect of the drug compared to non-liposomal formulations. Due to the versatility and usefulness of liposomes, it is of high interest to study the fate of the liposomes in eye.

1.2 Hypothesis/Problem Statement

The *in vivo* fate of liposomes is influenced by the properties of the liposomes, such as size, charge, incorporation of cholesterol and chain saturation.

1.3 Objectives and Scope

The objective of this project is to study the *in vivo* fate of liposomes, in the eye after sub-conjunctival injection and the factors that affect the *in vivo* distribution of the liposomes in the eye. Factors that will be studied include size, charge, incorporation of cholesterol and chain saturation.

The influence of the size, charge, incorporation of cholesterol and chain saturation on the *in vivo* fate of the liposomes after subconjunctival injection will be studied in mice.

1.4 Dissertation Overview

This thesis is organized into 7 chapters:

Chapter 1 briefly introduces the limitations of ocular drug delivery and the advantages sub-conjunctival injection has over other routes of ocular drug administration. The importance of knowing the fate of drug delivery systems was discussed. This thesis aims to test the hypothesis that *in vivo* fate of nano-carriers is influenced by the properties of the nano-carriers, such as size, charge, incorporation of cholesterol and chain saturation.

Chapter 2 aims to provide readers with background information and related research on the work done in this thesis. The chapter introduces the anatomy of the eye followed and various routes of ocular drug administration. Barriers to ocular delivery were discussed and a compilation of previous findings on the fate of liposomes in the eye after subconjunctival injection was made. Factors affecting clearance of liposomes were summarized.

Chapter 3 covers the materials, fabrication methodology and experimental methodology used in this work. Preparation of liposomes (both fluorescent and radioactive liposomes) and characterization methods were described in this chapter. *In vivo* studies which forms the bulk of this project was also described in detail. This includes the use of Cellvizio® Dual Band for *in vivo* confocal imaging of the eye to qualitatively study the effect of various factors on the distribution of liposomes in the eye after subconjunctival injection. The use of liquid scintillation counter to quantify the amount of liposomes in ocular tissues and sample preparation for analysis was also described in detail.

Chapter 4 discusses the feasibility of using Cellvizio® Dual Band for studying the fate of liposomes in the eye after subconjunctival injection. As the Cellvizio® Dual Band has not been used in a similar way elsewhere, optimization of the dyes used to identify tissue landmarks was required. Two different dyes, Evans Blue and DRAQ5, were studied for their suitability to be used in the study. A pilot study was then conducted with the optimized staining conditions. The results of the pilot study and feasibility of

using this imaging method to study the distribution of the liposomes in the eye was discussed.

Chapter 5 describes the use of Cellvizio® to qualitatively study the effect of various liposomal properties on the distribution of liposomes in the eye after subconjunctival injection in the mice. Charge and size of the liposomes were found to affect the location at which the liposomes were distributed to while incorporation of cholesterol and chain saturation affected how fast the liposomes were cleared from the eye.

Based on the outcome of the qualitative studies done in Chapter 5, a few types of liposomes were chosen to quantitatively study factors like charge (POPC/DOTAP liposome), size (POPC MLVs) and effect of chain saturation (DPPC liposomes) in Chapter 6. Radioactively labelled liposomes were injected into the subconjunctival space of the eye and the radioactivity levels in various ocular tissues were quantified. It was observed that the main factors that can control the clearance of the liposomes from the eye were charge and size. The data obtained in this chapter complemented the results from the imaging studies in chapter 5.

Chapter 7 covers a discussion of the results achieved in this research with a conclusion of this work. Through the studies done in this work, it could be established that the ocular distribution of liposomes after subconjunctival injection is affected by charge and size of the liposomes. Limitations of this work was discussed and future work that could answer or solve these limitations was explored.

1.5 Findings and Outcomes/Originality

This research led to several novel outcomes by:

1. Establishing the use of Cellvizio® imaging system to study the distribution of liposomes in the eye as this is the first time this confocal imaging system was used for such type of study
2. Achieving a relation between liposomal properties and distribution in the eye after subconjunctival injection.

References

- [1] Kim, Y.C., et al., *Ocular delivery of macromolecules*. Journal of Controlled Release, 2014. **190**: p. 172-181.
- [2] Kang-Mieler, J.J., C.R. Osswald, and W.F. Mieler, *Advances in ocular drug delivery: emphasis on the posterior segment*. Expert Opinion on Drug Delivery, 2014. **11**(10): p. 1647-1660.
- [3] Kuno, N. and S. Fujii, *Recent Advances in Ocular Drug Delivery Systems*. Polymers, 2011. **3**(1): p. 193.
- [4] Voss, K., et al., *Development of a novel injectable drug delivery system for subconjunctival glaucoma treatment*. Journal of Controlled Release, 2015. **214**: p. 1-11.
- [5] Wong, T.T., et al., *Nanomedicine for glaucoma: sustained release latanoprost offers a new therapeutic option with substantial benefits over eyedrops*. Drug Delivery and Translational Research, 2014. **4**(4): p. 303-309.
- [6] Natarajan, J.V., et al., *Sustained Drug Release in Nanomedicine: A Long-Acting Nanocarrier-Based Formulation for Glaucoma*. ACS Nano, 2014. **8**(1): p. 419-429.
- [7] Natarajan, J.V., et al., *Nanomedicine for glaucoma: liposomes provide sustained release of latanoprost in the eye*. International Journal of Nanomedicine, 2012. **7**: p. 123-131.
- [8] Giarmoukakis, A., et al., *Biodegradable nanoparticles for controlled subconjunctival delivery of latanoprost acid: In vitro and in vivo evaluation. Preliminary results*. Experimental Eye Research, 2013. **112**: p. 29-36.
- [9] Fedorchak, M.V., et al., *28-day intraocular pressure reduction with a single dose of brimonidine tartrate-loaded microspheres*. Experimental Eye Research, 2014. **125**: p. 210-216.
- [10] Amrite, A.C. and U.B. Kompella, *Size-dependent disposition of nanoparticles and microparticles following subconjunctival administration*. Journal of Pharmacy and Pharmacology, 2005. **57**(12): p. 1555-1563.
- [11] Bourges, J.-L., et al., *Ocular Drug Delivery Targeting the Retina and Retinal Pigment Epithelium Using Polylactide Nanoparticles*. Investigative Ophthalmology & Visual Science, 2003. **44**(8): p. 3562-3569.
- [12] Bassyouni, F., et al., *Advances and new technologies applied in controlled drug delivery system*. Research on Chemical Intermediates, 2015. **41**(4): p. 2165-2200.
- [13] Jesorka, A. and O. Orwar, *Liposomes: Technologies and Analytical Applications*.

- Annual Review of Analytical Chemistry, 2008. **1**(1): p. 801-832.
- [14] Sercombe, L., et al., *Advances and Challenges of Liposome Assisted Drug Delivery*. Frontiers in Pharmacology, 2015. **6**.
- [15] Koning, G.A. and G. Storm, *Targeted drug delivery systems for the intracellular delivery of macromolecular drugs*. Drug Discovery Today, 2003. **8**(11): p. 482-483.
- [16] Metselaar, J.M. and G. Storm, *Liposomes in the treatment of inflammatory disorders*. Expert Opinion on Drug Delivery, 2005. **2**(3): p. 465-476.
- [17] Ding, B.S., et al., *Advanced drug delivery systems that target the vascular endothelium*. Mol Interv, 2006. **6**(2): p. 98-112.
- [18] Hua, S. and S.Y. Wu, *The use of lipid-based nanocarriers for targeted pain therapies*. Frontiers in pharmacology, 2013. **4**: p. 143-143.
- [19] Sharma, A. and U.S. Sharma, *Liposomes in drug delivery: Progress and limitations*. International Journal of Pharmaceutics, 1997. **154**(2): p. 123-140.
- [20] Siler-Marinkovic, S., *Liposomes as Drug Delivery Systems in Dermal and Transdermal Drug Delivery*, in *Percutaneous Penetration Enhancers Chemical Methods in Penetration Enhancement: Nanocarriers*, N. Dragicevic and H.I. Maibach, Editors. 2016, Springer Berlin Heidelberg: Berlin, Heidelberg. p. 15-38.
- [21] Habib, F.S., et al., *Liposomes as an ocular delivery system of fluconazole: in-vitro studies*. Acta Ophthalmologica, 2010. **88**(8): p. 901-904.
- [22] Abrishami, M.M., et al., *Preparation, Characterization, and in vivo Evaluation of Nanoliposomes-encapsulated Bevacizumab (Avastin) for Intravitreal Administration*. Retina, 2009. **29**(5): p. 699-703.
- [23] Pleyer, U., et al., *Ocular absorption of cyclosporine A from liposomes incorporated into collagen shields*. Current Eye Research, 1994. **13**(3): p. 177-181.
- [24] Shen, Y. and J. Tu, *Preparation and ocular pharmacokinetics of ganciclovir liposomes*. The AAPS Journal, 2007. **9**(3): p. E371-E377.

Chapter 2

Literature Review

This chapter aims to provides readers with background information and related research on the work done in this thesis. The chapter starts off by introducing the anatomy of the eye followed and various routes of ocular drug administration. Barriers to ocular delivery were discussed and a compilation of previous findings on the fate of liposomes in the eye after subconjunctival injection was made. Factor affecting clearance of liposomes were summarized.

2.1 Eye

Being an immune privileged organ [1], the eye is well protected from exogenous substances and external stress. Therapeutic drugs must pass through various barriers regardless of the route of administration, such as topical, subconjunctival or intravitreal injection [2]. This is often done with the help of nano-carriers which aim to aid the movement of the drugs to the intended site.

The eye can be divided to two segments, the anterior segment which occupies 1-third of the eye and the posterior segment which occupies the remaining 2-thirds of the eye [3]. As seen in Figure 2.1, the anterior segment is comprised of the cornea, the ciliary body, the iris, aqueous humor and lens, while the posterior segment includes the vitreous humor, retina, choroid and sclera [4].

Figure 2.1: Structure of human eye [1].

The cornea is clear and avascular [3, 4]. It continues with the sclera at the limbus which is highly vascularized [3-6]. The aqueous humor is a clear watery fluid found between the cornea and the lens. This fluid is secreted by the ciliary body and drained by the trabecular meshwork and Schlemm's canal [7]. It provides nutrients and removes waste from avascular tissues. Most importantly, it regulates the intraocular pressure (IOP) which is required to maintain the curvature of the cornea [4, 7]. The conjunctiva (not shown in the figure) is a thin transparent membrane on the sclera that extends up to the corneal limbus [5].

The sclera is a fibrous tissue that provides protection to the posterior ocular tissues [4, 5] while the retina is a membrane that is in contact with the vitreous on its inner surface and adheres to the choroid through the retinal pigment epithelium on its outer surface [4].

The sclera comprises of irregularly arranged dense collagen fibers which contributes to its opacity and elasticity. This also aids in maintaining the shape of the eyeball. It supports and protects the inner parts of the eye when there is change in intraocular pressure or sudden eye movements [8].

The extracellular matrix of the sclera consists of 95% of Collagen type I and 5% of collagen types III, V, VI, and VII. Proteoglycans which are highly hydrophilic and negatively charged can be found in abundance in the collagen fibrils [9, 10].

The sclera can be divided into three layers: the episclera, the scleral stroma and the lamina fusca [9, 11]. The densely vascularized episclera consists of a thin layer of connective tissue comprising of collagen fibers, melanocytes, and a few macrophages [10]. The scleral stroma, which makes up most of sclera tissue, contains irregularly arranged collagen bundles, elastic fibers, fibroblasts, proteoglycans, and glycoproteins. It is not vascularized and obtains its nutrition from the episcleral and choroidal vascular networks. The innermost scleral layer is made up of pigmented cells and loosely arranged collagen bundles. A thin suprachoroidal space separates the lamina fusca from the choroid [9, 10, 12].

The vitreous is a hydrogel which comprises mainly of water in a collagen-glycosaminoglycan network (CGN). The CGN is made up of collagen fibers separated by random coil glycosaminoglycans [8].

The inner wall of the posterior segment is composed of photosensitive retinal tissue which comprises of photoreceptors. These photoreceptors convert light energy and transfer electrical impulses to the brain. They are functionally related to the retinal pigment epithelium (RPE) which is a stable epithelial cell layer characterized by hexagonal-shaped cells, tight junctions and sheet-like microvilli. [1, 13]

The circulation of blood in the posterior segment of the eye is dependent largely on the choroid vessels. Other than providing the retina and the anterior chamber with nourishment and blood circulation, the choroid is also known to regulate eye temperature and maintain the IOP. [1, 14].

2.2 Eye Diseases

Various diseases can affect the function of the eye. These diseases can be either caused by inflammations, bacterial and viral infections, or simply due to aging [6]. Diseases affecting anterior segment include conjunctivitis, anterior uveitis and cataract while those affecting the posterior segment include diabetic retinopathy, age-related macular degeneration (AMD) and glaucoma [1, 15]. These three posterior segment diseases total up to 14% of the causes of blindness in the world [1, 16].

Diabetic retinopathy occurs when there is damage at the retina due to unwanted blood vessel proliferation as a result of abnormal vascular endothelial growth factor (VEGF) production triggered by blood vessels weakened by hyperglycemia [1, 17].

AMD occurs in dry and wet forms with dry AMD accounting for 90% of AMD cases. Dry AMD is believed to be caused by cellular debris that is accumulated between the choroid and RPE in the macula. This accumulation disrupts the epithelium and eventually results in loss of central vision. Dry AMD progresses to wet AMD when this disruption of the epithelium triggers abnormal VEGF production [1].

Glaucoma is often associated with an increase in intraocular pressure (IOP) which usually occurs due to impaired aqueous humor outflow. This damages the inner layer of the retina and the optic nerve, leading to irreversible blindness [1, 18, 19]. Most IOP lowering drugs stimulate alpha and/or beta receptor which leads to vasoconstriction of ciliary body followed by decrease in aqueous humor production [20]. These receptors can be found in the ciliary, corneal and conjunctiva epithelium [21].

2.3 Routes of drug administration

Topical administration of eye drops is usually used to treat anterior segment diseases while systemic administration, periocular or intraocular injections of drugs are usually employed for therapeutics that are required to reach the posterior segment of the eye [2, 22, 23]. Figure 2.3 shows the three main modes of ocular delivery: topical, periocular and intravitreal.

Figure 2.3: Ocular drug delivery routes [24].

2.3.1 Topical

Being the most convenient and acceptable mode of ocular drug delivery, a wide variety of topical formulations are available in the market. Topical instillation has many advantages over systemic administration. It is non-invasive, easy to administer and results in low absorption into systemic circulation [3]. However, it also has many disadvantages such as rapid drainage and drug removal due to fast tear turnover rate and blinking [3]. The drug clearance typically occurs within 15–30s resulting in the intraocular bioavailability of <5% to the anterior segment (even <1% for many cases) for topically applied drugs [25, 26]. One major drawback of eye drops is poor patient compliance which is particularly evident in elderly people. Approximately 20% of glaucoma patients have problems with routine eye drops which often results in a more severe loss of vision [18, 27, 28]. Common preservatives used in eye drops could also

result in ocular discomfort [18].

2.3.2 Intravitreal

Intravitreal injection or implant is the most invasive type of ocular drug delivery as it involves puncturing of the eye with the injector. Although intravitreal injection deliver the drug directly to the retina and vitreous, it carries the risk of possible hemorrhage, retinal detachment and endophthalmitis [25, 29]. Trans-scleral drug delivery administrations such as subconjunctival or sub-Tenon's injections, on the other hand, can avoid such complications [30].

2.3.3 Periocular

Periocular delivery includes a whole range of delivery routes that are around the eye such as injections and implants at the peribulbar, retrobulbar, sub tenon, suprachoroidal, and subconjunctival locations [29]. These routes are more invasive than topical administration but less invasive than intravitreal injections. Among the periocular injection methods, there is much interest in sub-conjunctival injection due to its ability to utilize the trans-scleral route. By taking up 95% of the total surface area of the globe, the sclera provides a large avenue for drug diffusion. Differences in scleral thickness in particular regions of the eye could also be used to further optimize trans-scleral drug diffusion if the DDS can act as a depot for sustained delivery [23, 25, 31].

Figure 2.3.3: Subconjunctival Injection [32].

The subconjunctival route (shown in Figure 2.3.3) can be used to deliver drugs to both the anterior and posterior segments of the eye. By injecting into the subconjunctival space, the formulation is placed between the conjunctiva and the sclera. This allows

drug molecules to be transported directly through the sclera without having to pass through the conjunctival epithelial tight junctions [33]. Despite this, other conjunctival and episcleral flow mechanisms still play a major role in the subconjunctival route. [9, 34]

Sustained lowered IOP was observed for 90 days in rabbits [35] and up to 120 days in ocular hypertensive nonhuman primates [36] after sub-conjunctival injection of Latanoprost-loaded EggPC liposomes. Significant IOP reduction was also observed for 3 months after injection of the same formulation in patients diagnosed with either ocular hypertension or primary open-angle glaucoma [37]. In other studies, sustained release of Latanoprost was also observed for ~5 months in rabbits using a hyaluronic acid-polymeric system [18] and 6 days using PLA-PEG nanoparticles [38]. IOP reduction was also observed for 8 days with the PLA-PEG nanoparticles [38]. Although trans-scleral delivery is comparatively easy and patient compliant, static and dynamic barriers present in the eye still pose a challenge to drug permeation [15, 39]. These barriers will be further discussed in section 2.4.

2.4 Barriers to ocular delivery

There are three main types of barriers that hinder ocular drug delivery: static, dynamic and metabolic [3, 24]. Static barriers comprise of physical barriers in the eye while dynamic barriers include clearance through blood circulation, lymphatic drainage and bulk fluid flow. Metabolic barriers reduce drug penetration by degrading the drugs [3, 40]. In addition, physiological processes such as blinking and tear drainage also contribute to the poor efficacy of conventional drug formulations [41, 42].

2.4.1 Static barriers

Static barriers in the anterior segment of the eye comprise mainly of the corneal epithelium, corneal stroma, and blood–aqueous barrier [3] while the sclera, Bruch’s membrane and choroid and retinal pigmented epithelium (RPE) are barriers present in the posterior segment (Figure 2.4.1) [43].

Figure 2.4.1: Static barriers [43].

In order to maintain corneal transparency, the corneal epithelium is sealed by specialized structures, such as tight junctions and desmosomes [42, 44]. This prevents almost any substance larger than 500 Da from passing through the corneal epithelium [42, 45]. Most topical drugs are thus unable to permeate through the cornea [42, 44]. Presence of proteoglycans and collagen fibers in the sclera can hinder the diffusion of drug molecules through the tissue while the lipids and lipoproteins of the choroid and the Bruch's membrane can also interfere with the delivery of drug molecules [43].

Compared to the cornea, the sclera is highly permeable due to its relatively large surface area. Transscleral permeation is influenced both by the physicochemical properties of the drug and scleral properties [9]. At physiological pH, the scleral matrix has an overall negative charge which can result in charge-based interactions between the permeant and the sclera. Electrostatic repulsion of negatively charged drugs can drive the drugs further into the eye while positively charged drugs can bind and be retained close to the surface of the sclera [46]. This was previously confirmed in bovine and porcine sclera, where negatively charged solutes showed higher permeability than those with a positive charge [22]. Joseph et al also demonstrated through *ex vivo* porcine sclera transport experiments that positive charged DPPC-DPTAP liposomes localized on the episcleral region due to electrostatic attraction with the negatively charged sclera. Negatively charged DPPC-DPPG liposomes and neutral liposomes, on the other hand, were observed to have a certain extent of scleral diffusion in the *ex vivo* condition. As the negatively charged liposomes are also subjected to repulsion with the negatively

charged sclera, the extent of sclera diffusion was lower than neutral liposomes. [47]

Scleral permeation can also be influenced by the lipophilicity of drug molecules. In a study to show the correlation between drug lipophilicity and human scleral permeation, Wen, H. et al demonstrated that molecules with higher lipophilicity exhibited stronger binding to the sclera, resulting in a longer transport time across the tissue [46]. However, it was also stated that this factor might be insignificant when the drug is incorporated into a depot formulation [9, 46].

Being a highly porous tissue, permeability across the sclera is also dependent on the permeant molecular weight and radius. For hydrophilic molecules more than 100kDa, the radius is the dominating factor affecting their transport across the sclera [48]. When comparing proteins and polysaccharides of similar molecular weights, polysaccharides with flexible structures displayed higher permeability compared to rigid proteins. [9, 49]

After permeation across the sclera, the drug/carrier has to overcome the blood retinal barrier (BRB). [9] The BRB is composed of the inner and outer BRB. The outer BRB is made up of tight junctions of RPE cells while the inner BRB is made up of retinal capillary endothelial cells [5]. Together, both RPE and the endothelial cells provide a cellular barrier which is highly effective in preventing paracellular movement. It only allows entry of very small molecules such as CO₂, O₂ and lipophilic molecules from the choroid to inner retinal tissues [5, 24, 50]. Therefore, the BRB highly restricts the entry of drugs.

Using the 2-chamber Ussing-type apparatus, these static barriers have been widely studied *ex vivo* [43].

2.4.2 Dynamic barriers

Most of the dynamic barriers are created by clearance mechanisms through blood and/or lymphatic vessels found at the conjunctiva, episclera and choroid. Dynamic barriers also include clearance by the bulk fluid flow, uveoscleral outflow and presence of transporter proteins, efflux pumps and ion transporters [43].

2.4.2.1 Clearance through lymphatics and blood vessels

The conjunctiva is highly vascularized and contains many lymph vessels [43]. Topically administered drugs may be cleared by the systemic blood circulation and lymph vessels while making their way through the conjunctiva [51], thus lowering drug permeation to ocular tissues. Dating back to the 1970s, several studies have shown that drugs present in the conjunctival and episcleral tissues may also be cleared by the same manner, as seen in Figure 2.4.2.1 [5].

Figure 2.4.2.1: Clearance pathways [43].

This was confirmed by higher amount of intraocular drug penetration found in rabbits after conjunctival/episcleral clearance mechanisms were selectively eliminated through an incised conjunctival window [43, 51]. Micro-particles were found to have higher retention compared to nanoparticles after sub-conjunctival injection [43, 52] and higher half-life was observed for albumin compared to sodium molecules [53] in subconjunctival tissue, suggesting that molecular size may affect the rate of clearance.

Drugs/carriers can also be eliminated through the choroid which is also highly vascularized and supplied with lymph vessels [5, 43, 51, 54]. Due to the lack of reports on factors affecting drug clearance through lymphatic and blood vessels, further investigations are required for better understanding of these dynamic barriers [43].

2.4.2.2 Clearance by fluid flow

Fluid flow may also limit transport of ocular therapeutics. Drug molecules may be cleared through the choroidal or conjunctival vasculature after being carried along by bulk fluid flows present in ocular tissues. As mentioned briefly in section 2.1, aqueous

humor flows from the ciliary body to the posterior chamber and into the anterior chamber through the pupil. It is then drained either through the conventional pathway (through trabecular meshwork into Schlemm's canal and aqueous veins) or directly through the ciliary body before entering the suprachoroidal space (uveoscleral outflow pathway) [43]. The aqueous humor then flows through the sclera [55] and is cleared through vasculature in the conjunctiva. Drug molecules/carriers may be carried along with this convective current and eventually be cleared in a similar way [5, 43].

While most of the fluid leave the eye by the same routes as the aqueous humor, some passes through the posterior ocular tissues [43]. Fluid flow is also generated towards the choroid due to the differences in osmotic pressure of the choroid and the vitreous [56] and outward bulk fluid flow occurs due to pressure difference between the suprachoroid and the episcleral tissue [43, 57].

2.4.2.3 Presence of transporters and efflux pumps

As the RPE regulates the transport of molecules into the sub-retinal space, various transporter proteins are present in the RPE. These include ion transporters, amino acid transporters and drug efflux pumps. Drug penetration to the retina may be obstructed by these transporter proteins [43, 58-60].

2.4.2.4 Metabolic barriers

Being an exposed organ, the eye possesses metabolizing enzymes as a defense mechanism for entry of xenobiotics into the body [40, 43]. Drug-metabolizing enzymes are present in many ocular tissues, but are most active in the ciliary body and the RPE due to their responsibility for detoxifying drugs [40, 43]. During trans-scleral delivery, drug molecules may be degraded by these metabolizing enzymes thus impeding their transport to the retina [40, 43].

2.5 Distribution in the eye after subconjunctival injection

While many studies have been done on the pharmacokinetics of therapeutics after subconjunctival injection [18, 52, 61], very few have focused on the distribution of the carriers or sometimes even the ocular distribution of drugs itself in the eye. Most studies

focused on drug levels in blood, the aqueous humor and vitreous humor [18, 38, 61] and physiological effects (e.g. reduction of IOP for glaucoma drugs) [18, 36, 38, 62].

Amrite and Kompella et al. proposed that the fate of sub-conjunctivally injected particles can include 1) entry into the intraocular tissues through the sclera, 2) entry into the systemic circulation through local intraocular circulation or through conjunctival or episcleral vasculature and 3) retention at the injection site till the particles degrade or are cleared by the lymphatic system [63, 64]. They investigated the influence of size and charge of polymeric nanoparticles on the fate in the eye [63] by sub-conjunctivally injecting fluorescent polystyrene particles of various sizes (~ 20 nm, ~ 200 nm and ~ 2 μ m). Fluorescence in ocular tissues (sclera, retina, vitreous, lens and cornea) were detected with fluorescence spectrophotometry. Micro-particle (~ 2 μ m, actual zeta size: 1.5 μ m) and ~ 200 nm (actual zeta size: 205-290 nm) particles remained at the injection site for up to 60 days while only 15% of 20 nm (actual zeta size: 25-37 nm) nanoparticles were found on day 1 with no detectable traces of it on day 60. This was speculated to be due to either the rapid bulk flow or the leakage of particles into the pre-corneal area through the needle track. Other than significant levels of ~ 20 nm nanoparticles being detected in the sclera-choroid on day 1, none of the particles were observed in the tissues. As the sclera can be considered as a porous membrane, it was concluded that passive diffusion could be the principle mechanism of penetration into the sclera [63, 65]. The effect of surface properties such as hydrophobicity/hydrophilicity was studied and effect of surface charges was also investigated in the same study and it was concluded that surface properties only affect the distribution for ~ 20 nm particles. However, the comparison was done between particles of different actual sizes (25 nm and 37 nm). Furthermore, negatively charged, hydrophilic particles were compared with neutral, hydrophobic particles. It was unclear if the results were due to the slight differences in size or charge differences or hydrophobicity/hydrophilicity. Therefore, a more robust study is required to understand how the surface properties affect the distribution of the particles.

Recently, Wong, C.W., et al studied the kinetics of liposomes in the eye in while evaluating subconjunctival liposomal steroids for the treatment of experimental uveitis. The liposomes used were PEGylated DPPC liposomes with fluorescence lipid incorporated into the lipid bilayer. The liposomes were seen initially to be rapidly

cleared from the injection site post injection followed by slow elimination for over 31 days. Histology and immunostaining at day 1 also showed that the liposomes were present in the ciliary body and injection site. Macrophage uptake of liposomes was observed. Liposomes were detected in the aqueous humor from day 1 till the end of the study (day 31). [66]

Other studies on ocular distribution focused only on the delivered pharmaceuticals/agent. Hirnle et al. studied the distribution of liposomal drugs in rabbit eyes by incorporating 6-carboxyfluorescein (6-CF) in the aqueous space of the liposomes. By quantifying the amount of 6-CF in sclera, retina, choroid and cornea, they were able to map out the distribution of the model drug up to 7 days. 6-CF was detected in the whole eye, and was slowly released with the highest amount observed in the sclera 30 minutes post injection. Liposomal 6-CF was still detectable at the injection site, sclera, retina, choroid and cornea 7 days post injection [67].

Moon et al. studied the intraocular and systemic absorption of liposome-bound low-molecular-weight heparin (LMWH) (~546 nm, positively charged) after subconjunctival injection in rabbits that had undergone induction of subconjunctival hemorrhages. Plasma, aqueous humor, vitreous humor, conjunctiva and sclera were measured for LMWH activity up to 5 days post injection. The liposomes were believed to have acted as a depot which resulted in a sustained release of LMWH up to 5 days [68]. Interestingly, it was observed from the graphs in the paper that the LMWH level was always higher in the aqueous humor compared to vitreous humor and in sclera compared to conjunctiva. This suggested that the LMWH might have been carried to the ciliary body and subsequently to the posterior and anterior chambers through fluid flow from the subconjunctival depot. However, as the authors did not make any conclusions on these differences, it is unclear if the differences were significant.

Feng et al. investigated the distribution of sub-conjunctivally injected pRNA nanoparticles in mice using whole body imaging and histology examination. pRNA were observed to be distributed to various ocular tissues (conjunctiva, cornea, retina, and sclera) before rapidly clearing from the eye. The presence of the nanoparticles in the cervical lymph nodes and not the free dye indicated that the extent of clearance from lymph circulation is different for nanoparticles and small polar molecules. pRNA were

also internalized by cells in conjunctiva (highest in superior conjunctiva), cornea and sclera by 6 hours, up to 20 hours after injection. However, as the techniques used in this study could only either detect fluorescence in the whole eye or particles that have been internalized in the cells, the amount of nanoparticles in each ocular tissue were not determined [39]. Similarly, in a MRI study done on subconjunctival injection of model drugs Gd-DTPA (<1 nm) and Gd-Albumin (4 nm), the drug solutions acted as depot in the subconjunctival space immediately after injection. The drug concentration in the subconjunctival bleb decreased dramatically and was undetectable about 3 to 4 hours post-injection. Comparisons made with post mortem experiments identified that *in vivo* clearance mechanisms such as blood circulation and lymphatic drainage were in play [69].

Li, Hao et al. investigated ocular penetration and clearance of a contrast agent, Magnevist, a model hydrophilic probe, in rabbit eyes by using MRI. Within 20 minutes after sub-conjunctival injection, the hydrophilic probe was found to be present in the ciliary body and anterior chamber. Presence of the probe at the ciliary body away from the injection site suggested the influence of blood circulation on the delivery to the anterior segment of the eye while the presence of the probe observed near the periocular fat suggested transport through circulation around the eye. Instead of diffusing to other ocular tissues away from the globe, the contrast agent was found to have diffused into the retina and to the vitreous in postmortem experiments, indicating the importance of active elimination mechanism when trans-passing the sclera [70]. This was also evident in clearance of NaFl in rats. 5.4% of injected NaFl was found in the lymph node 60 minutes after subconjunctival injection in rats, with at least 10% of NaFl cleared through the lymphatic system within that hour [30, 70]. A compilation of various studies on the clearance of particle/drug after subconjunctival injection found in literature is listed on Table 2.5.

It can be concluded from these studies that carriers/molecules of sizes smaller than 40 nm are rapidly cleared from the injection site while carriers above 200 nm in size tend to be retained at the site of injection. As a complete comparison of effect of properties other than size has not been done, a more robust and comprehensive study is required to understand the distribution of nano-carriers in the eye.

Table 2.5: Compilation of studies done on fate of sub-conjunctivally injected molecules.

Nano-carrier/Drug	Clearance/distribution	Animal	Method of detection	
Liposomes				
Pegylated DPPC liposomes ~110-130 nm	Remained at the site of injection and cornea for up to 31 days. Liposomes was detected in aqueous humor from 1 day post injection up to 31 days. Liposomes were detected in the ciliary body and injection site 1 day post injection. Fluorescence was detected within macrophages.	Rabbit	Fluorotron Master, Immunostaining	[66]
Polymeric Particles				
1.5 μm	Remained at the site of injection for up to 60 days.	Rat	Spectro-fluorometer	[63]
205-290 nm	Remained at the site of injection for up to 60 days.	Rat	Spectro-fluorometer	[63]
25-37 nm	Significant levels in the sclera-choroid on day 1. Rapidly cleared by day 1, undetectable on day 60.	Rat	Spectro-fluorometer	[63]
Model Drugs/Probes				
Fluorescein	At least 10% of NaFl was cleared through the lymphatic drainage within the first hour. 5.4% of injected NaFl was found in the lymph node 1 hour after injection.	Rat	Spectro-fluorometer, Histology	[30]
Alexa647	Cleared from injection site in 4.5 hours. No lymphatic clearance through the cervical lymph nodes detected. No cell internalization.	Mouse	Whole body imaging and Fluorescent Microscopy	[39]

Magnevist (Gd-DTPA)	<p>Penetration into the ciliary body and anterior chamber was observed within 20 min.</p> <p>Probe found at the ciliary body away from the subconjunctival depots, indicating blood circulation clearance.</p> <p>Clearance to the regions near the periocular fat behind the eye, indicating systemic clearance.</p>	Rabbit	MRI	[70]
6-CF in aqueous space of liposome	<p>Highest concentration of 6-CF in the sclera 30 minutes post injection.</p> <p>6-CF still detectable at the injection site, sclera, retina, choroid and cornea 7 days post injection.</p>	Rabbit	Spectro-fluorometer	[67]
Liposome bound Low Molecular Weight Heparin (LMWH) (~546 nm, positively charged)	<p>LMWH concentration in free LMWH > liposome-bound LMWH in the plasma, aqueous humor, vitreous humor, conjunctiva and sclera at day 1 and day 2</p> <p>LMWH concentration in free LMWH $\leq$ liposome-bound LMWH on the subsequent days up to day 5.</p>	Rabbit	Colorimetric method	[68]
pRNA Nanoparticles (8-12 nm)	<p>Cleared from injection site approximately 6-9hours after injection.</p> <p>Eliminated by lymph circulation in 2 hours.</p> <p>pRNA(18kDa) and dsRNA (6 nm, 11 kDa) were internalized by cells in conjunctiva (highest in superior conjunctiva), cornea and sclera at 6 h, up to 20 h after injection.</p> <p>pRNA-X (22 kDa) was also internalized in retina cells at 6 hours.</p>	Mouse	Whole body imaging, fluorescent microscopy (Whole eye and flat mount)	[39]

Macromolecule (Bevacizumab 12 nm, 149 kD)	1h: Detectable in ocular structure near injection site 6h: Detectable in posterior tissues and cornea. Intra corneal diffusion of into the corneal periphery adjacent to the injection site detected 24h-day 21: Detected in all layers of cornea stroma.	Mouse	Fluorescence immuno- histology	[71]
DTPA, Albumin	Forms a localized depot in the subconjunctival space immediately after injection Cleared about 3-4 h post-injection.	Rabbit	Dynamic contrast- enhanced magnetic resonance imaging	[69]

2.6 Liposomes for Ocular Drug Delivery

Liposomes are the most extensively investigated among various colloidal carriers [72]. Liposomes are phospholipid vesicles with discrete aqueous space enclosed by one or more concentric lipid bilayers [73]. This gives liposomal systems the ability to entrap both lipophilic (in the bilayer membrane) and hydrophilic (in the aqueous center) compounds, allowing encapsulation of a diverse range of drugs [72, 74-78]. Liposomes offer several advantages such as biocompatibility and the freedom to control their characteristics by modifying their physicochemical and biophysical properties [72, 74-79]. Liposomes have been widely studied for their use as an ocular delivery system both as ophthalmic applications and injectable formulations. When compared with topical application of drug solution, Fluconazole loaded liposomal formulations were found to show better improvements in rabbits infected with *Candida* keratitis [80]. Liposomal formulations of Bevacizumab [81], Cyclosporine [82] and Ganciclovir [83] have also shown longer half-life and prolonged effect of the drug compared to non-liposomal formulations. Furthermore, positively charged liposomes have shown to result in improved corneal permeability for topical applications due to the negatively charged cornea [26].

Natarajan et al. showed that the IOP lowering effect of a liposomal formulation of

Latanoprost could be sustained up to 90 days after a single sub-conjunctival injection in rabbits [35] and up to 120 days in ocular hypertensive nonhuman primates [36]. Wong et al. also observed significant IOP reduction through 3 months after injection of the same formulation in patients who were diagnosed with either ocular hypertension or primary open-angle glaucoma [37]. While liposomal formulations have shown improved delivery of the drugs to the eye, little is known of the fate of these liposomes in the ocular tissues. Knowledge of where the liposomes are distributed after administration and where they reside to be able to achieve sustained delivery of drugs could lead to better improvisation of the formulations.

The fate of liposomes and the effect of factors such as charge, size and fluidity on its distribution after subconjunctival injection will be investigated for the first time in this thesis.

2.7 Factors affecting clearance of liposomes

Despite the lack of studies on the fate of liposomes in the eye, systemic clearance of liposomes and protein-liposomes interactions (both *in vitro* and *in vivo*) has been studied over the years. As the liposomes injected into the eye enters the blood circulation (both ocular and systemic) eventually, liposomal properties that affect systemic clearance are also of relevance when understanding the fate of liposomes.

It has been known that, upon entering the blood stream, liposomes are prone to rapid elimination [84, 85]. This is due to opsonization of plasma components and uptake by fixed macrophages of the reticuloendothelial system (RES), mainly the liver and spleen. [78, 84]

Opsonization of liposomes occurs when there is adsorption of plasma proteins onto the phospholipid membrane. Although some *in vitro* studies have shown that liposomal clearance via macrophages can occur in the absence of plasma proteins, the interaction between the liposomal systems and plasma proteins is important in determining overall nanocarrier biodistribution [84]. As binding of protein on liposomes can cause destabilization of the lipid layer and leakage of entrapped contents, high levels of protein binding are associated with rapid clearance of liposomes from the circulation.

[86, 87] Opsonization of liposomes by serum proteins depends on a variety of factors including size, surface charge, packing of the lipid bilayers and stability. [84, 86, 88]

The extent of opsonization of liposomes has been shown to decrease with liposome size from 800 to 200 nm in diameter. Due to more proteins being adsorbed on large vesicles, large unmodified liposomes are eliminated more rapidly than small, neutral, or positively charged liposomes. [88] When comparing particles of the same size, the presence of high electrostatic charge can promote the interaction of liposomes with opsonins. More proteins were observed to be adsorbed to charged liposomes compared to neutral liposomes. [84, 88]

When tested in mice, cationic liposomes were found to have significant protein binding (400-1100 g protein/mol lipid) and are cleared extremely rapidly from the circulation. [89-91] This can be attributed to the net negative charge of most plasma proteins at physiological pH. However, the effect of negative charge on clearance was dependent on factors other than charge itself. In studies done by S.C. Semple et al., the incorporation of different anionic phospholipids trigger different clearance behavior in CD1 mice [92]. LUVs containing phosphatidyl serine (PS), phosphatidic acid (PA) or cardiolipin (CL) surface were cleared more rapidly from the circulation compared to liposomes containing phosphatidylglycerol (PG) or phosphatidylinositol (PI). There was also a difference in the interaction of these anionic liposome membranes with blood proteins indicating that presence of surface charge alone does not necessarily facilitate protein-membrane interactions. Specific lipid head group and acyl chain composition were also observed to have an effect on liposome-protein interactions. LUVs containing bovine liver PI have protein binding values of 158 g protein/mol lipid and are cleared from the circulation in minutes, while LUVs containing plant PI have a circulation half-life of approximately 90 min and protein binding value of 27 g protein/mol lipid. The major acyl chain species in plant PI and bovine liver PI is 18:2 and 18:0, indicating that saturated lipids have higher interaction with protein and are cleared faster compared to unsaturated lipids. [90, 91]

Upon investigating the influence of acyl chain length on a series of saturated PC derivatives, Sean C. Semple et al discovered that for saturated lipids with acyl chain lengths greater than 16 carbons, the protein binding values was 90 g protein/mol lipid,

and the liposomes were very rapidly cleared while DMPC (14:0/14:0) had protein binding values of 30 g protein/mol lipid and were cleared slower compared to longer chain versions. The team attributed these effects to the presence of defects in membrane packing that occur in saturated PC liposomes at temperatures below the phase transition temperature of the constituent lipid. These defects result in the occurrence of hydrophobic domains at the surface of the vesicles. As plasma proteins have strong affinities for hydrophobic domains, they opsonized rapidly. [88, 90]

The inclusion of cholesterol in liposomes has been an important factor for increasing liposomal stability, minimizing lipid-protein interactions and decreasing membrane permeability. Presence of cholesterol in the liposomal membrane reduces possible lipid exchange with other circulating structures (e.g., red blood cells and lipoproteins) which can cause the replacement of the lipids with less physiologically stable components. Incorporating cholesterol into small (approximately 100 nm), neutral liposomes has been shown to prolong circulation. [84, 88, 93] It was also demonstrated that liposomes rich in cholesterol have lower affinity for uptake by hepatic Kupffer cells, suggesting that cholesterol does inhibit opsonization of liposomes [87].

The RES is the main site of liposome accumulation following their systemic administration and primary organs associated include liver, spleen, kidney, lungs, bone marrow, and lymph nodes [94]. The liver has the largest capacity for uptake of liposomes followed by the spleen [95]. The fenestrations in the microvasculature of these organs gives them the ability to sequester liposomes from the circulation. Pore diameters in these capillaries can range from 100 to 800 nm. This is large enough for the extravasation and removal of most liposomes [96]. Liposomes are cleared in the RES by resident macrophages via direct interactions with the phagocytic cells. [84, 95] The distribution to other tissues of the body is substantially affected by this rapid uptake of liposomes into the RES. [97]

The findings of how liposomal properties affect protein interaction and systemic clearance *in vivo* is summarized in Table 2.7.

Table 2.7: Summary of effect of liposomal properties on protein interaction and *in vivo* systemic clearance.

Liposomal properties	Effect on protein interaction and clearance
Size	<p>Extent of opsonization of liposomes decreases with liposome size from 800 to 200 nm in diameter.</p> <p>Large unmodified liposomes are eliminated more rapidly than small, neutral, or positively charged liposomes [88]</p> <p>↑ size, ↑ blood clearance</p>
Charge	<p>Cationic liposomes were found to have significant higher protein binding and are cleared extremely rapidly from the circulation compared to neutral and negatively charged liposomes. [89-91]</p> <p>Positively charged liposomes, ↑ blood clearance</p>
Chain Length	<p>For neutral saturated lipids, an increase in acyl chain length resulted in higher protein binding and faster clearance. [90]</p> <p>↑ acyl chain length, ↑ blood clearance</p>
Chain Saturation	<p>Liposomes containing lipid with saturated acyl chain (18:0) have higher protein binding values and are cleared faster from the circulation compared to unsaturated lipid. [90-92]</p> <p>↑ saturation, ↑ blood clearance</p>
Incorporation of Cholesterol	<p>Inclusion of cholesterol in liposome membranes decreased binding of proteins and increased circulatory lifetimes of liposomes [87]</p> <p>Incorporation of cholesterol, ↓ blood clearance</p>

References

- [1] Delplace, V., S. Payne, and M. Shoichet, *Delivery strategies for treatment of age-related ocular diseases: From a biological understanding to biomaterial solutions*. Journal of Controlled Release, 2015. **219**: p. 652-668.
- [2] Kuno, N. and S. Fujii, *Recent Advances in Ocular Drug Delivery Systems*. Polymers, 2011. **3**(1): p. 193.
- [3] Cholkar, K., et al., *Novel Strategies for Anterior Segment Ocular Drug Delivery*. Journal of Ocular Pharmacology and Therapeutics, 2012. **29**(2): p. 106-123.
- [4] Achouri, D., et al., *Recent advances in ocular drug delivery*. Drug Development and Industrial Pharmacy, 2013. **39**(11): p. 1599-1617.
- [5] Cholkar, K., et al., *I - Eye: anatomy, physiology and barriers to drug delivery*, in *Ocular Transporters and Receptors*. 2013, Woodhead Publishing. p. 1-36.
- [6] Schermer, A., S. Galvin, and T.T. Sun, *Differentiation-related expression of a major 64K corneal keratin in vivo and in culture suggests limbal location of corneal epithelial stem cells*. The Journal of Cell Biology, 1986. **103**(1): p. 49-62.
- [7] Goel, M., et al., *Aqueous Humor Dynamics: A Review*. The Open Ophthalmology Journal, 2010. **4**: p. 52-59.
- [8] Eriksen, A.Z., et al., *The diffusion dynamics of PEGylated liposomes in the intact vitreous of the ex vivo porcine eye: A fluorescence correlation spectroscopy and biodistribution study*. International Journal of Pharmaceutics, 2017. **522**(1): p. 90-97.
- [9] Agban, Y., et al., *Depot formulations to sustain periocular drug delivery to the posterior eye segment*. Drug Discovery Today, 2019.
- [10] Watson, P.G. and R.D. Young, *Scleral structure, organisation and disease. A review*. Experimental Eye Research, 2004. **78**(3): p. 609-623.
- [11] Wang, C., Y. Xie, and G. Wang, *The elastic modulus and collagen of sclera increase during the early growth process*. Journal of the Mechanical Behavior of Biomedical Materials, 2018. **77**: p. 566-571.
- [12] Kakizaki, H., et al., *Anatomy of Tenons capsule*. Clinical & Experimental Ophthalmology, 2012. **40**(6): p. 611-616.
- [13] la Cour, M. and T. Tezel, *The Retinal Pigment Epithelium*, in *Advances in Organ Biology*, J. Fischbarg, Editor. 2005, Elsevier. p. 253-272.
- [14] Kiilgaard, J.F. and P.K. Jensen, *The Choroid and Optic Nerve Head*, in *Advances in Organ Biology*, J. Fischbarg, Editor. 2005, Elsevier. p. 273-290.

- [15] Patel, A., et al., *Ocular drug delivery systems: An overview*. World J Pharmacol, 2013. **2**(2): p. 47-64.
- [16] *Global Data On Visual Impairments 2010*, in <http://www.who.int/blindness/publications/globaldata/en/>. 2012, World Health Organization.
- [17] Curtis, T.M., T.A. Gardiner, and A.W. Stitt, *Microvascular lesions of diabetic retinopathy: clues towards understanding pathogenesis[quest]*. Eye, 2009. **23**(7): p. 1496-1508.
- [18] Voss, K., et al., *Development of a novel injectable drug delivery system for subconjunctival glaucoma treatment*. Journal of Controlled Release, 2015. **214**: p. 1-11.
- [19] Johnson, M., 'What controls aqueous humour outflow resistance?'. Experimental Eye Research, 2006. **82**(4): p. 545-557.
- [20] Schmidl, D., et al., *Pharmacotherapy of glaucoma*. Journal of ocular pharmacology and therapeutics: the official journal of the Association for Ocular Pharmacology and Therapeutics, 2015. **31**(2): p. 63-77.
- [21] Woldemussie, E., M. Wijono, and D. Pow, *Localization of alpha 2 receptors in ocular tissues*. Visual Neuroscience, 2007. **24**(5): p. 745-756.
- [22] Lee, S.S., P.M. Hughes, and M.R. Robinson, *Recent advances in drug delivery systems for treating ocular complications of systemic diseases*. Current Opinion in Ophthalmology, 2009. **20**(6): p. 511-519.
- [23] Geroski, D.H. and H.F. Edelhauser, *Drug Delivery for Posterior Segment Eye Disease*. Investigative Ophthalmology & Visual Science, 2000. **41**(5): p. 961-964.
- [24] Kang-Mieler, J.J., C.R. Osswald, and W.F. Mieler, *Advances in ocular drug delivery: emphasis on the posterior segment*. Expert Opinion on Drug Delivery, 2014. **11**(10): p. 1647-1660.
- [25] Meng, T., et al., *Therapeutic implications of nanomedicine for ocular drug delivery*. Drug Discovery Today, 2019.
- [26] Mishra, G.P., et al., *Recent Applications of Liposomes in Ophthalmic Drug Delivery*. Journal of Drug Delivery, 2011. **2011**: p. 14.
- [27] Reardon, G., S. Kotak, and G.F. Schwartz, *Objective assessment of compliance and persistence among patients treated for glaucoma and ocular hypertension: a systematic review*. Patient preference and adherence, 2011. **5**: p. 441-463.
- [28] Rotchford, A.P. and K.M. Murphy, *Compliance with timolol treatment in glaucoma*. Eye, 1998. **12**(2): p. 234-236.

- [29] Lorentz, H. and H. Sheardown, *Ocular Delivery of Biopharmaceuticals*, in *Mucosal Delivery of Biopharmaceuticals: Biology, Challenges and Strategies*, J. das Neves and B. Sarmiento, Editors. 2014, Springer US: Boston, MA. p. 221-259.
- [30] Lee, S.J., et al., *Evaluation of Clearance Mechanisms with Transscleral Drug Delivery*. Investigative Ophthalmology & Visual Science, 2010. **51**(10): p. 5205-5212.
- [31] Urtti, A., *Challenges and obstacles of ocular pharmacokinetics and drug delivery*. Advanced Drug Delivery Reviews, 2006. **58**(11): p. 1131-1135.
- [32] LLC, E.N. *Animal Injection*. 2018 19012020]; Available from: <https://www.clodrosome.com/animal-injection/>.
- [33] Gaudana, R., et al., *Ocular Drug Delivery*. The AAPS Journal, 2010. **12**(3): p. 348-360.
- [34] Weijtens, O., et al., *Dexamethasone concentration in the subretinal fluid after a subconjunctival injection, a peribulbar injection, or an oral dose*. Ophthalmology, 2000. **107**(10): p. 1932-1938.
- [35] Natarajan, J.V., et al., *Nanomedicine for glaucoma: liposomes provide sustained release of latanoprost in the eye*. International Journal of Nanomedicine, 2012. **7**: p. 123-131.
- [36] Natarajan, J.V., et al., *Sustained Drug Release in Nanomedicine: A Long-Acting Nanocarrier-Based Formulation for Glaucoma*. ACS Nano, 2014. **8**(1): p. 419-429.
- [37] Wong, T.T., et al., *Nanomedicine for glaucoma: sustained release latanoprost offers a new therapeutic option with substantial benefits over eyedrops*. Drug Delivery and Translational Research, 2014. **4**(4): p. 303-309.
- [38] Giarmoukakis, A., et al., *Biodegradable nanoparticles for controlled subconjunctival delivery of latanoprost acid: In vitro and in vivo evaluation. Preliminary results*. Experimental Eye Research, 2013. **112**: p. 29-36.
- [39] Feng, L., et al., *Ocular Delivery of pRNA Nanoparticles: Distribution and Clearance After Subconjunctival Injection*. Pharmaceutical Research, 2014. **31**(4): p. 1046-1058.
- [40] Reimondez-Troitiño, S., et al., *Nanotherapies for the treatment of ocular diseases*. European Journal of Pharmaceutics and Biopharmaceutics, 2015. **95**, Part B: p. 279-293.
- [41] Pal Kaur, I. and M. Kanwar, *Ocular Preparations: The Formulation Approach*. Drug Development and Industrial Pharmacy, 2002. **28**(5): p. 473-493.

- [42] Diebold, Y. and M. Calonge, *Applications of nanoparticles in ophthalmology*. Progress in Retinal and Eye Research, 2010. **29**(6): p. 596-609.
- [43] Kim, S.H., et al., *Transport Barriers in Transscleral Drug Delivery for Retinal Diseases*. Ophthalmic Research, 2007. **39**(5): p. 244-254.
- [44] Keister, J.C., et al., *Limits on Optimizing Ocular Drug Delivery*. Journal of Pharmaceutical Sciences, 1991. **80**(1): p. 50-53.
- [45] Hämäläinen, K.M., et al., *Characterization of paracellular and aqueous penetration routes in cornea, conjunctiva, and sclera*. Investigative Ophthalmology & Visual Science, 1997. **38**(3): p. 627-634.
- [46] Wen, H., J. Hao, and S.K. Li, *Influence of Permeant Lipophilicity on Permeation Across Human Sclera*. Pharmaceutical Research, 2010. **27**(11): p. 2446-2456.
- [47] Joseph, R.R., et al., *Characterization of liposomal carriers for the trans-scleral transport of Ranibizumab*. Scientific Reports, 2017. **7**(1): p. 16803.
- [48] Prausnitz, M.R. and J.S. Noonan, *Permeability of cornea, sclera, and conjunctiva: A literature analysis for drug delivery to the eye*. Journal of Pharmaceutical Sciences, 1998. **87**(12): p. 1479-1488.
- [49] Wen, H., J. Hao, and S.K. Li, *Characterization of Human Sclera Barrier Properties for Transscleral Delivery of Bevacizumab and Ranibizumab*. Journal of Pharmaceutical Sciences, 2013. **102**(3): p. 892-903.
- [50] Yasukawa, T., et al., *Drug delivery systems for vitreoretinal diseases*. Progress in Retinal and Eye Research, 2004. **23**(3): p. 253-281.
- [51] Robinson, M.R., et al., *A rabbit model for assessing the ocular barriers to the transscleral delivery of triamcinolone acetonide*. Experimental Eye Research, 2006. **82**(3): p. 479-487.
- [52] Nida Sen, H., et al., *Subconjunctival Sirolimus for the Treatment of Chronic Active Anterior Uveitis: Results of a Pilot Trial*. American Journal of Ophthalmology, 2012. **153**(6): p. 1038-1042.
- [53] Baum, J.L., *The kinetics of subconjunctival injections*. Survey of Ophthalmology. **24**(2): p. 129.
- [54] Sasaki, H., et al., *Drug absorption behavior after periocular injections*. Biol Pharm Bull, 1999. **22**(9): p. 956-60.
- [55] Inomata, H. and A. Bill, *Exit sites of uveoscleral flow of aqueous humor in cynomolgus monkey eyes*. Experimental Eye Research, 1977. **25**(2): p. 113-118.
- [56] Marmor, M.F., *Chapter 19 - Mechanisms of Normal Retinal Adhesion A2 - Ryan*,

- Stephen J, in *Retina (Fifth Edition)*, S.R. Sadda, et al., Editors. 2013, W.B. Saunders: London. p. 447-464.
- [57] Bill, A., *Blood circulation and fluid dynamics in the eye*. Physiological Reviews, 1975. **55**(3): p. 383-417.
- [58] Mannermaa, E., K.-S. Vellonen, and A. Urtti, *Drug transport in corneal epithelium and blood–retina barrier: Emerging role of transporters in ocular pharmacokinetics*. Advanced Drug Delivery Reviews, 2006. **58**(11): p. 1136-1163.
- [59] Constable, P.A., et al., *P-Glycoprotein expression in human retinal pigment epithelium cell lines*. Experimental Eye Research, 2006. **83**(1): p. 24-30.
- [60] Aukunuru, J.V., et al., *Expression of Multidrug Resistance-Associated Protein (MRP) in Human Retinal Pigment Epithelial Cells and Its Interaction with BAPSG, a Novel Aldose Reductase Inhibitor*. Pharmaceutical Research, 2001. **18**(5): p. 565-572.
- [61] Weijtens, O., et al., *High concentration of dexamethasone in aqueous and vitreous after subconjunctival injection*. American Journal of Ophthalmology, 1999. **128**(2): p. 192-197.
- [62] Natarajan, J.V., et al., *Sustained Release of an Anti-Glaucoma Drug: Demonstration of Efficacy of a Liposomal Formulation in the Rabbit Eye*. PLoS ONE, 2011. **6**(9): p. e24513.
- [63] Amrite, A.C. and U.B. Kompella, *Size-dependent disposition of nanoparticles and microparticles following subconjunctival administration*. Journal of Pharmacy and Pharmacology, 2005. **57**(12): p. 1555-1563.
- [64] Bourges, J.-L., et al., *Ocular Drug Delivery Targeting the Retina and Retinal Pigment Epithelium Using Polylactide Nanoparticles*. Investigative Ophthalmology & Visual Science, 2003. **44**(8): p. 3562-3569.
- [65] Amrite, A.C., et al., *Effect of circulation on the disposition and ocular tissue distribution of 20 nm nanoparticles after periocular administration*. Molecular Vision, 2008. **14**: p. 150-160.
- [66] Wong, C.W., et al., *Evaluation of subconjunctival liposomal steroids for the treatment of experimental uveitis*. Scientific reports, 2018. **8**(1): p. 6604-6604.
- [67] Hirnle, E., P. Hirnle, and J.K. Wright, *Distribution of liposome-incorporated carboxyfluorescein in rabbit eyes*. Journal of Microencapsulation, 1991. **8**(3): p. 391-399.
- [68] Moon, J.-W., et al., *Effect of Subconjunctivally Injected, Liposome-Bound, Low-*

- Molecular-Weight Heparin on the Absorption Rate of Subconjunctival Hemorrhage in Rabbits*. Investigative Ophthalmology & Visual Science, 2006. **47**(9): p. 3968-3974.
- [69] Kim, S.H., et al., *Drug Elimination Kinetics Following Subconjunctival Injection Using Dynamic Contrast-Enhanced Magnetic Resonance Imaging*. Pharmaceutical Research, 2008. **25**(3): p. 512-520.
- [70] Li, S.K., et al., *MRI study of subconjunctival and intravitreal injections*. Journal of Pharmaceutical Sciences, 2012. **101**(7): p. 2353-2363.
- [71] Dastjerdi, M.H., et al., *Corneal Penetration of Topical and Subconjunctival Bevacizumab*. Investigative Ophthalmology & Visual Science, 2011. **52**(12): p. 8718-8723.
- [72] Bassyouni, F., et al., *Advances and new technologies applied in controlled drug delivery system*. Research on Chemical Intermediates, 2015. **41**(4): p. 2165-2200.
- [73] Jesorka, A. and O. Orwar, *Liposomes: Technologies and Analytical Applications*. Annual Review of Analytical Chemistry, 2008. **1**(1): p. 801-832.
- [74] Sercombe, L., et al., *Advances and Challenges of Liposome Assisted Drug Delivery*. Frontiers in Pharmacology, 2015. **6**.
- [75] Koning, G.A. and G. Storm, *Targeted drug delivery systems for the intracellular delivery of macromolecular drugs*. Drug Discovery Today, 2003. **8**(11): p. 482-483.
- [76] Metselaar, J.M. and G. Storm, *Liposomes in the treatment of inflammatory disorders*. Expert Opinion on Drug Delivery, 2005. **2**(3): p. 465-476.
- [77] Ding, B.S., et al., *Advanced drug delivery systems that target the vascular endothelium*. Mol Interv, 2006. **6**(2): p. 98-112.
- [78] Hua, S. and S.Y. Wu, *The use of lipid-based nanocarriers for targeted pain therapies*. Frontiers in pharmacology, 2013. **4**: p. 143-143.
- [79] Sharma, A. and U.S. Sharma, *Liposomes in drug delivery: Progress and limitations*. International Journal of Pharmaceutics, 1997. **154**(2): p. 123-140.
- [80] Habib, F.S., et al., *Liposomes as an ocular delivery system of fluconazole: in-vitro studies*. Acta Ophthalmologica, 2010. **88**(8): p. 901-904.
- [81] Abrishami, M.M., et al., *Preparation, Characterization, and in vivo Evaluation of Nanoliposomes-encapsulated Bevacizumab (Avastin) for Intravitreal Administration*. Retina, 2009. **29**(5): p. 699-703.
- [82] Pleyer, U., et al., *Ocular absorption of cyclosporine A from liposomes incorporated into collagen shields*. Current Eye Research, 1994. **13**(3): p. 177-181.

- [83] Shen, Y. and J. Tu, *Preparation and ocular pharmacokinetics of ganciclovir liposomes*. The AAPS Journal, 2007. **9**(3): p. E371-E377.
- [84] Sercombe, L., et al., *Advances and Challenges of Liposome Assisted Drug Delivery*. Frontiers in Pharmacology, 2015. **6**(286).
- [85] Gregoriadis, G., *Fate of Liposomes in vivo and its Control: A Historical Perspective*, in *Stealth Liposomes*, F.J.M. Danilo D. Lasic, Editor. 1995.
- [86] Abu Lila, A.S. and T. Ishida, *Liposomal Delivery Systems: Design Optimization and Current Applications*. Biological and Pharmaceutical Bulletin, 2017. **40**(1): p. 1-10.
- [87] Semple, S.C., A. Chonn, and P.R. Cullis, *Influence of Cholesterol on the Association of Plasma Proteins with Liposomes*. Biochemistry, 1996. **35**(8): p. 2521-2525.
- [88] Foteini, P., et al., *Physicochemical study of the protein–liposome interactions: influence of liposome composition and concentration on protein binding*. Journal of Liposome Research, 2018: p. 1-9.
- [89] Oku, N., et al., *Effect of serum protein binding on real-time trafficking of liposomes with different charges analyzed by positron emission tomography*. Biochimica et Biophysica Acta (BBA) - Biomembranes, 1996. **1280**(1): p. 149-154.
- [90] Semple, S.C., A. Chonn, and P.R. Cullis, *Interactions of liposomes and lipid-based carrier systems with blood proteins: Relation to clearance behaviour in vivo*. Advanced Drug Delivery Reviews, 1998. **32**(1): p. 3-17.
- [91] Senior, J.H., K.R. Trimble, and R. Maskiewicz, *Interaction of positively-charged liposomes with blood: implications for their application in vivo*. Biochimica et Biophysica Acta (BBA) - Biomembranes, 1991. **1070**(1): p. 173-179.
- [92] Chonn, A., S.C. Semple, and P.R. Cullis, *Association of blood proteins with large unilamellar liposomes in vivo. Relation to circulation lifetimes*. Journal of Biological Chemistry, 1992. **267**(26): p. 18759-18765.
- [93] Geng, S., et al., *Two cholesterol derivative-based PEGylated liposomes as drug delivery system, study on pharmacokinetics and drug delivery to retina*. Nanotechnology, 2014. **25**(27): p. 275103.
- [94] Poste, G., D. Papahadjopoulos, and W.J. Vail, *Chapter 4 Lipid Vesicles as Carriers for Introducing Biologically Active Materials into Cells*, in *Methods in Cell Biology*, D.M. Prescott, Editor. 1976, Academic Press. p. 33-71.
- [95] Chrai, S.S., R. Murari, and I. Ahmad, *Liposomes (a review): Part two: Drug*

delivery systems. BioPharm, 2002. **15**: p. 40-43+49.

[96] Sapra, P. and T.M. Allen, *Ligand-targeted liposomal anticancer drugs*. Progress in Lipid Research, 2003. **42**(5): p. 439-462.

[97] Allen, T.M. and P.R. Cullis, *Liposomal drug delivery systems: From concept to clinical applications*. Advanced Drug Delivery Reviews, 2013. **65**(1): p. 36-48.

Chapter 3

Experimental Methods

This chapter describes the materials, fabrication methodology and experimental methodology used in this work. Preparation of liposomes (both fluorescent and radioactive liposomes) and characterization method is described in this chapter. In vivo studies which forms the bulk of this project is also described in detail. This includes the use of Cellvizio® Dual Band for in vivo confocal imaging of the eye to qualitatively study the effect of various factors on the distribution of liposomes in the eye after subconjunctival injection. The use of liquid scintillation counter to quantify the amount of liposomes in ocular tissues and sample preparation for analysis are also described in detail.

3.1 Materials

1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-stearoyl-2-linoleoyl-sn-glycero-3-phosphocholine (SLPC), 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine (PLPC), 1-stearoyl-2-oleoyl-sn-glycero-3-phosphocholine (SOPC), L- α -phosphatidylcholine (Egg, Chicken)($>99\%$ purity)(EggPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt) (POPG), were obtained from Avanti Lipids.

Radiolabeled lipids, Phosphatidylcholine, L- α -1-palmitoyl-2-oleoyl [$1\text{-}^{14}\text{C}$] (^{14}C -POPC) was purchased from American Radiolabeled Chemical (ARC) while Avanti EggPC (^{14}C -EggPC) were custom-radiolabeled by ARC upon request. L- α -DiPalmitoyl Phosphatidylcholine, [DiPalmitoyl-1- ^{14}C] (^{14}C -DPPC), Soluene® 350 and Ultima Gold were purchased from PerkinElmer.

Fluorescein DHPE (N-(Fluorescein-5-Thiocarbamoyl)-1,2-Dihexadecanoylsn-Glycero-3-Phosphoethanolamine, Triethylammonium Salt) and DRAQ5™ Fluorescent Probe were purchased from ThermoFisher. Evans Blue was purchased from Sigma, along with Chloroform, Methanol, Sodium phosphate dibasic anhydrous (Na_2HPO_4), potassium phosphate monobasic anhydrous (KH_2PO_4), potassium chloride (KCl), and sodium chloride (NaCl) salts. The salts were used for preparation of phosphate buffered saline (PBS) without further purification. The water used in all the experiments was from a MiliQ purification system with a resistivity of at least $18.2 \pm 0.2 \text{ m}\Omega \text{ cm}$. Polycarbonate filters (0.2 and 0.08 μm) were purchased from Whatman.

3.2 Methods

Figure 3.2: Overview of experiments done.

An overview of the experiments done is shown in the flow chart in Figure 3.2 and will be further elaborated in the following sub-sections.

3.2 Liposomal Synthesis

3.2.1 Preparation of Liposomes

Liposomes was prepared using the thin film hydration method. Lipids were weighed and dissolved in Chloroform and Methanol at a ratio of 2:1. The solvent was allowed to evaporate at a controlled rate using a rotary evaporator at the speed of 150 rpm at 40°C for an hour. This resulted in the formation of a thin lipid film which was hydrated with PBS at 60 °C to form multi-lamellar vesicles (MLV). In order to prepare large uni-lamellar vesicles (LUV) with a desired size of 100 nm, the MLVs were passed through an extruder (Northern Lipids Inc, Canada) multiple times. The MLVs were extruded 5 times through 0.2 µm and 10 times through 0.08 µm polycarbonate filters sequentially. The liposome samples were filtered through a 0.22 µm syringe filter (Fisherbrand™ PVDF 13 mm, Fisher Scientific) to achieve sterility for *in vivo* studies.

Fluorescent neutral liposomes were prepared with 5% Fluorescein DHPE and 95% POPC lipids. Positively charged liposomes were prepared with POPC and DOTAP at a molar ratio of 60:40 and negatively charged liposomes were prepared with POPC and POPG at a molar ratio of 60:40, POPC/Cholesterol liposomes were prepared with POPC and Cholesterol at molar ratio of 60:40, along with 5% Fluorescein DHPE as shown in Table 3.2.1.1.1.

Table 3.2.1.1: No. of moles of lipids used for each type of liposome.

	Concentration (in 3ml PBS)	Main Lipid (moles)	DOTAP or POPG (moles)	Cholesterol	Fl-DHPE (moles)
Neutral Liposomes	26.3 mM	7.5×10^{-5}	-		4.0×10^{-6}
Positively charged liposomes (POPC/DOTAP) (40:60)	26.3 mM	4.5×10^{-5}	3.0×10^{-5}		4.0×10^{-6}
Negatively charged liposomes (POPC/POPG) (40:60)	26.3 mM	4.5×10^{-5}	3.0×10^{-5}		4.0×10^{-6}
POPC/Cholesterol (60:40) Liposomes	26.3 mM	4.5×10^{-5}		3.0×10^{-5}	4.0×10^{-6}
DPPC Liposomes	26.3 mM	7.5×10^{-5} DPPC			4.0×10^{-6}
EggPC Liposomes	26.3 mM	7.5×10^{-5}			4.0×10^{-6}
SLPC Liposomes	26.3 mM	7.5×10^{-5}			4.0×10^{-6}
PLPC Liposomes	26.3 mM	7.5×10^{-5}			4.0×10^{-6}

Radioactive liposomes were prepared by mixing 93.84% of cold (non-labelled) solution of the lipid with 6.16% of hot (radiolabeled) solution of the lipid to achieve a dosing solution of 1 μ Ci/10 μ l. Cold lipid solution was prepared by measuring 100 mg of lipid and dissolving in 1ml of chloroform. Hot lipid solution was used as received from ARC or Perkin Elmer depending on the lipid. For 14 C POPC, 14 C EggPC, the lipids were received as a solution in toluene: ethanol (1:1) (55 mCi/mmol) packaged in silanized vials under argon. Similarly, 14 C DPPC was also received as a solution in toluene: ethanol (1:1) (112.0 mCi/mmol) packaged in a silanized vial under argon. Both lipids were radiolabeled on the tail group. The molecular structure of the radiolabeled lipids can be seen in Table 3.2.1.2.

Table 3.2.1.2: Molecular structure of radiolabeled lipids provided by ARC and Perkin Elmer respectively. ^{14}C is indicated by *.

	Molecular structure of radiolabeled lipids
^{14}C POPC	<p>M.W. 760.1</p>
^{14}C DPPC	<p>M.W. 734.0</p>

3.2.2 Zeta Size and Zeta Potential Measurements

In order to determine the size and charge of the liposomes, the zeta size and zeta potential were measured using a Malvern Zetasizer Nano ZS (UK). The samples were diluted 100 times in de-ionized water (for size measurements) and PBS (for zeta potential measurements) and placed in the respective cuvettes for measurements. The zeta size of the liposomes was measured based on dynamic light scattering theory. The scattered light intensity of the particles was measured at a fixed detector angle of 173° with a laser power intensity of 633 nm. The hydrodynamic size of the liposomes was then calculated from the measured intensity based on Brownian motion of particles in suspension. Zeta potential (ζ -potential) was obtained from using the Smoluchowski function $\zeta = \mu\eta/\epsilon$ where η and ϵ were the viscosity and permittivity of the solvent respectively μ , the electrophoretic mobility. For charged liposomes, the surrounding ions near the liposomes will distribute into an electrical double layer, resulting in potential at the slipping plane which is the zeta potential value. A total of 3 readings were measured for each sample.

3.3 *In vivo* Study

3.3.1 Animals

6 to 8 weeks-old female BALB/C mice were used for the optimization of dyes and the pilot study. 6 to 8 weeks-old female C57BL/6J mice were used for the rest of the studies. The animals were treated according to the guidelines from the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals in ophthalmic and vision research. The protocols utilized were approved by the institutional animal care and use committee (IACUC) at SINGHEALTH and DUKE-NUS.

3.3.2 Subconjunctival administration

Prior to the subconjunctival injection, all mice were anesthetized by either an intra-peritoneal (IP) injection of 10 mg/kg Xylazine and 80 mg/kg Ketamine or inhalational isoflurane under a flow of oxygen (5% for induction and 2-2.5% for maintenance). A drop of lidocaine 1% was also administered to the eye of each mouse as a form of topical anesthesia. 10 μ L of fluorescent liposomes/fluorescein was injected into the subconjunctival space in the mice eyes using a 32-gauge needle (Hamilton, 0.5" PT4) by gently pulling the conjunctiva. The needle was inserted at temporal region (4T) about 0.2 mm away from the limbus, towards the superior region (4S) of the eye. A drop of Fucithalmic® Eye Drop (LEO Pharma) was given to each mouse after the injection and each mouse was monitored until it regained consciousness.

3.4 Qualitative Imaging Studies

3.4.1 Histological assessment

The mice were sacrificed with an overdose of Pentobarbital sodium solution and their eyes were enucleated after confirming that the mice have expired. The eyeballs were washed in PBS and fixed in 4% PFA overnight after enucleation. The eyeballs were trimmed and embedded in optimal cutting temperature (OCT) medium. Once frozen, the blocks containing the eyeballs were loaded on to the cryostat (maintained at a temperature lower than -20°C) and cut into 5 μ m sections using a sharp blade. Sections were then washed and imaged after the slides were air dried to remove any remnants of water droplets.

3.4.2 In vivo Confocal Imaging

In vivo confocal fluorescence imaging was performed with Cellvizio® Dual Band (Figure 3.4.2a) from Mauna Kea Technologies, France. Cellvizio® Dual Band is a confocal laser imaging system designed for *in vivo* visual inspection of tissues at the microscopic level. It can generate two color laser excitation (488 nm and 660 nm) and dual band fluorescence detection (502-633 nm and 673-800 nm) in real time at the same location in the tissue using the same probe which consists of a bundle of optical fibers. [1]

Figure 3.4.2: a) Cellvizio® Dual Band Imaging System [1] and b) Image of Cellvizio® probe on mouse eye.

The probe that was used is a S1500 probe with a tip diameter of 1.5 mm, a 600x500 μm field of view, 15 μm axial resolution and a 3.3 μm lateral resolution, intended for surface scan of tissues. It can be placed in direct contact with the tissue of interest (as shown in Figure 3.4.2b) and signal originating from the first tens of microns immediately in front of it can be acquired using this probe [2].

The probes were calibrated and images were acquired for a control mouse (not injected with any fluorescence dyes/liposomes) prior to image acquisition of any experimental mice. The fluorescence in the images acquired for the control mouse is considered as autofluorescence and serves as the minimum gain to be used for analysis of experimental groups.

3.4.2.1 Eye Map

In order to identify the region scanned and maintain consistency in the scans, an eye map was created as shown in Figure 3.4.2.1. The eye was divided to three areas of interest: 1- Cornea/pupil, 2 - Cornea/iris, 3 - Limbus, 4 - Sclera. As the probe covers the whole of the pupil, 1A refers to the cornea right in front of the pupil. Regions 2, 3 and 4 were further divided according to their position: superior (S), nasal (N), inferior (I), and temporal (T).

Figure 3.4.2.1: Eye map for CellVizio Scans. Image of eye retrieved from *Dorland's Medical Dictionary for Health Consumers*. (2007) [3].

During each time point, videos were taken (frame rate: 9 Hz) and flags were made when the probe is on each region of interest (1A, 2S, 2N, 2I, 2T, 3S, 3N, 3I, 3T, 4S, 4N, 4I, 4T). Each flagged frame is then exported from the video as an image.

3.4.2.2 Choice of Dyes

As CellVizio® Dual Band can generate two color laser excitation (488 nm and 660 nm) and allow dual band fluorescence detection at the same time, the fluorescent liposomes can be detected in one channel and vessels or cells can be stained and detected in another channel. This can allow better identification of landmarks in the region that is

being imaged. As green fluorescence is detectable in almost all imaging systems, the 488 nm channel was reserved for liposomes. All the liposomes used in the distribution studies were tagged with green fluorophore by incorporating the phospholipid, Fluorescein-DHPE in the structure.

In order to use the 660 nm channel to detect vasculature or cells that will help in identifying ocular landmarks, the suitability of two dyes were tested.

Table 3.4.2.2: Description of dyes tested for use with CellVizio® system.

Dyes tested	Description	Administration
Evans Blue (Vasculature)	Potent inhibitor of L-glutamate uptake for circulation. Ex/Em: 540/680 nm [4]	Intravenous injection [5-7]
DRAQ5 (Cell nuclei)	Lipophilic and membrane permeable dye effective for live-cell imaging. Ex/Em: 646/697 nm [4, 8]	Instillation on the eye

3.4.2.2.1 Evans Blue Dye

Evans Blue Dye (EBD) is non-toxic and has been widely used to study blood vessels. It is identified by its blue color and can be administered as an intravital dye. Under fluorescence microscopy, it is observed by red fluorescence. [5-7, 9].

In order to determine the concentration of EBD that can allow ocular vasculature to be visibly seen and imaged using the CellVizio probe, two commonly used concentrations of EBD were tested. 1% and 2% EBD (w/v) solution were prepared in PBS and sterilized by filtering through a 0.22 µm syringe filter (Fisherbrand™ PVDF 13 mm, Fisher Scientific). EBD was administered in the mice through intravenous (i.v.) injection of the tail using a 27G needle. The eyes of the mice were imaged at day 5 and day 7 to determine if the vasculature stain is still visible at these time points.

3.4.2.2.2 DRAQ5 dye

DRAQ5 is an anthraquinone derivate [10, 11] that has a high affinity for double-stranded DNA. It is able to provide a bright nuclear signal, making it ideal for visualizing living cells [8]. DRAQ5 is able to penetrate very quickly into the nucleus and stains the DNA stoichiometrically without causing any severe cytotoxic effects [11-13].

As DRAQ5 has not been commonly used as an intravital dye, a few studies were done to assess the suitability of this dye for imaging cells in the eye and the duration required for complete staining. In order to determine the concentration of DRAQ5 that can allow cells to be visibly seen *in vivo* with the CellVizio® system, three concentrations of DRAQ5 were tested. As the working concentration for *in vitro* cell staining are from 5-20 μM at 10 μL for 1×10^6 cells [14, 15], 10 μM , 20 μM and 40 μM were chosen for the *in vivo* staining. 10 μL of 10 μM , 20 μM and 40 μM of DRAQ5 were instilled onto the mice eyes as a ‘eye drop’ and washed away after 10 minutes. The eyes were imaged at 25 minutes, 90 minutes, 180 minutes and 24 hours after instillation on the eye to determine the time required for complete staining of the cells in the eye. The mice were observed for any discomfort and were weighed up to 7 days after daily instillation of the dye.

3.4.3 Pilot study: Feasibility of CellVizio® for eye imaging

As this is the first time the CellVizio® system is being used on the ocular surface, a pilot study was done using 5 mice (one control mouse + 4 experimental mice). EBD was used as the vasculature stain and DRAQ5 dye was used as the cell nuclei stain. The mice were anesthetized by inhalational isoflurane prior to EBD injection and by intra peritoneal (i.p.) injection of Xylazine/Ketamine prior to subconjunctival injection. Mice were given an i.v. injection of 2% EBD through the tail vein at least 2 hours before the first time point and instilled with 10 μL of 40 μM DRAQ5 prior to each time point. The mice were imaged at 1 hour, 6 hours, 12 hours, 1 day, 3 days, 5 days, 7 days, 10 days, 12 days, 14 days, 21 days, 28 days and 35 days after sub-conjunctival injection of the liposomes. The mice were sacrificed at day 35 after the last imaging time point. The steps taken for the experiment are shown in Figure 3.4.3.

Figure 3.4.3: Flow chart highlighting main procedures in the CellVizio imaging protocol.

3.4.4 Distribution study using CellVizio®

After the completion and studying the outcome of the pilot studies, improvements were made for the actual experimental studies:

1. Instead of using two types of anesthetic agents, only inhalational isoflurane was used throughout the procedures.
2. Instead of using both Evans Blue and DRAQ5 for the 660 nm (red) channel only Evans Blue was used.

Table 3.4.4: Liposomal properties studied using CellVizio®.

Factors		
Charge (100 nm liposomes)	Neutral	n=4
	Positively charged	n=5
	Negatively charged	n=5
Size (Neutral liposomes)	100 nm	n=5
	250 nm	n=5
	MLVs	n=5
Incorporation of Cholesterol (100 nm neutral liposomes)	POPC/Cholesterol	n=5
Chain Saturation (100 nm neutral liposomes)	DPPC	n=5
Natural Lipid (100 nm neutral liposomes)	EggPC	n=5
EggPC Components (100 nm neutral liposomes)	SLPC	n=5
	PLPC	n=5

The effect of liposomal properties on the distribution in the eye was studied in a total of 67 mice (59 experimental, 4 control, 4 Fluorescein) in batches as listed in Table 3.4.4. 1 mouse was kept as control for each batch studied while experimental mice were injected with fluorescent liposomes. Initial studies included a group of 4 mice which were injected with equivalent concentration of Fluorescein to confirm that the distribution observed is due to the nature of liposomes and not the dye. The liposomal properties that were studied include charge, size, incorporation of cholesterol, chain saturation. Natural lipid, EggPC, were also studied to observe how it differs from its synthetic substitute, POPC.

3.5 Quantitative Radioactive Studies

3.5.1 Preparation of radiolabeled liposomes

Liposomes were prepared by the same thin film hydration method as the non-radioactive liposomes. To make radioactive liposomes, non-radiolabeled lipids were doped with ~6% of the radiolabeled lipid and mixed in a 2:1 mixture of Chloroform and Methanol. The solvent was allowed to resulting in the formation of a thin lipid film which was hydrated with PBS to form MLVs. In order to prepare large uni-lamellar vesicles (LUV) with a desired size of 100 nm, the MLVs were passed through a handheld extruder (Avanti Lipids) multiple times. The MLVs were extruded 30 times through 0.08 μm polycarbonate filter. Table 3.5.1 lists the timepoints studied for each type of liposomes.

Table 3.5.1: Liposomes for quantitative studies.

Liposomes	Timepoints studied	No. of mice per timepoint
Neutral Liposomes (100 nm)	6, 24, 72, 120 h	5
Neutral Liposomes (MLV)	24, 72, 120, 168 h	5
Positively charged liposomes (DOTAP-POPC) (40:60)	24, 72, 120, 168 h	5
DPPC Liposomes	6, 24, 72, 120 h	5
EggPC Liposomes	24, 72, 120, 168 h	5
EggPC + ^{14}C POPC Liposomes	24, 72, 120, 168 h	5

3.5.2 Biodistribution Studies

10 μl of liposomes ($\sim 0.8 \mu\text{Ci}$) was injected into the subconjunctival space in the mice eyes using a 32-gauge needle (Hamilton, 0.5" PT4) by gently pulling the conjunctiva. The needle was inserted at temporal region (4T) about 0.2 mm away from the limbus, towards the superior region (4S) of the eye. A drop of Fucithalmic® Eye Drop (LEO Pharma) was given to each mouse after the injection and the mice were monitored until it regained consciousness.

Depending on the outcome of the CellVizio® imaging studies, animals were sacrificed at 6 h (for POPC and DPPC liposomes), 24 h, 72 h, 120 h and 168 h (for POPC-DOTAP,

MLVs and EggPC liposomes) after the subconjunctival injection. Before sacrificing the animals, blood samples were obtained by cardiac puncture. The conjunctiva was harvested right after sacrificing the mice. The eyes were enucleated and fixed in 10% Formalin for 30 minutes prior to dissection. Dissection of the eye was done right after the stipulated fixing time. After removing the optic nerve, the eye ball was cut along the limbus to separate the cornea and sclera. Vitreous Humor, Retina, RPE, Sclera, Iris, Cornea, Lens, Optic Nerve were collected.

Other tissues, Right Eye, Axillary Lymph Nodes, Cervical Lymph Nodes, Inguinal Lymph Nodes, Heart, Lungs, Liver, Kidneys, Spleen, Stomach, Small Intestine and Brain were collected and kept frozen at -20°C until processed. Other than ocular tissues, all the tissues harvested were weighed right after collection.

3.5.3 Tissue Solubilization Protocol

Harvested tissues were dissolved in Soluene® 350 and kept in 37°C incubator for the tissues to be fully dissolved. The original protocol for use of Soluene® for dissolving tissues requires heating above 60°C. However, as it was not feasible in the facility due to safety issues regarding radioactive chemicals, the tissue solubilization method was optimized for 37°C. Table 3.5.3. shows the optimized solubilization protocol for each tissue analyzed and the amount mixed with the scintillant, Ultima Gold, for quantification.

Table 3.5.3: Solubilization protocol for tissues.

Tissue		Amount of Soluene®	Duration to dissolve tissue	Amount of dissolved tissue mixed with 3ml of Ultima Gold
Ocular Tissues	Conjunctiva	200 µl	2 days	200 µl
	Optic Nerve	200 µl	2 days	200 µl
	Retina	200 µl	2 days	200 µl
	RPE	200 µl	2 days	200 µl
	Sclera	200 µl	2 days	200 µl
	Cornea	200 µl	2 days	200 µl
	Iris	200 µl	2 days	200 µl

	Lens	200 μ l	2 days	200 μ l
	Vitreous Humor	Collected in tissue paper		
	Right Eye	1 ml	2 days	1 ml
Blood	Plasma	-	-	50 μ l
Lymph	Axillary Lymph Node	1 ml	4 days	1 ml
Nodes	Cervical Lymph Nodes	1 ml	4 days	1 ml
	Inguinal Lymph Nodes	1 ml	4 days	1 ml
Organs	Spleen	1 ml	4 days	1 ml
	Kidneys	1 ml/100 mg tissue	4-7 days	500 μ l
	Liver	1 ml/100 mg tissue	7 days	200 μ l

3.5.4 Determination of radioactivity

In order to separate the plasma from the blood, the collected blood was centrifuged at 200rpm for 20 minutes at 4°C. 50ul of plasma was collected for analysis. The radioisotope content of the plasma (50ul), vitreous humor and the formalin used to fix the eyes (to detect if liposomes were lost during fixation) were measured by mixing the aliquots directly with Ultima Gold (Perkin Elmer). The rest of the ocular tissues, Conjunctiva, Optic Nerve, Retina, RPE, Sclera, Iris, Cornea, Lens were mixed with 200ul Soluene® and left to dissolve in a 37°C incubator for 48 hours. The dissolved tissues were mixed with Ultima Gold (scintillation cocktail) and the radioactivity was quantified by using the TriCarb liquid scintillation counter (Perkin Elmer).

Ionizing radiation from the radionuclide was converted to photons of light with the help of the scintillator (a component of the scintillation cocktail) through a process called scintillation. The intensity of the photons of light is proportional to the initial energy of a Beta particle. When the vial containing a radionuclide and scintillation cocktail is placed in the instrument's dark detection enclosure, photon intensity can be measured by the scintillation counter. The light emitted from the sample vials is amplified by a photosensitive device and is converted to pulses of electrical energy which are registered as counts. The signal amplitude determines the energy channel (keV) and the counts accumulated was sorted into separate channels to create a sample spectrum. By using the spectrum, Counts Per Minute (CPM) for each sample can be determined by various count correlation calculations done by the system. Disintegrations Per Minute

(DPM) was calculated by determining the counting efficiency of each sample. Using a quench curve, the instrument then compares the quench index for the samples for quench standards to determine a sample counting efficiency, and then calculate DPM and corresponding radioactive quantity in terms of μCi for the unknown samples. [16] An overview of the process can be seen in Figure 3.5.4.

For every tissue that was analyzed, the values from the corresponding tissues harvested from a control mouse was considered as background noise and deducted from the sample values.

Figure 1-1. Overview of Scintillation Counting Process (1 of 2).

Figure 1-2 Overview of Scintillation Counting Process (2 of 2).

Figure 3.5.4: Overview of Scintillation Counting Process [16]

3.6 Statistical Analysis

Data are expressed as mean $\pm$ standard deviation (SD). Where only two conditions were compared, the significance of differences between the two conditions was determined by the two-tailed Student's t-test with significance at $p < 0.05$. Where more than two conditions were compared, the significance of differences between the conditions was determined by one-way ANOVA using Origins software.

References

[1] Technologies, M.K., *Cellvizio Dual Band Instructions for Use, Part A – May 2014*.

2014.

- [2] Technologies, M.K., *Microprobes User Guide –June2013*. 2013.
- [3] *limbus*. (n.d.) *Dorland's Medical Dictionary for Health Consumers*. (2007). Retrieved August 9 2016 from <http://medical-dictionary.thefreedictionary.com/limbus>.
- [4] Technologies, M.K., *Fluorescent Dyes List V.2014-1.01*. 2014.
- [5] Levi, H., et al., *Stimulation of the Sphenopalatine Ganglion Induces Reperfusion and Blood-Brain Barrier Protection in the Photothrombotic Stroke Model*. PLoS ONE, 2012. **7**(6): p. e39636.
- [6] Prager, O., et al., *Dynamic in vivo imaging of cerebral blood flow and blood–brain barrier permeability*. NeuroImage, 2010. **49**(1): p. 337-344.
- [7] Bar-Klein, G., et al., *Losartan prevents acquired epilepsy via TGF- β signaling suppression*. Annals of Neurology, 2014. **75**(6): p. 864-875.
- [8] Progatzy, F., M.J. Dallman, and C. Lo Celso, *From seeing to believing: labelling strategies for in vivo cell-tracking experiments*. Interface Focus, 2013. **3**(3).
- [9] Hamer, P.W., et al., *Evans Blue Dye as an in vivo marker of myofibre damage: optimising parameters for detecting initial myofibre membrane permeability*. Journal of Anatomy, 2002. **200**(1): p. 69-79.
- [10] Smith, P.J., et al., *Characteristics of a novel deep red/infrared fluorescent cell-permeant DNA probe, DRAQ5, in intact human cells analyzed by flow cytometry, confocal and multiphoton microscopy*. Cytometry, 2000. **40**(4): p. 280-291.
- [11] Smith, P.J., et al., *A novel cell permeant and far red-fluorescing DNA probe, DRAQ5, for blood cell discrimination by flow cytometry*. Journal of Immunological Methods, 1999. **229**(1–2): p. 131-139.
- [12] Mari, P.-O., et al., *Influence of the live cell DNA marker DRAQ5 on chromatin-associated processes*. DNA Repair, 2010. **9**(7): p. 848-855.
- [13] Edward, R., *Use of DNA-specific anthraquinone dyes to directly reveal cytoplasmic and nuclear boundaries in live and fixed cells*, in *Molecules and Cells*. 2009. p. 391-396.
- [14] Abcam, *Data sheet: DRAQ5™ (5mM)*.
- [15] Scientific, T., *Instructions: DRAQ5™ Fluorescent Probe* 2010.
- [16] PerkinElmer, I., *Tri-Carb® Liquid Scintillation Analyzer*. PerkinElmer, Inc.: U.S.A.

Chapter 4

Using Cellvizio® to study the fate of liposomes in the eye

This chapter describes the feasibility of using Cellvizio® Dual Band for studying the fate of liposomes in the eye after subconjunctival injection. As the Cellvizio® Dual Band has not been used in a similar way elsewhere, optimization of the dyes used to identify tissue landmarks was required. Two different dyes, Evans Blue and DRAQ5, were studied for their suitability to be used in the study. A pilot study was then conducted with the optimized staining conditions. The results of the pilot study and feasibility of using this imaging method to study the distribution of the liposomes in the eye was discussed.

4.1 Characterization of liposomes

Table 4.1: Size of liposomes used.

Type	Composition	Size (nm)	PDI
Neutral (POPC)	95% POPC, 5% Fl-DHPE	109±2	0.04-0.07

For the pilot study on feasibility for Cellvizio® for longitudinal tracking of liposomes in the eye, neutral 100 nm POPC liposome were used. The composition and size of the liposomes studied can be seen in Table 4.1.

4.2 Feasibility of the CellVizio dual band for ocular distribution studies

4.2.1 Choice of Dye used for CellVizio Imaging

As this is the first time the Cellvizio® system is used for ocular imaging, an optimization of the dyes used for the imaging is very important. Two dyes were chosen according to the recommendation from Mauna Kea technologies. Evans blue serves as a vasculature stain and is expected to be detected at regions 3 (limbus) and 4 (sclera). As region 3 covers both the cornea and the sclera which are separated by the limbus, effective vasculature staining will allow the identification of the cornea, sclera and limbus as the blood vessels are absent in the cornea. DRAQ5, on the other hand, serves as a stain for cell nuclei. As the cornea is made up of various cell layers, DRAQ5 nuclei staining is expected to be more prominent at region 1 (cornea/pupil) and 2 (cornea/iris) and on the corneal side on region 3 (limbus).

4.2.1.1 Optimizing Evans Blue Dye Concentration

Figure 4.2.1.1.1: CellVizio images of mice eyes taken 5 days after i.v. injection of 1% and 2% EVD. Evans Blue was not injected in the control mouse. (3S: superior limbus, 3N: Nasal limbus, 3I: Inferior limbus, 3T: Temporal limbus)

Figure 4.2.1.1.2: CellVizio images of mice eyes taken 7 days after i.v. injection of 1% and 2% Evans Blue. Evans Blue was not injected in the control mouse. (3S: superior limbus, 3N: Nasal limbus, 3I: Inferior limbus, 3T: Temporal limbus)

Figure 4.2.1.1.1 shows the compilation of images taken at the limbus region at day 5 (3S: superior limbus, 3N: Nasal limbus, 3I: Inferior limbus, 3T: Temporal limbus) while Figure 4.2.1.1.2 shows the compilation of images taken at the limbus region at day 7. From the images captured on both day 5 and day 7, it can be seen that 1% Evans Blue was not sufficient for the vasculature staining to be detected by the Cellvizio® probe. The vasculature staining by 2% Evans Blue was prominent and a distinction between

the cornea and sclera region can be observed. 2% Evans Blue also persisted up to day 7 which was the length of study planned, making it suitable for use for Cellvizio® imaging of the eye.

4.2.1.2 Optimizing DRAQ5 concentration

Figure 4.2.1.2.1: Effect of DRAQ5 concentration cell nuclei staining. Compilation of CellVizio images of mice eyes taken 25 minutes after instillation of 10 μ M, 20 μ M and 40 μ M DRAQ5. Images were taken at the cornea (1A: Cornea/Pupil, 2S: Superior Cornea/Iris, 2N: Nasal Cornea/Iris, 2I: Inferior Cornea/Iris, 2T: Temporal Cornea/Iris). DRAQ5 was not administered in the control mouse.

Figure 4.2.1.2.2: Effect of DRAQ5 concentration on cell nuclei staining. Compilation of CellVizio images of mice eyes taken 90 minutes after instillation of 10 µM, 20 µM and 40 µM DRAQ5. Images were taken at the cornea (1A: Cornea/Pupil, 2S: Superior Cornea/Iris, 2N: Nasal Cornea/Iris, 2I: Inferior Cornea/Iris, 2T: Temporal Cornea/Iris). DRAQ5 was not administered in the control mouse.

From Figures 4.2.1.2.1 and 4.2.1.2.2, it can be seen that 40 µM of DRAQ5 allowed complete staining of the cells of the cornea while the lower DRAQ5 concentrations were not sufficient for cell nuclei staining with the given time of 25 minutes and 90 minutes. When the staining duration was extended for 180 minutes and 1 day for 40 µM of DRAQ5, it can be seen that 40 µM of DRAQ5 persisted for 180 minutes but not 1 day. This indicates that the DRAQ5 staining has to be done prior to every time point.

Figure 4.2.1.2.3: Effect of time on DRAQ5 staining. Compilation of CellVizio images of a mouse eye taken at 25 minutes, 90 minutes, 180 minutes after instillation of 40 μ M DRAQ5. Images were taken at the cornea (1A: Cornea/Pupil, 2S: Superior Cornea/Iris, 2N: Nasal Cornea/Iris, 2I: Inferior Cornea/Iris, 2T: Temporal Cornea/Iris) of the same mouse.

The cell nuclei stains at 90 minutes were observed to be brighter than 180 minutes and the extent of staining is similar to that at 180 minutes. It can be concluded that for sufficient cell nuclei staining of the eye, DRAQ5 is to be used at concentration of 40 μ M and has to be given at least 90 minutes for the stain to work. The mice also did not show any alarming weight changes or observable discomfort after frequent instillation of 40 μ M DRAQ5 (as shown in Figure 4.2.1.2.4), indicating that 40 μ M is safe for use in the mice.

Figure 4.2.1.2.4: Effect of DRAQ5 on the weight of mice. 10 μ L of 40 μ M of DRAQ5 was directly instilled on the eye on Day 0, 1, 2, 5 and 6. Weight was taken on Day 1, 2, 5, 6 and 7.

From the optimization study of Evans Blue and DRAQ5, it can be concluded that 2% Evans Blue can be used for vasculature staining up to at least 7 days and 40 μ M DRAQ5 can be used for cell nuclei staining if administered on the eye 90 minutes prior to the imaging time point.

4.2.2 Pilot study

With the optimized dye concentration and staining duration, a pilot study on the distribution of neutral liposomes was done with the use of CellVizio dual band system. The study was planned for 7 days but it was extended to 35 days as the fluorescent liposomes could still be well imaged by the Cellvizio® system up to 35 days.

Figure 4.2.2.1: Photographs of mice eyes after subconjunctival injection of fluorescent liposomes. The arrows indicate the bulge in the subconjunctival space which contains the injected liposomes.

Figure 4.2.2.2: Compilation of the distribution of the FL-POPC liposomes in the limbus region of the eye over 35 days (3S: Superior limbus, 3T: Temporal limbus). Images were only taken from the 488 channel.

From the images taken of the four mice at the limbus region (Figure 4.2.2.2), it can be seen that the fluorescent liposomes are distributed at both the cornea and sclera side of

the limbus up to 1 day. From day 7 onwards, the liposomes tend to stick to the limbus and are more prominent in the temporal region of the eye (which is in close proximity to the injection site). The staining for vasculature and cell nuclei did not turn out as good as expected (Figure 4.2.2.3). As the cell nuclei were freshly stained with DRAQ5 every time point, the intensity of the stain remains bright while the vasculature stain faded with each time point. This resulted in the vasculature stain being overpowered by the cell nuclei stain (especially from day 7 onwards). It was impossible to differentiate the corneal and scleral side of the limbus. Another problem was the inconsistency of the DRAQ5 cell nuclei staining. On certain days, it was complete while it was not complete on certain days even though the same protocol was followed for every time point. It might be due to the cells requiring certain duration for recovery before being stained again. In order to identify the limbus region sufficiently, 2% Evans Blue should be used for future studies.

Figure 4.2.2.3: Compilation of the distribution of the FL-POPC liposomes in the cornea, limbus and sclera region of the eye over 28 days with both EBD vasculature staining and DRAQ5 cell nuclei staining for one representative mouse. S represents the sclera and C represents the cornea side of the limbus when identifiable.

Figure 4.2.2.4: Histology images of the mouse eye 1 day post injection. Arrows point to the cornea where most of the liposomes can be observed at this time point.

Compared to the histology sections taken at day 1 after injection of the liposomes (Figure 4.2.2.4), the fluorescent signals can be seen with better resolution when imaged with Cellvizio®. Liposomes at the sclera can barely be seen in the histology images but can clearly be detected using the Cellvizio® system. The washing and sectioning procedures done in order to obtain the histology sections might also have affected the retention of the liposomes in the tissue sections. Although the Cellvizio® dual band system does have limitations in terms of user variability and locating the exact tissue layer in which the liposomes are distributed, it is a feasible method for longitudinal study of nano-carrier distribution in the eye.

Figure 4.2.2.5: Effect of Cellvizio® imaging protocol on the weight of mice. Weight was taken on all the days the CellVizio imaging was done.

The use of the Cellvizio® imaging system also did not cause any distress the mice, as observed by minimal changes in weight (Figure 4.2.2.5) and absence of physical signs of stress in the mice.

It can be concluded from the pilot study that the Cellvizio® system can indeed be a suitable imaging system for longitudinal study of distribution of liposomes in the eye and 2% Evans Blue is suitable for use as a vasculature stain.

4.3 Conclusion

In conclusion, imaging using Cellvizio® system is a feasible method to image ocular distribution of nano-carriers in the eye. This is also the first time Cellvizio® imaging system is used for longitudinal study of ocular distribution of liposome *in vivo*. Although Cellvizio® imaging system only allows imaging of the surface of the eye, it was able to give an insight on how the liposomes are distributed in the eye without having to sacrifice the animals. This makes it suitable to be used as a pre-screening step for nano-carriers before more intensive quantification studies are done. By trying out two different dyes to identify different landmarks on the eye, it was observed that it is better to just focus on only using Evans Blue dye for vasculature staining than using it together with DRAQ5 which is a cellular stain.

Chapter 5

Qualitative study on effect of liposomal properties on ocular distribution

This chapter describes the use of Cellvizio® to qualitatively study the effect of various liposomal properties on the distribution of liposomes in the eye after subconjunctival injection in the mice. With optimization of dyes and improvisation of anesthesia methods used during the pilot study, factors like charge, size, incorporation of cholesterol, chain saturations are discussed in this chapter. Charge and size of the liposomes were found to affect the location at which the liposomes were distributed to while incorporation of cholesterol and chain saturation affected how fast the liposomes were cleared from the eye. Positively charged liposomes were observed to stay at the negatively charged sclera region while negatively charged and neutral liposomes moved away from the sclera region towards the limbus region of the eye. The size of the MLVs affected the mobility of the liposomes resulting them to be retained at the injection site until clearance. Liposomes with cholesterol were cleared slower while saturated liposomes, DPPC, were cleared faster.

5.1 Effect of charge of liposome

Table 5.1: Zeta size and zeta potential of the liposomes

Type	Composition	Size (nm)	PDI	Average Zeta Potential (mV)
Neutral (POPC)	95% POPC, 5% FI-DHPE	109±2	0.04-0.07	-12
Positively Charged (DOTAP-POPC)	95% 60:40 POPC:DOTAP, 5% FI-DHPE	108±2	0.03-0.07	24
Negatively Charged (POPG-POPC)	95% 60:40 POPC:POPG, 5% FI-DHPE	106±1	0.17-0.19	-40

Figure 5.11: Photographs of mouse taken right after subconjunctival injection of 10µl of fluorescent liposomes: neutral, POPC (A), positively charged, POPC-DOTAP (B), negatively charged, POPC-POPG (C), and Fluorescein (D). Arrows indicate the injected liposomes/fluorescein.

Neutral, positively charged and negatively charged liposomes of the same size (~100 nm) were injected into the subconjunctival space of the eye. The composition and characterization of the liposomes studied can be seen in Table 5.1. To ensure that the what was observed from the images were due to fluorescence from the liposomes and not the dye which might have dislodged from the liposomes, mice were also injected with Fluorescein dye to observed the distribution of the dye itself. The distribution of the liposomes in the eye were monitored up to 14 days using confocal probe microscopy.

Figure 5.12: Distribution of neutral liposomes at the limbus (3S: Superior, 3N: Nasal, 3I: Inferior, 3T: Temporal) and sclera (4S: Superior, 4N: Nasal, 4I: Inferior, 4T: Temporal) region of 1 representative mouse over 14 days. C: Cornea, S: Sclera.

Figure 5.13: Average Fluorescence intensity of neutral POPC liposomes at the limbus and sclera region over 7 days. * and ^ represents significant difference $p < 0.05$.

From Figure 5.12 and 5.13, it can be seen that POPC liposomes can be observed in both the limbus region and sclera region at day 1. As the liposomes were injected from temporal region toward the superior regions, more liposomes were detected at both temporal and superior regions of the limbus and sclera. By day 3, we observe a significant reduction of liposomes in the sclera (66% from day 1 to day 3 and 93% from day 3 to day 7 for superior region and 60% and 88% drop for temporal region). The liposomes in the limbus were reduced at a lower rate, indicating that the limbus is the preferred location for the neutral liposomes. The liposomes on the sclera were probably cleared through lymphatic or blood circulation or might have gotten deeper into the sclera to the point that it cannot be detected by the probe.

Figure 5.14: Distribution of positively charged liposomes at the limbus (3S: Superior, 3N: Nasal, 3I: Inferior, 3T: Temporal) and sclera (4S: Superior, 4N: Nasal, 4I: Inferior, 4T: Temporal) region of 1 representative mouse over 14 days. C: Cornea, S: Sclera.

Figure 5.15: Average Fluorescence intensity of positively charged liposomes at the limbus and sclera region over 7 days. * and ^ represents significant difference $p < 0.05$.

For positively charged liposomes, their affinity for the negatively charged sclera was observed as they were seen on the sclera until they were cleared. Significant reduction is observed mainly between day 1 and day 3 for all regions. The highest drop was at limbus (94%) and sclera (83%) at nasal regions. Upon injection, the liposomes probably reached the nasal regions due to the flow of the liposomes in the subconjunctival space and did not have the chance to bind to the tissues.

Figure 5.16: Distribution of negatively charged liposomes at the limbus (3S: Superior, 3N: Nasal, 3I: Inferior, 3T: Temporal) and sclera (4S: Superior, 4N: Nasal, 4I: Inferior, 4T: Temporal) region of 1 representative mouse over 14 days. C: Cornea, S: Sclera.

Figure 5.17: Average Fluorescence intensity of negatively charged liposomes at the limbus and sclera region over 7 days. * and ^ represents significant difference $p < 0.05$.

Negatively charged liposomes acted in a similar way to neutral liposomes. They can be seen residing the limbus region over time. Significant reduction in fluorescence intensity was mainly between day 1 and 3 (74-98% for all regions).

Figure 5.18: CellVizio images showing disposition of neutral, positively charged, negatively charged liposomes and fluorescein dye at limbus (3S: Superior, 3N: Nasal, 3I: Inferior, 3T: Temporal) and sclera (4S: Superior, 4N: Nasal, 4I: Inferior, 4T: Temporal) region at day 7 of one representative mouse from each group. C: Cornea, S: Sclera.

Figure 5.19: Comparison of Fluorescence intensity of liposomes of different charges at the limbus and sclera region over 7 days. * and ^ represents significant difference $p < 0.05$.

While the fluorescence signal for the liposomes was the highest at the temporal and superior region of the eye which was in close proximity to the injection site, the liposomes can be seen to be distributed to both the cornea and sclera at least up to 1 day post injection. This was probably due to clearance through blood circulation, diffusion mechanisms, convective fluid flow and lymphatic drainage in the eye. Liposomes could also have entered the cornea through the naso-lacrimal duct and blood circulation through the vessels in the choroid and conjunctiva could have transported the liposomes towards both the posterior segment of the eye (as seen in presence of liposome on opposite ends, nasal and inferior, from the site of injection at day 1) and the anterior segment of the eye before being cleared out through the systemic circulation. The liposomes could also have entered the intraocular tissues via the sclera through passive diffusion and been brought along towards the anterior segment of the eye through convective fluid flow along with the aqueous humor or fluid flow between the ocular tissues. This can be seen by the presence and the retention of the liposomes in the cornea periphery adjacent to the injection site/limbus from day 3 onwards (for neutral and negatively charged liposomes). Lymphatic clearance from the lymph vessels present in the conjunctiva and choroid could be responsible for the reduction in the liposomes observed over time.

The effect of the charge on the liposome distribution was evident from day 3 post injection. At Day 3, most of the negatively charged liposomes have moved away from the injection site, while neutral and positively charged liposomes can still be seen in the sclera region. By day 5, the liposomes have already migrated to their preferred locations,

as no further changes in the location of the liposomes were seen on day 7 and day 14. Neutral liposome and negatively charged liposomes settled at the limbus zone (away from the sclera) while positively charged liposomes remained at the sclera.

The disposition of positively charged liposomes at the sclera can be due to the presence of collagen fibrils in the sclera. The collagen in the sclera is associated with glycosaminoglycans (GAGs) which are highly negative in charge. The negatively charged environment of the sclera could have held on to the positively charged liposomes, resulting in higher retention of the positively charged liposomes on the sclera compared to the neutral and negatively charged liposomes. Both neutral and negatively charged liposomes are similar in their disposition. After day 3, they remained in the corneal periphery/limbus up till they were cleared or no longer be detected. The neutral liposomes remained longer on the sclera compared to the negatively-charged liposomes. This could be due to the repulsive interaction between the negatively-charged sclera and the negatively-charged liposome which drove the negatively-charged liposome away from the sclera. The fluorescein dye, being a small molecule, was no longer detectable 1 day post injection, indicating that it has been cleared out of the eye. This is in agreement with previous studies done in literature on small fluorescent probes

5.2 Effect of size of liposome

Table 5.2: Size of the liposomes

Size	Composition	Size (nm)	PDI
100 nm	95% POPC, 5% FI-DHPE	109±2	0.04-0.07
250 nm	95% POPC, 5% FI-DHPE	244±4	0.21-0.024
MLVs	95% POPC, 5% FI-DHPE	Micron size	

Neutral POPC liposomes of 3 sizes (100 nm, 250 nm and micron sized) were studied

for the effect of size on distribution of liposomes in the eye. The composition and size properties of the liposomes are listed in Table 5.2. 100 nm POPC liposomes were discussed in the previous section.

Similar to 100 nm liposomes (discussed in 5.1), at day 1, 250 nm liposomes can be seen at the cornea and the sclera side of the limbus in all the regions, even away from the injection site. This indicates the existence of clearance by blood circulation and diffusion between ocular tissues (from sclera to cornea through the limbus) (Figure 5.21). After day 3, there was still presence of 250 nm liposomes at the superior and temporal limbus while the liposomes at the nasal and inferior regions have been cleared. 250 nm liposomes could still be seen in the sclera though the majority of the liposomes in nasal and inferior regions have been cleared. By day 7, there was only faint fluorescence observed in the superior limbus and superior sclera regions, indicating that most of the liposomes have been cleared. Consistent significant drop in fluorescence values in sclera regions, superior (49%, 90%), inferior (39%, 93%) and temporal (56%, 79%) from day 1 to day 3 to day 7 could be observed.

Figure 5.21: Distribution of 250 nm POPC neutral liposomes at the limbus (3S: Superior, 3N: Nasal, 3I: Inferior, 3T: Temporal) and sclera (4S: Superior, 4N: Nasal, 4I: Inferior, 4T: Temporal) region of 1 representative mouse over 7 days. C: Cornea, S: Sclera.

Figure 5.22: Average Fluorescence intensity of positively charged liposomes at the limbus and sclera region over 7 days. * and ^ represents significant difference $p < 0.05$.

Figure 5.23: Distribution of MLV POPC neutral liposomes at the limbus (3S: Superior, 3N: Nasal, 3I: Inferior, 3T: Temporal) and sclera (4S: Superior, 4N: Nasal, 4I: Inferior, 4T: Temporal) region of 1 representative mouse over 7 days. C: Cornea, S: Sclera.

Figure 5.24: Average Fluorescence intensity of positively charged liposomes at the limbus and sclera region over 7 days. * and ^ represents significant difference $p < 0.05$.

MLV liposomes, being much bigger, can be observed in higher amounts in day 7 compared to smaller sized 100 nm and 250 nm liposomes. Unlike the smaller liposomes, the MLVs were observed mainly at the temporal, superior and inferior regions of the eye. They tend to stay at the sclera region and crowd at the limbus (Figure 4.22). This was due to the fact that they are too big to be mobile enough to be transported towards the nasal region of the eye as the injection was done at the temporal region of the eye. Their size also prevents them from diffusing through the tissues to the cornea side of the limbus. Although some fluorescence was still observed on the cornea side of the limbus, it was of a lesser extent compared to the smaller liposomes. This could be due to the presence of smaller sized vesicles in the MLVs injected. There was no significant reduction from day 1 to day 3 for superior, inferior and temporal sclera.

Figure 5.25: CellVizio images showing disposition of 100 nm, 250 nm and MLV POPC liposomes at limbus (3S: Superior, 3N: Nasal, 3I: Inferior, 3T: Temporal) and sclera (4S: Superior, 4N: Nasal, 4I: Inferior, 4T: Temporal) region at day 7 of one representative mouse from each group. C: Cornea, S: Sclera.

Figure 5.26: Comparison of Fluorescence intensity of liposomes of different sizes at the limbus region and sclera region over 7 days. * and ^ represents significant difference $p < 0.05$.

In conclusion, size does affect the distribution of liposomes in the eye. Smaller liposomes (100 nm and 250 nm) were able to move towards the limbus and reside there over time until clearance while micron sized liposomes were not able to move over to the limbus and stayed at the sclera region until fully cleared.

5.3 Effect of incorporation of cholesterol

Table 5.3: Size of the liposomes

Liposome	Composition	Size (nm)	PDI
POPC	95% POPC, 5% Fl-DHPE	109±2	0.04-0.07
POPC/Chol (60:40)	95% 60:40 POPC:Cholesterol, 5% Fl-DHPE	108±1	0.03-0.08

Incorporation of cholesterol in unsaturated liposomes have shown to influence the fluidity of the liposomes. Cholesterol molecules fill in the free space that was formed due to the kink in the chain of the unsaturated lipids and this decreases the flexibility of the surrounding lipid chains. In this section, the distribution of POPC/cholesterol (60:40) liposomes was studied (Table 5.3). As POPC/Cholesterol liposomes were of neutral charge, they did not have affinity to the negatively charged sclera. The liposomes tend to move towards the limbus and stay there until they were cleared. (Figure 5.3.1)

Figure 5.31: Distribution of POPC/Chol (60:40) liposomes (100 nm) at the limbus (3S: Superior, 3N: Nasal, 3I: Inferior, 3T: Temporal) and sclera (4S: Superior, 4N: Nasal, 4I: Inferior, 4T: Temporal) region of 1 representative mouse over 7 days. C: Cornea, S: Sclera.

Figure 5.32: Average Fluorescence intensity of POPC/Cholesterol liposomes at the limbus and sclera region over 7 days. * and ^ represents significant difference $p < 0.05$.

Significant reduction in fluorescence intensity could be seen in the sclera side from day 1 to 3 to 7 (51%, 79% for superior region and 35%, 45% for temporal region respectively). This was similar to what was observed in POPC liposomes. When compared to POPC liposomes without cholesterol, the only difference would be that higher amount of fluorescence was observed at day 7 for POPC/Cholesterol liposomes indicating that POPC/Cholesterol liposomes tend to persist longer in the eye. This can be supported with previous studies done in literature on the effect of cholesterol on liposomes clearance *in vivo*. The inclusion of cholesterol in liposomes have been an important factor for increasing liposomal stability, minimizing lipid-protein interactions and decreasing membrane permeability. Presence of cholesterol in the liposomal membrane reduces possible lipid exchange with other circulating structures (e.g., red blood cells and lipoproteins) which can cause the depletion of lipids and their replacement with less physiologically stable components. Integrating cholesterol into small (approximately 100 nm), neutral liposomes has been shown to prolong circulation. [1-3] It was also demonstrated that cholesterol-rich liposomes possess a lower affinity for uptake by hepatic Kupffer cells, suggesting that cholesterol inhibits opsonization of liposomes [4]. As POPC liposomes containing cholesterol were less likely to be taken

by the blood circulation, they were also cleared slower than POPC liposomes without cholesterol as seen in Figure 5.33 and 5.34.

Figure 5.33: Comparison of Fluorescence intensity of liposomes with and without cholesterol at the limbus region and sclera region over 7 days. * and ^ represents significant difference $p < 0.05$.

Figure 5.34: CellVizio images showing disposition of POPC and POPC/Chol (60:40) liposomes at **limbus** (3S: Superior, 3N: Nasal, 3I: Inferior, 3T: Temporal) and sclera (4S: Superior, 4N: Nasal, 4I: Inferior, 4T: Temporal) region at day 7 of one representative mouse from each group. C: Cornea, S: Sclera. All the liposomes are 100 nm in size.

5.4 Effect of chain saturation

Table 5.4: Size of the liposomes.

Liposome	Composition	Size (nm)	PDI	T _m
POPC	95% POPC, 5% FI-DHPE	109±2	0.04-0.07	-2 °C
DPPC	95% DPPC, 5% FI-DHPE	96±1	0.13-0.17	41 °C

To study the effect of chain saturation, DPPC liposomes (~100 nm) (Table 5.4) were injected into 5 mice and monitored over 7 days.

Figure 5.41: Distribution of DPPC liposomes (100 nm) at the limbus (3S: Superior, 3N: Nasal, 3I: Inferior, 3T: Temporal) and sclera (4S: Superior, 4N: Nasal, 4I: Inferior, 4T: Temporal) region of 1 representative mouse over 7 days. C: Cornea, S: Sclera.

From Figure 5.41, it can be seen that DPPC liposomes, like the neutral 100 nm liposomes that were studied in previous sections, tend to move away from the injection site towards the limbus over time. However, unlike the POPC liposomes, DPPC liposomes were observed at a much lower amount even at Day 1.

In addition, unlike the previous experiments, the observation was not consistent in all 5 mice (Figure 5.42). Other than the liposomes being detected in the limbus for 2 mice, the DPPC liposomes can barely be observed in the limbus region for the other 3 mice. The liposomes detected near the injection site (sclera superior and temporal) were also low, indicating that clearance of DPPC liposomes was much faster than POPC

liposomes. This could be due to saturated lipids having higher interaction with proteins which resulted them to be cleared faster compared to unsaturated lipids. [5, 6] Other than faster uptake into the blood circulation, there could also be a possibility that the DPPC liposomes could have went in deeper into the eye to the extent that the probe was unable to detect or reach.

Figure 5.42: CellVizio images showing disposition of DPPC the limbus (3S: Superior, 3T: Temporal) and sclera (4S: Superior, 4T: Temporal) region at over time for 5 mice. All the liposomes are 100 nm in size.

Figure 5.43: CellVizio images showing disposition of POPC and DPPC at the limbus (3S: Superior, 3N: Nasal, 3I: Inferior, 3T: Temporal) and sclera (4S: Superior, 4N: Nasal, 4I: Inferior, 4T: Temporal) region at day 7 of one representative mouse from each group. C: Cornea, S: Sclera. All the liposomes are 100 nm in size.

5.5 Natural Lipid versus synthetic lipid

POPC has most commonly been used as a synthetic alternative for EggPC. [7] However, as both the synthetic lipid and natural lipid differ in composition, the distribution of EggPC liposomes was studied in the eye.

100nm EggPC Liposomes

Figure 5.51: Distribution of EggPC liposomes (100 nm) at the limbus (3S: Superior, 3T: Temporal) and sclera (4S: Superior, 4T: Temporal) region of 1 representative mouse over 14 days. C: Cornea, S: Sclera.

The distribution of EggPC liposomes can be seen in Figure 5.51. Unlike POPC liposomes, the EggPC liposomes resided in the eye for more than 14 days. EggPC liposomes were initially observed in both the limbus and sclera region but were found to stay at the sclera region over time. As the distribution and residence time was very different from POPC liposomes, further study was done on other constituents of EggPC to find out the contributing factor. Table 5.51 and 5.52 lists the main components of EggPC and the properties of the liposomes that were studied.

Table 5.51: List of main components of EggPC

EggPC constituents	
1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phosphocholine (POPC) 16:0-18:1 PC	36.57%
palmitoyl-2-linoleoyl- <i>sn</i> -glycero-3-phosphocholine (PLPC) 16:0-18:2 PC	27.74%
1-Stearoyl-2-linoleoyl- <i>sn</i> -glycero-3-phosphatidylcholine (SLPC) 18:0-18:2 PC	7.34%

Table 5.52: Size of the liposomes prepared

Liposome	Lipid Structure	Composition	Size (nm)	PDI
EggPC	Contains ~37% POPC, 28% PLPC, 7% SLPC and other PC lipids	95% EggPC, 5% FI-DHPE	98±1	0.05-0.10
POPC (16:0-18:1 PC)		95% POPC, 5% FI-DHPE	109±2	0.04-0.07
PLPC (16:0-18:2 PC)		95% POPC, 5% FI-DHPE	98±1	0.15-0.19
SLPC (18:0-18:2 PC)		95% POPC, 5% FI-DHPE	95±2	0.05-0.08

Figure 5.52: CellVizio images showing disposition of EggPC liposomes and its main constituents, POPC, PLPC and SLPC liposomes at the limbus (3S: Superior 3T: Temporal) and sclera (4S: Superior, 4T: Temporal) region at day 7 of one representative mouse from each group. C: Cornea, S: Sclera. All the liposomes are neutral and 100 nm in size.

Two constituents of EggPC, PLPC (16:0-18:2 PC) and SLPC (18:0-18:2 PC) were studied for their distribution in the eye. In comparison of the images taken at day 7, SLPC liposomes were found to move over to the limbus, while still retaining some of the liposomes in the sclera. PLPC liposomes were observed more at the sclera region, similar to that observed in EggPC, indicating that it could be the lipid that is giving EggPC its unique distribution. PLPC differs to POPC by an additional double bond in the chain. This could have contributed to the difference in distribution and residence time in the eye. As discussed in section 4.4, unsaturated lipids have lower interaction with proteins which resulted them to be cleared slower compared to saturated lipids. [5, 6] As POPC has a lower degree of unsaturation compared to PLPC, it is probably also more likely to have higher interaction with blood plasma proteins resulting in a faster clearance than PLPC.

5.6 Conclusion

Effect of charge, size, cholesterol and chain saturation were studied on the distributed of liposomes in the eye. The main factors that affect the location at which liposomes were distributed to were charge and size. Due to the negatively charged sclera, positive charged liposomes tend to stick to the sclera until they were fully cleared. Both neutral and negatively charged liposomes do not have affinity for the negatively charged sclera so they tend to move away from the sclera and reside in the limbus region.

Size of the liposomes affected their mobility in the subconjunctival space. Despite being neutral in charge, MLVs were too big to move away from the injected site and stayed at the sclera region until they were cleared.

Liposomes with cholesterol were cleared at a slower rate compared to liposomes without cholesterol. This could be attributed to cholesterol providing stability to the lipid bilayers thus reducing protein interactions which in turn reduce uptake and clearance by the circulation. As observed from DPPC liposomes, chain saturation in lipids increased protein-lipid interactions and results in faster clearance of liposomes from the eye.

It was also observed that the distribution of EggPC liposomes was very different from POPC liposomes. EggPC liposomes were found to be cleared at a much slower rate than POPC liposomes and they tend to stay around at the sclera region over time. This was found to be attributed by PLPC, another constituent of EggPC.

References

- [1] Sercombe, L., et al., *Advances and Challenges of Liposome Assisted Drug Delivery*. Frontiers in Pharmacology, 2015. **6**(286).
- [2] Geng, S., et al., *Two cholesterol derivative-based PEGylated liposomes as drug delivery system, study on pharmacokinetics and drug delivery to retina*. Nanotechnology, 2014. **25**(27): p. 275103.
- [3] Foteini, P., et al., *Physicochemical study of the protein–liposome interactions: influence of liposome composition and concentration on protein binding*. Journal of Liposome Research, 2018: p. 1-9.
- [4] Semple, S.C., A. Chonn, and P.R. Cullis, *Influence of Cholesterol on the Association of Plasma Proteins with Liposomes*. Biochemistry, 1996. **35**(8): p. 2521-2525.
- [5] Semple, S.C., A. Chonn, and P.R. Cullis, *Interactions of liposomes and lipid-based carrier systems with blood proteins: Relation to clearance behaviour in vivo*. Advanced Drug Delivery Reviews, 1998. **32**(1): p. 3-17.
- [6] Senior, J.H., K.R. Trimble, and R. Maskiewicz, *Interaction of positively-charged liposomes with blood: implications for their application in vivo*. Biochimica et Biophysica Acta (BBA) - Biomembranes, 1991. **1070**(1): p. 173-179.
- [7] van Hoogevest, P. and A. Wendel, *The use of natural and synthetic phospholipids as pharmaceutical excipients*. European Journal of Lipid Science and Technology, 2014. **116**(9): p. 1088-1107.

Chapter 6

Quantitative Study on fate of liposomes in the Eye

This chapter describes the quantitative study of effect of various liposomal properties on the distribution of liposomes in the eye after subconjunctival injection in the mice. Based on the outcome of the qualitative studies done in Chapter 5, a few types of liposomes were chosen to study factors like charge (POPC/DOTAP liposome), size (POPC MLVs) and effect of chain saturation (DPPC liposomes). Liposomes that contained radioactively labelled lipids were injected into the subconjunctival space of the eye and studied for 6, 24, 72 and 168 hours. The amount of radioactivity in the ocular tissues and various organs were quantified and discussed in detail. It was observed that the main factors that can control the clearance of the liposomes from the eye was charge and size. Positively charge liposomes and MLVs were observed at higher quantities, particularly in the conjunctiva and sclera. By being able to quantitate the actual amount of liposomes in the ocular tissues, the data obtained in this chapter complements the results from the imaging studies in chapter 5.

6.1 Neutral POPC liposomes

Figure 6.1: Distribution of 100 nm POPC liposomes after subconjunctival injection into the mice eye. (* $p < 0.05$ in comparison to previous timepoint)

It can be seen from the graphs in Figure 6.1-6.4 that all liposomes were found in highest quantity in the conjunctiva and sclera at all time points. For 100 nm POPC liposomes, significant differences ($p < 0.05$) in drop of radioactive levels were observed in most ocular tissues for the duration of the study.

POPC liposomes were detected in the vitreous humor at 6 hours, indicating that some of the injected liposomes were able to pass through the sclera, RPE and Retina to reach the vitreous humor. This is also evident in the radioactivity detected in the sclera and RPE and retina.

In non-ocular tissues, POPC liposomes were detected in the liver, lymph nodes, spleen and kidneys. At 6 hours, $15.8 \pm 3.6\%$ of injected dose of liposomes was detected in the liver, followed by lower amounts in cervical lymph node, spleen, kidneys, axillary and inguinal lymph nodes. This indicates an uptake of the liposomes by the RES and the lymphatic system, with the liver uptake being the main mode of clearance. By 120 hours, the liposomes in the liver was significantly reduced, indicating that they have left the body by excretion.

6.2 Positively charged liposomes (POPC/DOTAP)

As positively charged liposomes were seen to have significant difference in distribution in the eye compared to neutral liposomes in chapter 5, positively charged liposomes were studied in detail quantitatively.

Figure 6.2: Distribution of 100 nm POPC/DOTAP (positively charged) liposomes after subconjunctival injection into the mice eye. (* $p < 0.05$ in comparison to previous timepoint)

As seen in Figure 6.2, positively charged liposomes were observed to be in highest quantity in the conjunctiva ($12.6 \pm 2.3\%$ injected dose) followed by the sclera ($1.1 \pm 0.3\%$ injected dose) at 24 h post injection. This relates to what was observed in the imaging studies in chapter 5 where liposomes were found sticking on the sclera region until clearance. When conducting the imaging studies, there wasn't a way to differentiate how much of the liposomes were on conjunctiva and the sclera but it was evident from the quantitative studies that there was indeed a substantial amount that was taken up to the conjunctiva. Liposomes on the conjunctiva are more susceptible to clearance by the circulation as the conjunctiva is highly vascularized. This can also be observed by the drop in the quantity of liposomes detected in the conjunctiva over time. The low quantity of liposomes detected in the retina, RPE and vitreous humor indicated that only minimal amount of the positively charged liposomes were able to have made their way through the sclera into the inner layers of the eye. This is due to the liposome being attracted to negatively charged sclera so instead of moving through the sclera, they tend to stick on the sclera. Another reason could also be that, since positively charged particles are also known to have higher binding to blood plasma protein, the liposomes which do manage to go through the sclera despite the attractive forces, would have been cleared by the choroidal circulation.

POPC/DOTAP liposomes were also taken up by the RES as seen by the values in the liver and spleen. This was also due to their high protein binding potential. Up to 120h, the radioactivity detected was the highest in the liver followed by lymph nodes, kidneys and spleen. By 168h, the radioactivity detected in the liver was lowest compared to other organs, indicating that the liposomes have probably left the body through excretion.

6.3 Neutral POPC MLVs

Figure 6.3: Distribution of POPC MLVs (micron size) after subconjunctival injection into the mice eye. (* $p < 0.05$ in comparison to previous timepoint)

Neutral MLVs were observed in high levels in the conjunctiva and sclera at all time points studied. As size is a factor in sclera diffusion, it would be a challenge for MLVs to be diffused into the sclera. However, there was still radioactivity detected in the RPE and Vitreous humor, indicating that there could either be presence of smaller sized liposomes in the MLVs or the MLVs could have degraded after injection. Liposomes were found to be highest in the liver at 24 h followed by lymph nodes, spleen and kidneys, indicating that they were eventually taken up the RES and cleared through excretion.

6.4 DPPC liposomes

Figure 6.4: Distribution of 100 nm DPPC liposomes after subconjunctival injection into the mice eye. (* $p < 0.05$ in comparison to previous timepoint)

From the longitudinal imaging studies in Chapter 5, it was observed that the clearance of DPPC liposomes was much higher than other POPC liposomes studied. To understand this phenomenon, radioactively labeled DPPC liposomes were injected into the subconjunctival space mice eyes to quantify the amount of liposomes present in ocular tissue and various tissues at 6, 24, 72 and 120 hours. It can be seen in Figure 6.4 that despite the low amount observed in imaging studies, DPPC liposomes were able to be quantified in the ocular tissue. For all the time points, DPPC liposomes were found to be in highest quantity in conjunctiva, followed by sclera, cornea, RPE and vitreous humor. It could be possible that the liposome went further in to the layers of the sclera and were beyond the depth that could be captured by the imaging probe. The liposomes could also have gone further to the back of the eye in the subconjunctival space (which is also a spot which cannot be reached by the probe). As observed by liposomes of the same size, DPPC liposomes were also detected mostly in the liver, lymph nodes and kidney and spleen. The lack of values for liver at 6 h was due to a technical issue and not that it was not detectable. DPPC liposomes were found in highest quantity in the liver followed by kidneys and lymph nodes and spleen at all the timepoints.

6.5 EggPC liposomes

The imaging studies done in chapter 5 showed that there was a big difference in the distribution and residence duration of EggPC liposomes compared to POPC liposomes. Two attempts were made to study the distribution of EggPC liposomes but it was unsuccessful due to challenges in radiolabeling EggPC lipids. This was due to EggPC constituting of various different types of phosphatidylcholine. Radioactive EggPC liposomes were initially prepared by requesting for custom labeling of the EggPC lipids from the same company that provided the radioactive lipids. However, the results achieved from the distribution studies were almost the same as that for POPC lipids. The final constituents of EggPC lipid might have been changed during the labelling and purification process. Another attempt to prepare radiolabeled EggPC liposomes was done by mixing EggPC lipids with radiolabeled POPC lipids (as POPC is a constituent of EggPC) to ensure that EggPC lipids constituents were not lost during the fabrication. However, the distribution profile was still the same as that for POPC lipids, indicating that the radiolabeled POPC lipids might have formed liposomes on their own.

6.6 Effect of liposomal properties on the distribution

The radioactivity of the individual tissues or organs were compared against each other in order to conclude on how various factors affect the distribution of liposomes in the eye. The comparisons can be found in Figure 6.61-6.63.

Figure 6.6.1: Comparison of quantity of liposomes detected in ocular tissues for 100 nm POPC, 100 nm POPC/DOTAP, POPC MLV and 100 nm DPPC liposomes after subconjunctival injection into the mice eye. (* $p < 0.05$ in comparison to POPC liposomes).

For the ocular tissues, it can be seen that positively charged liposomes and MLVs were observed at higher quantities for all the major ocular tissues such as conjunctiva, cornea, RPE and vitreous humor. As the imaging studies were only able to capture the liposomes nearer to the surface of the eye, much more information was achieved from the quantitative studies. Higher quantity of POPC/DOTAP and POPC MLV liposomes in the conjunctiva and sclera were expected as both liposomes were found to stay at the sclera region during the imaging studies. This is due to the sclera being more negatively charged for POPC/DOTAP liposomes and lack of mobility to other tissues for MLVs. Although the amount of POPC/DOTAP and MLV liposomes were significantly higher in the cornea, RPE, retina and vitreous humor compared to POPC liposomes, the amounts were much lower than those detected in sclera and conjunctiva. This could probably be due a higher quantity of POPC/DOTAP and MLV liposomes acting as depot in the subconjunctival space. A small quantity of liposomes could still have been transported to these tissues due to fluid flow (for cornea) and permeation through sclera (for retina, RPE and vitreous humor) despite the positive charge (for POPC/DOTAP). As for MLVs, the liposomes could either have degraded or smaller sized vesicles that were formed during the synthesis could have passed through the sclera. There is also possible of the liposomes being transported by the tears to the cornea through the nasolacrimal route. Positively charged liposomes could be retained at a higher amount on the cornea due to the attraction to the negatively charged cornea.

For DPPC, it can be seen that there was no significant difference in the radioactivity detected in all ocular tissues at all timepoints in comparison to POPC liposomes (except for retina at 120h). Significant higher values were detected for DPPC liposomes at 120h in the retina compared to POPC liposomes. This could imply that DPPC liposomes were more permeable through the sclera compared to POPC.

Figure 6.6.2: Comparison of quantity of liposomes detected in lymphatic system for 100 nm POPC, 100 nm POPC/DOTAP, POPC MLV and 100 nm DPPC liposomes after subconjunctival injection into the mice eye. (* $p < 0.05$ in comparison to POPC liposomes).

Figure 6.6.3: Comparison of quantity of liposomes detected in the blood and excretory organs for 100 nm POPC, 100 nm POPC/DOTAP, POPC MLV and 100 nm DPPC liposomes after subconjunctival injection into the mice eye. (* $p < 0.05$ in comparison to POPC liposomes).

In terms of clearance from the eye, POPC/DOTAP liposomes were observed at significant higher quantity at 24 h compared to other liposomes. Positively charged liposomes were expected to be cleared faster through blood circulation due to their higher binding affinity with plasma proteins. This lag in clearance could be due to the fact that the liposomes were stuck to the sclera of the eye and not taken up to the blood circulation. Another finding was that DPPC liposomes were observed in higher amounts in lymphatic tissues (especially axillary lymph nodes, inguinal lymph nodes and spleen) and kidneys indicating that the DPPC liposomes were mainly cleared by lymphatic clearance and blood circulation. This is with agreement with previous findings in literature that saturated lipids tend to be cleared faster due to their interactions with proteins *in vivo*.

6.7 Conclusion

From the quantitative distribution studies, it can be concluded that the main factors that affect ocular distribution are charge and size. All liposomes were found mostly in the conjunctiva and sclera after injection into the eye. The amount of positively charged liposomes and MLVs found in the eye were much higher than POPC and DPPC liposomes. This is in agreement with the findings from the imaging studies. Liposomes were also able to be observed in the RPE, retina and vitreous humor, indicating that they were able to pass through the sclera towards the inner layers. The liposomes were eventually cleared through the systemic blood circulation and lymphatic drainage with DPPC liposomes being detected at higher amounts in the lymphatic tissues and kidneys. This could be due to the higher binding affinity that saturated lipids have for proteins.

It can be concluded that positively charged liposomes and MLVs can act as depot for drug to be delivered to both the posterior segment and anterior segments of the eye. This can be considered when designing drug delivery systems to the eye.

Chapter 7

Discussion, conclusion and future work

This chapter covers a discussion of the results achieved in this research with a conclusion of this work. Through the studies done in this work, it could be established that the ocular distribution of liposomes after subconjunctival injection is affected by charge and size of the liposomes. Limitations of this work will be discussed and future work that could answer or solve these limitations will be explored.

7.1 Discussion and conclusion

Due to its ability to utilize the trans-scleral route, there has been much interest in administration of ocular drugs through sub-conjunctival injection. Sustained therapeutic effect for glaucoma medications such as Latanoprost [1-5] and Brimonidine Tartrate [6] have been achieved through sub-conjunctival administration. However, the fate of the drug delivery systems after sub-conjunctival injection is not clearly known. To have a therapeutic effect, sub-conjunctivally administered particles could have either entered the intraocular tissues through the sclera or through intraocular circulation or been retained at the site of administration to slowly release the drug until the drug delivery system (DDS) degrades [7, 8]. In order to achieve and maintain therapeutic drug concentrations in the eye, it is important to know the *in vivo* fate of the DDS in the eye after administration.

Amrite and Kompella et al. observed that micro-particle (1.5 μm) and ~ 200 nm (actual zeta size: 205-290 nm) particles remained at the injection site for up to 60 days while only 15% of 20 nm (actual zeta size: 25-37 nm) nanoparticles were found on day 1 with no detectable traces of it on day 60. [7, 8]. In a study by Moon et al. on liposome-bound low-molecular-weight heparin (LMWH) (~ 546 nm, positively charged) after sub-conjunctival injection in rabbits, it was believed that liposomes acted as a depot which resulted in a sustained release of LMWH up to 5 days [9]. An investigation by Feng et al. on sub-conjunctivally injected pRNA nanoparticles showed that pRNA were observed to be distributed to various ocular tissues (conjunctiva, cornea, retina, and sclera) before rapidly clearing from the eye. The presence of the nanoparticles in the cervical lymph nodes indicated clearance from lymph circulation. Similarly, in a MRI study done on subconjunctival injection of model drugs Gd-DTPA (<1 nm) and Gd-Albumin (4 nm), the drug solutions acted as depots in the subconjunctival space immediately after injection before being undetectable about 3 to 4 hours post-injection. Comparisons made with post mortem experiments identified that *in vivo* clearance mechanisms such as blood circulation and lymphatic drainage were in play [10].

Magnevist, a model hydrophilic probe, was found to be present in the ciliary body and anterior chamber within 20 minutes after sub-conjunctival injection in rabbit eyes by using MRI. Li, Hao et al. suggested that this presence of the probe at the ciliary body

away from the injection site suggested the influence of blood circulation on the delivery to the anterior segment of the eye. Presence of the probe observed near the periocular fat also suggested transport through circulation around the eye. Instead of diffusing to other ocular tissues away from the globe, the contrast agent was found to have diffused into the retina and to the vitreous in postmortem experiments, indicating the importance of active elimination mechanism when trans-passing the sclera [11].

It can be concluded from findings in literature that carriers/molecules of sizes smaller than 40 nm are rapidly cleared from the injection site while carriers above 200 nm in size tend to be retained at the site of injection. Particles injected in the subconjunctival space could be delivered to the anterior segment of the eye through ocular blood circulation the ciliary body and subsequently to the posterior and anterior chambers through fluid flow from the subconjunctival depot. There could also be possibly of the liposomes being transported to the cornea by the tears through the naso-lacrimal route. On the other hand, passive diffusion could be the main mechanism for delivery through the sclera to the RPE, retina and vitreous humor.

As a complete comparison of effect of properties other than size has not been done, a more robust and comprehensive study is required to understand the distribution of nano-carriers in the eye. Furthermore, a comprehensive study the effect various liposomal properties have on ocular distribution after sub-conjunctival injection was also not conducted before.

In this work, effect of various liposomal properties was studied on their distribution after subconjunctival injection. The initial part of the study comprised of qualitative studies to give an insight on how various factors affect the distribution. It was done by using Cellvizio® imaging system, a confocal laser imaging system, designed for *in vivo* visual inspection of tissues at the microscopic level. As the imaging system has not been used for ocular imaging, optimization of the imaging protocol was conducted to choose the right dye to identify landmarks in the eye (such as vasculature which could differentiate cornea region from the sclera region). A pilot study was done to ensure the feasibility of using this imaging system for the study. With improvements to the imaging protocol, longitudinal ocular distribution studies were conducted using the confocal imaging system for the first time. This imaging technique allowed the tracking

of liposomes in the eye without sacrificing the animal. This also eliminates the loss of liposomes detected due to histological preparation techniques and drop in fluorescence intensity during the preparation step to image the histology slides. The limit of this imaging system was that it could only be able to image the surface of the eye with depth of up to a few microns.

With the optimized imaging protocol, liposomal properties such as charge (neutral, positive and negative), size (100 nm, 250 nm, micron-size), incorporation of cholesterol, chain saturation (DPPC) and origin of the lipid (natural versus synthetic) were studied on their influence on the distribution of the liposomes after subconjunctival injection into the eye. It was seen that while liposomes can be seen both in the limbus and sclera region on day 1, the liposomes have moved to their most preferred locations by day 3 and resided there till they were cleared. Positively charged liposomes were observed to have an affinity to the sclera region due to their attraction to the negatively charged sclera. Neutral and negatively charged liposomes have no affinity to the sclera so they ended up moving away towards the limbus and stayed at the limbus region till clearance. 250 nm neutral liposomes were distributed in the similar way as 100 nm liposomes. Micron sized liposome were seen to be residing in the sclera region due to their inability to move around. Incorporation of cholesterol did not affect the distribution profile of the liposomes but affected the clearance of the liposomes. Higher levels of fluorescence were detected for POPC/cholesterol liposomes at day 7 compared to POPC liposomes. The inclusion of cholesterol increased the stability of the liposomes and minimize lipid-protein interactions which in turn reduced their clearance via blood circulation. Saturated liposomes (DPPC) were observed to be cleared faster in the eye compared to POPC liposomes. The distribution of EggPC liposomes was significantly different from POPC liposomes. EggPC liposomes were found to reside in the sclera region and were still detected in abundance at day 14 post injection. It was found that PLPC, another constituent of EggPC, has the same distribution profile and could have contributed to the distribution of EggPC liposomes. As POPC is often used as the synthetic alternative for EggPC, such difference must be taken into consideration when substituting the lipids for each other. Other than giving an insight to distribution of liposomes in eye, the imaging study also provided information on the time frame to focus on for more in depth quantitative studies.

From the outcomes of the qualitative study, POPC, POPC/DOTAP, POPC MLVs, DPPC and EggPC liposomes were chosen to be studied in depth using radiolabeled lipids. With radioactive liposomes prepared by mixing the lipids with their radiolabeled analogues, the distribution of liposomes was quantified by measuring the radioactivity in the ocular tissues. For POPC, POPC/DOTAP, POPC MLVs and DPPC liposomes, the results from the quantitative study were in agreement with results achieved from the qualitative study. Higher amount of liposomes were detected in POPC/DOTAP and POPC MLVs in the eye, mainly in conjunctiva ($12.6 \pm 2.3\%$ for POPC/DOTAP, $11.14 \pm 3.07\%$ for MLVs), and sclera ($1.1 \pm 0.3\%$ for POPC//DOTAP, $2.45 \pm 1.67\%$ for MLVs) at 24 h, compared to $0.83 \pm 0.30\%$ in conjunctiva and $0.34 \pm 0.04\%$ in sclera for POPC liposomes. Radioactivity measured at 168 h after injection of the POPC/DOTAP and POPC MLVs were similar to the levels measured for POPC liposomes at 24 h, indicating that these liposomes acted as a depot in the injection site. Liposomes were also detected in the cornea suggesting that they could have been taken up through ocular blood circulation to the ciliary body and through fluid flow towards the anterior segment. The liposomes could also have been transported to the cornea through tears as it would have when applied as topical eye drops. Positively charged liposomes could be retained at a higher amount on the cornea due to the attraction to the negatively charged cornea. Presence of liposomes in RPE and retina and vitreous humor (though minimal) indicated that a very minimal portion of the liposomes were indeed able to pass through the sclera by passive diffusion and also escape the uptake by choroidal circulation. Radioactivity measured in the liver, kidneys, lymph nodes and spleen indicated that blood circulation and lymphatic drainage were the main routes of clearance from the eye. Radioactivity measurements for DPPC liposomes also showed that their distribution did not differ much from the POPC liposomes. The quantity of liposomes detected in the ocular tissues were almost similar or slightly higher than that of POPC liposomes. As they were not observed during the imaging studies which was limited to the surface of the eye, it can be concluded that DPPC liposomes tend to be able to move to deeper layers of the tissue. DPPC liposomes were also observed to be taken up by the lymphatic system, liver and kidneys in higher amounts compared to POPC liposomes. This could be attributed to the higher binding affinity of saturated lipid to proteins *in vivo*. Due to challenges in radiolabeling EggPC liposomes without losing their original composition, the quantitative distribution of EggPC liposomes could not be studied.

It can be concluded that the main factors that affect the distribution of liposomes in the eye are charge and size of the liposomes. 100 nm positively charged liposomes and micron sized neutral liposomes can act as a depot in the injection site and be cleared from the eye at a slower rate compared to 100 nm neutral liposomes. This can be a crucial factor to consider when designing drug delivery system for subconjunctival injection.

7.2 Future work

Although the work in the thesis was conducted with the best ability possible, there were still a few unanswered questions and limitations in the studies done:

- 1) Like most studies done on liposomes, it cannot be fully determined whether the liposomes that were detected were of liposomes in a complete vesicle or if the liposomes have degraded and the detected amounts were of the free lipids.
- 2) The imaging studies provided insight to where the liposomes were distributed and quantification studies did provide a breakdown of the liposome distribution in the ocular tissues. However, the depth of permeation of the liposomes into the sclera or cornea could not be identified.
- 3) As the study was done in mice, due to the size of the mouse eye, a few other important structures of the eye could not be extracted. These include, the aqueous humor and ciliary body. From the imaging studies done, it can be seen that neutral 100 nm liposomes tend to reside at the limbus region over time. It is possible that they could be residing in the ciliary body of the eye. Similar studies can be conducted in rabbits where it would be possible to separate the ciliary body for quantification. Furthermore, in terms of size and structure, distribution in the rabbit eye would be more relatable to the human eye.
- 4) Other than following the fate of the liposomes in the eye, it would be meaningful and beneficial to study both the fate of encapsulated drug and the liposomes in the eye.

Therefore, the following studies were proposed:

- 1) Similar study of distribution of liposomes in bigger animals e.g. rabbits or monkeys
- 2) Study of integrity of liposomes *in vivo*

- 3) Study of fate of liposomes and drugs in the eye by using both a radioactive counter and radioactive imaging

7.2.1 Study the integrity of liposomes *in vivo*

Although the liposomes can be quantified through levels of radioactivity, it cannot be confirmed if what was measured was of intact liposomes or of lipids that have left the liposomes upon degradation. Studies can be done to test the integrity of liposomes *in vivo*. Thin layer chromatography can be employed to differentiate lipids from liposomes.

Initial experiments would require optimization of TLC methods to differentiate lipids from liposomes. The optimized method can be used to quantify the amount of liposomes and free lipids *in vivo*. However, it must be noted that this study can only be done for blood samples. For tissue samples, any process done to extract the liposomes (either physical homogenization or solubilization of tissue as used in this work) could destroy the liposomes and intact liposomes will not be detected.

7.2.2 Study of fate of liposomes and drugs

As this work done only focused on distribution of liposomes, it would also be important to study the fate of both the liposomes and loaded drugs in the eye. As MLVs and positively charged liposomes have been shown to act as a depot in the injected site, it would be interesting to know how these depot systems affect the release of the drug into the ocular layers.

Joseph et al had previously demonstrated through *ex vivo* porcine sclera transport experiments that positive charged liposomes localized on the episcleral region while negatively charged liposomes and neutral liposomes were observed to have a certain extent of scleral diffusion in the *ex vivo* condition. While the liposomes did not permeate fully across the sclera, the drug that was encapsulated was able to be released and diffused across the sclera. It was also observed that charged liposomes slowed down the release of liposomes through the sclera. [12]

With this, it would be beneficial to be able to detect and image the liposomes and the

drug released in the eye after subconjunctival injection. Radioactive liposomal formulations can be prepared by radiolabeling the liposomes and drug with different radioactive ligands (For example, ^{14}C for lipids and ^1H or ^{125}I for drugs/proteins). The liposomal formulation can be injected in the animal eyes and detected for radioactivity after the animals are sacrificed. Radioactivity levels can be quantified using the liquid scintillation counter or gamma counter depending on the radioactive ligand used. In addition, the eyes can also be harvested to be imaged using Digital Autoradiography (DAR). DAR is a powerful method to image and determine quantitatively (sub-mm scale) the distribution of a radiotracer within a tissue section. However, the spatial resolution of the DAR image is limited due to blurring [13]. Although it might not be useful for quantification, it can be used to image the eye sections to give an idea of where the liposomes and drugs are located. Two formulations, POPC/Latanoprost Formulation and POPC/Lucentis Formulation, that have been optimized by our group can be studied. Histology sections can also be further analyzed by immuno-staining to study if liposomes were taken up by specific cells/ macrophages or leucocytes.

Proposed study plan:

- Liposomal drug formulations can be synthesized with ^{14}C radiolabeled lipid and $^1\text{H}/^{125}\text{I}$ radiolabeled drug (^1H for Latanoprost, ^{125}I for Lucentis)
- Formulations are to be injected into mice/rabbit eyes. Animals (n=5) will be sacrificed at various timepoints (3h, 6h, 24h, 72h, 120h, 168h) to quantify the radioactivity left in the ocular tissues using Scintillation Counter/Gamma Counter. (Scintillation counter for ^{14}C and ^1H and Gamma Counter for ^{125}I)
- Animals (n=5) will be sacrificed at various timepoints (3h, 6h, 24h, 72h, 120h, 168h) to obtain samples for DAR imaging. Eyes will be enucleated and fixed in formalin for at least 12 hours. The fixed eyes will be trimmed and embedded in OCT and kept frozen in -80°C . The frozen blocks containing the eye balls will be sectioned. Images of the histology sections of the eyes can be taken using both a bright field microscope and DAR. The images can be merged to identify the location of the liposomes and drugs in the eye.

This study could also be extended to disease models - Glaucoma for Latanoprost and age-related macular degeneration (AMD) for Lucentis, as it would be more meaningful to study the kinetics of the liposome and drugs in actual disease setting.

For Glaucoma, ocular hypertensive nonhuman primates [2] can be used for the study. Intraocular Pressure (IOP) levels which are indicative of efficiency of the drugs will be measured and compared with the liposome and drug kinetics to observe the interplay of the drug and recovery.

For AMD, the disease model can be achieved by either 1) laser induced choroidal neovascularization [14] or 2) suprachoroidal implantation of a non-biodegradable 1 mm Hydron pellet to provide sustained release of both VEGF and bFGF [15]. The extent of neovascularization can be measured by fluorescence microscopy of flat mounts of retina extracted from the eyes. A reduction in neovascularization indicates the efficiency of the formulation. As the measurement requires sacrifice of the animals, the study should start from small animals like rats before moving to bigger animal models.

References

- [1] Wong, T.T., et al., Nanomedicine for glaucoma: sustained release latanoprost offers a new therapeutic option with substantial benefits over eyedrops. *Drug Delivery and Translational Research*, 2014. **4**(4): p. 303-309.
- [2] Natarajan, J.V., et al., Sustained Drug Release in Nanomedicine: A Long-Acting Nanocarrier-Based Formulation for Glaucoma. *ACS Nano*, 2014. **8**(1): p. 419-429.
- [3] Natarajan, J.V., et al., Nanomedicine for glaucoma: liposomes provide sustained release of latanoprost in the eye. *International Journal of Nanomedicine*, 2012. **7**: p. 123-131.
- [4] Giarmoukakis, A., et al., Biodegradable nanoparticles for controlled subconjunctival delivery of latanoprost acid: In vitro and in vivo evaluation. Preliminary results. *Experimental Eye Research*, 2013. **112**: p. 29-36.
- [5] Voss, K., et al., Development of a novel injectable drug delivery system for subconjunctival glaucoma treatment. *Journal of Controlled Release*, 2015. **214**: p. 1-11.
- [6] Fedorchak, M.V., et al., 28-day intraocular pressure reduction with a single dose of brimonidine tartrate-loaded microspheres. *Experimental Eye Research*, 2014. **125**:

p. 210-216.

- [7] Amrite, A.C. and U.B. Kompella, Size-dependent disposition of nanoparticles and microparticles following subconjunctival administration. *Journal of Pharmacy and Pharmacology*, 2005. **57**(12): p. 1555-1563.
- [8] Bourges, J.-L., et al., Ocular Drug Delivery Targeting the Retina and Retinal Pigment Epithelium Using Polylactide Nanoparticles. *Investigative Ophthalmology & Visual Science*, 2003. **44**(8): p. 3562-3569.
- [9] Moon, J.-W., et al., Effect of Subconjunctivally Injected, Liposome-Bound, Low-Molecular-Weight Heparin on the Absorption Rate of Subconjunctival Hemorrhage in Rabbits. *Investigative Ophthalmology & Visual Science*, 2006. **47**(9): p. 3968-3974.
- [10] Kim, S.H., et al., Drug Elimination Kinetics Following Subconjunctival Injection Using Dynamic Contrast-Enhanced Magnetic Resonance Imaging. *Pharmaceutical Research*, 2008. **25**(3): p. 512-520.
- [11] Li, S.K., et al., MRI study of subconjunctival and intravitreal injections. *Journal of Pharmaceutical Sciences*, 2012. **101**(7): p. 2353-2363.
- [12] Joseph, R.R., et al., Characterization of liposomal carriers for the trans-scleral transport of Ranibizumab. *Scientific Reports*, 2017. **7**(1): p. 16803.
- [13] Zhang, M., et al., Image deconvolution in digital autoradiography: A preliminary study. *Medical Physics*, 2008. **35**(2): p. 522-530.
- [14] Pennesi, M.E., M. Neuringer, and R.J. Courtney, Animal models of age related macular degeneration. *Molecular aspects of medicine*, 2012. **33**(4): p. 487-509.
- [15] Wong, C.G., et al., Comparison of Lucentis and Avastin in the Suprachoroidal VEGF/bFGF-Implant Rabbit CNV Model Provides a Potential Standardized Pre-Clinical Approach in the Rational Development of More Effective QSAR-Based Drugs, Sustained Release Formulations, & Drug Delivery Systems for Ameliorating AMD. *Investigative Ophthalmology & Visual Science*, 2007. **48**(13): p. 1786-1786.