Studies on sustained release platforms for delivery of therapeutic agents to the posterior segment of the eye

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NANYANG
TECHNOLOGICAL
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**STUDIES ON SUSTAINED RELEASE PLATFORMS FOR
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SEGMENT OF THE EYE**

RINI RACHEL JOSEPH

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

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A thesis submitted to the Nanyang Technological University
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Doctor of Philosophy

2017

Statement of Originality

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

08/09/2017

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Date

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Abstract

For treatment of several disorders of the retinal area, the drug molecules need to be directly delivered to the posterior segment of the eye. The intravitreal route of drug delivery is the most common and effective route for delivering therapeutic amounts of drug to this area. However, the invasive nature of this route may cause several side effects. Trans-scleral route of ocular drug administration is a less invasive method, which may be used as an alternative route of drug delivery to target the posterior segment. Limitations of this route lie mainly in maintaining the required dose at the site of action, as well as overcoming several barriers (cell and systemic) to drug transport. Age Related Macular degeneration (AMD) is a disease of the posterior segment currently treated with monthly intravitreal injections of Anti-VEGF antibody, which are costly and invasive.

Encapsulating drugs in carriers, such as liposomes has the potential to sustain the duration of drug release and increase the bioavailability of the drugs at the site of action thus reducing the need for frequent drug administration. In this work, the focus was on understanding the mechanisms of transport, controlling and optimizing the various factors that affect the encapsulation and delivery of drugs and nanocarriers trans-sclerally using *ex vivo* models. The effect of the various properties of the nanocarriers on scleral distribution and transport were evaluated in this work. An *ex vivo* setup was designed and optimized for use in trans-scleral transport studies. Porcine scleral tissues were used as the animal tissue model for the *ex vivo* studies performed. Various nanocarriers with different properties were explored for their trans-scleral transport using the *ex vivo* setup. In particular, liposomes were studied for their size, charge and rigidity. Lucentis loaded liposomes were then studied for trans-scleral transport and distribution of the drug from the liposomal formulations.

The results obtained from these studies suggest that size, charge and flexibility of the liposomes determine their transport and scleral distribution properties. Smaller sized liposomes demonstrated higher intrascleral penetration compared to larger sized liposomes.

Positively charged liposomes were found to be episclerally localized, compared to neutral or negatively charged liposomes. Liposomes made of unsaturated lipids such as POPC, with a lower melting transition temperature demonstrated higher intrascleral diffusion compared to liposomes made of saturated lipids such as DPPC. Rigid PLGA nanocarriers were also found to be episclerally localized rather than diffuse intrasclerally. Trans-scleral transport of Lucentis from different liposome-encapsulated formulations was compared with trans-scleral transport of bare-Lucentis and found to be slowed down in the case of encapsulated formulations. Negatively charged liposomes consisting of DPPC-DPPG demonstrated the highest encapsulation efficiency as well as the slowest *ex vivo* trans-scleral transport of the encapsulated Lucentis, over the study period of 7-days. The carriers maybe considered to be acting as episcleral or intrascleral depots and releasing the encapsulated drug, which then diffuses trans-sclerally.

In conclusion, we have demonstrated the ability of nanocarriers of different properties to act as subconjunctival depot systems and providing sustained release. The findings from this work can be useful in developing successful sustained release therapeutics for diseases of the posterior ocular segment, and in particular, AMD.

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Abbreviations

AMD	Age Related Macular Degeneration
APTES	(3-Aminopropyl) triethoxysilane
BSA	Bovine Serum Albumin
CLSM	Confocal Laser Scanning Microscopy
CNV	Choroidal Neovascularization
DI	Deionized (water)
DLS	Dynamic Light Scattering
DMDAP	1,2-dimyristoyl-3-dimethylammonium-propane
DME	Diabetic Macular Edema
DMPC	1,2-Dimyristoyl-sn-glycero-phosphocholine
DMPG	1,2-Dimyristoyl-sn-glycero-3-phosphorylglycerol sodium salt
DOPC	1,2-Dioleoyl-sn-glycero-3-phosphocholine
DOTAP	1,2-dioleoyl-3-trimethylammonium-propane (chloride salt)
DPPC	Dipalmitoyl phosphatidylcholine
DPPG	1,2-Dipalmitoyl-sn-glycero-3-phosphorylglycerol sodium salt
DPTAP	1,2-dipalmitoyl-3-trimethylammonium-propane (chloride salt)
DSC	Differential Scanning Calorimetry
ELISA	Enzyme Linked Immunosorbent Assay
FA	Fluoresceinamine
FDA	Food and Drug Administration
FITC	Fluorescein Isothiocyanate
GCL	Ganglion Cell Layer
Hap	Hydroxy Apatite
HSA	Human Serum Albumin
HUVEC	Human Umbilical Vein Endothelial Cell
ILM	Inner Limiting Membrane
kDa	Kilo Daltons
LUV	Large Unilamellar Vesicles
MLV	Multi Lamellar Vesicles

MW	Molecular Weight
MWCO	Molecular weight cut off
NaF	Sodium Fluorescein
nm	Nanometer
NP	Nanoparticle
PBS	Phosphate Buffered Saline
PDI	Poly Dispersity Index
PEG	Poly Ethylene Glycol
POPC	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
POPG	1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt)
PLGA	Poly (Lactic-co-Glycolic Acid)
PS	Polystyrene
RNA	Ribo Nucleic Acid
RPE	Retinal Pigment Epithelium
siRNA	Small Interfering Ribonucleic Acid
SNA	Spherical Nucleic Acid
T _m	Melting transition temperature
TEM	Transmission Electron Microscopy
VEGF	Vascular Endothelial Growth Factor

Chapter 1

Introduction

This chapter describes the problem statement and hypotheses which forms the basis of this research work, in addition to describing the objective and scope of the research. An overview of the chapters in the dissertation is also described, and a pointer to the novelty and significance of results and outcome of this research work is provided at the end of this chapter. Briefly, this research was aimed at understanding the properties of nanocarriers which can make them potentially suitable candidates for a new, less invasive alternative route of ocular drug delivery, namely trans-scleral drug delivery, compared to the current intravitreal route of administration. This study can prove to be the basis for better design and development of sustained release formulations targeting posterior ocular segment diseases.

1.1 Background/Problem Statement

There have been several exciting new advancements in the field of ocular drug delivery, in the last few decades, both in development of new drug formulations for treatment, as well as in new modes of drug administration. Anatomically, the eye can be considered as consisting of two chambers: the anterior segment, and the posterior segment. The anatomical uniqueness of the eye permits that, depending on the disease and the target site of drug action, several modes of drug administration can be utilized. The typical routes of drug administration are represented in Figure 1.1 below.

Figure 1.1: Schematic of the different routes of drug administration to the eye. Adapted from: <http://www.medeuronet.com>

Topical administration via eye drops is the most common route of drug administration and effectively used in treatment of anterior segment ocular diseases. But this route has limited local drug availability and about 95% of the administered dose is cleared away

rapidly by circulation. Systemic administration has limited effectiveness in treatment of ocular disorders, due to the presence of blood-ocular barriers, which limit drug penetration to ocular tissues, and the high drug concentrations required, may in turn cause systemic side effects. High local drug concentrations can be achieved in the posterior ocular tissues by means of intravitreal injections, which are however, invasive and are associated with complications such as endophthalmitis and retinal detachment[1]. Periocular routes of drug administration, which do not damage the integrity of the eyeball, but deliver the drug in close proximity to the posterior ocular tissues, have been gaining importance in recent times as an alternative to the intravitreal drug delivery route[2-4].

Periocular routes of drug delivery comprise of subconjunctival, retrobulbar, sub-Tenon, suprachoroidal, and posterior juxtасcleral routes[5]. Of these, the subconjunctival route is considered the least invasive and easily accessible. In this route, the drug administered just beneath the conjunctiva, is transported through the sclera, choroid and RPE to reach the retinal tissues. Transport properties of small drugs, and macromolecules have been explored and the properties affecting trans-scleral transport have been identified. However, any drug introduced in the subconjunctival space has to encounter barriers to successful transport and delivery to the posterior segment, including the sclera, which is a tissue barrier, and the circulatory and lymphatic systems which play a role in clearing away the drug from the periocular spaces [6, 7]. This results in low local bioavailability of the drug and the need for repeated injections. Several diseases affecting the posterior ocular segment of the eye, such as Age related Macular Degeneration (AMD), Diabetic macular edema (DME), Diabetic Retinopathy (DR), etc. are chronic conditions requiring prolonged duration of treatment. Encapsulation of the drug in nanocarriers could protect the drugs from rapid washout and provide a more sustained and prolonged release profile in the posterior segment tissues, and also potentially reduce the frequency of such injections. There is a need to develop stable nano sized carriers for subconjunctival drug delivery that are safe, optically clear and which have sufficient drug loading ability for such sustained delivery systems.

Of the various diseases affecting the posterior ocular segment, Age related Macular Degeneration (AMD) alone is a leading cause of blindness in the developed world. AMD is a degenerative diseases causing progressive deterioration or breakdown of the eye's macula. The macula is the region of the retina responsible for best central vision. AMD can be of 2 types: Wet AMD and Dry AMD. About 10 percent of people who have macular degeneration have the wet form, but this form is more damaging to vision compared to the Dry Form of AMD. In Wet AMD, deposition of dead photoreceptor cells (called drusen) causes hypoxia, resulting in growth of abnormal blood vessels underneath the retina. This is known as Choroidal Neovascularization (CNV) and the newly formed blood vessels may exude fluid or blood, distorting central vision. The current standard of treatment for this is bimonthly intravitreal injection of an Anti-VEGF antibody, ranibizumab (marketed as Lucentis) or aflibercept (marketed as Eylea). This drug binds to the Vascular Endothelial Growth Factor (VEGF), and blocks its expression preventing growth of abnormal blood vessels, which in turn helps to slow vision loss, and in some cases, improve vision. This injection, however is invasive, and expensive, and can cause sight threatening complications in several cases [1].

Administering the drugs via periocular routes, such as the subconjunctival route is a potentially safer and viable route for treatment of posterior ocular disorders such as AMD. However, the subconjunctivally administered drug may be cleared away by circulatory and lymphatic drainage and may not have therapeutic concentrations required for action at target tissues. For AMD the site of action is the choroid/RPE, and the drug must cross several barriers from the site of administration. Encapsulating the drug in nano/micro carriers offers the possibility to act as a reservoir and prolong the drug release while retaining the bioactivity.

1.2 Hypotheses

It was hypothesized that sustained trans-scleral delivery is possible by the use of carriers, which can either act as an episcleral depot systems from the subconjunctival space, or an intrascleral depot by transporting to some extent inside the sclera. In order to verify this

hypothesis, trans-scleral transport and localization studies were performed with nanocarriers of varying properties, to study their depot forming ability. Properties affecting the distribution of nanocarriers have been studied for intravitreal delivery; but there have been no systematic studies on properties affecting transport and distribution of nanocarriers administered via the subconjunctival route. Sustained duration of action has been demonstrated by some formulations, delivered subconjunctivally [8-13]. A systematic analysis of the properties affecting the subconjunctival transport will help to elucidate the mechanism of action of the carriers and provide information on the carrier type best suited for each clinical need.

1.3 Objectives and Scope

Currently, a systematic study of the transport and distribution of nanocarriers post subconjunctival administration has yet to be carried out. This has been addressed in the current work which is divided into three main studies, as described in the scope and aims below. The information derived from the studies carried out as a part of this thesis, will help in the development of suitable *in vivo* sustained release platforms. These platforms can subsequently be used for development of sustained release formulations. Knowing the distribution and localization of the nanocarriers is imperative for the design of nanocarriers best suited for a particular application. In addition, fundamental studies such as this work act as stepping stones towards understanding carrier mediated trans-scleral drug delivery.

The primary objective of the project was to study and understand trans-scleral transport and distribution of several nanocarriers using an *ex vivo* model, and elucidation of nanocarrier properties which affect the transport. These nanocarriers may be potentially suitable for sustained delivery, for AMD or other posterior ocular disorders. The secondary objective of this work was to develop sustained release liposomal formulations for ranibizumab, for sustained trans-scleral delivery, targeting AMD, which can be used subsequently for translation to *in vivo* studies.

The scope and aims of this thesis are as follows:

- 1) Developing and optimizing an *ex vivo* setup for trans-scleral transport studies.

First, it was aimed to design and develop an *ex vivo* setup, which uses porcine sclera as a model animal tissue, for studying trans-scleral transport. Porcine sclera was chosen as the animal tissue model, due to its anatomical similarity to human sclera. This setup can be used for studying the diffusional transport of several drugs, proteins, antibodies, macromolecules or even nano/microcarrier systems. The setup serves as a useful preliminary tool for the screening of transport various drugs and carriers, before they could be used for *in vivo* studies.

- 2) Trans-scleral transport of the bare anti-VEGF antibody ranibizumab was studied.

Trans-scleral transport of the bare anti-VEGF ranibizumab was established to serve as a benchmark for transport studies involving the loaded nanocarriers. Transport of bare ranibizumab and the loaded-nanocarriers were subsequently compared to study the effect of the different carriers in providing sustained release.

- 3) Trans-scleral transport of bare nanocarriers was studied with nanocarriers of different properties.

Several nanocarriers which have the potential to be used for encapsulation of ranibizumab for providing sustained release were studied. In particular, liposomes were studied in detail for various properties such as size, charge and rigidity, and their effect on the transport. In addition, polymersomes, PLGA micro and nanoparticles, gold and liposomal Spherical Nucleic Acids (SNAs) were also explored for their scleral transport and distribution.

- 4) Different formulations of liposomes were studied for their ranibizumab loading ability.

Based on the studies on scleral distribution and localization of liposomes, different formulations of liposomes were examined for ranibizumab loading ability.

- 5) A dialysis method was used to study and understand the release of drugs from liposomes *in vitro*.

In vitro release studies on the liposomal formulations was conducted by a dialysis method, for studying release of ranibizumab from the liposomes.

- 6) Trans-scleral transport of the different formulations of ranibizumab loaded liposomes was studied.

Next, ranibizumab loaded liposomes were studied for trans-scleral transport. The transport of ranibizumab from each of the formulations, across the sclera was quantified, and comparisons were drawn with the transport of bare ranibizumab across the sclera.

1.4 Novelty of experimental objectives

This research has led to several novel outcomes by:

1. Developing a preliminary *ex vivo* screening tool for studying properties of nanocarriers which affects their trans-scleral transport and intrascleral distribution.
2. Establishing the concept of a subconjunctival minimally-invasive depot system which can be formed by certain nanocarriers.
3. Correlating the properties of nanocarriers to the nature and type of the depot formed.
4. Demonstrating a first of its kind *ex vivo* study of ranibizumab-loaded liposomes which has the potential ability to provide sustained delivery *in vivo*.

1.5 Dissertation overview

This thesis is divided into seven chapters.

In Chapter 1, background information on ocular drug delivery and motivation for this project is presented. The overall objectives of the project are presented along with the project hypothesis and novelty of the project.

Chapter 2 details the existing relevant literature pertaining to sustained posterior segment ocular delivery and gaps in knowledge, which are addressed in this work.

In Chapter 3, the experimental methods followed to fulfil the project objectives are described in detail. The methods followed for design, assembling and optimizing the *ex vivo* setup are described in detail. Description of preparation of several types of nanocarriers, which have been chosen in order to attempt to fully understand the mechanisms underlying trans-scleral transport properties are also included.

Chapter 4 is the first chapter in the results section, and contains experimental studies on transport of the bare ranibizumab antibody. The transport is explored in different buffers and pH conditions as well. These studies provide a valuable baseline for comparison against transport of nanocarriers encapsulating the drug.

Chapter 5 comprises the results from trans-scleral transport of nanocarriers, and their differential distribution and localization in the sclera. The different types of nanocarriers studied include liposomes, PLGA micro and nanoparticles, polymersomes, and gold and liposomal SNAs.

In Chapter 6, the transport of the ranibizumab loaded liposomes is described. The majority of the work described in this chapter focuses on loading and release of ranibizumab *in vitro* and *ex vivo*.

Chapter 7 ties up the threads of the overall project, summarizes the overall outcomes and major implications of the project and provides future recommendations based on our findings. The outcomes and research findings are explained in the light of the original hypothesis.

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Chapter 2

Literature Review

This chapter describes in detail, the background information and literature pertaining to work described in the thesis. Overall, this chapter is a review of knowledge and existing literature regarding ocular drug delivery and challenges associated with drug delivery, particularly for the posterior ocular segment. Specific information is also provided regarding the periocular mode of ocular drug delivery (particularly delivery via the trans-scleral route); what is already known and what remains to be explored, in the context of the hypothesis and areas covered in this thesis. The need for development and understanding of sustained delivery platforms is emphasized with regards to trans-scleral transport.

2.1 The eye

The eye is the sensory organ responsible for vision. The eye is more or less an anatomically and immunologically isolated organ, owing to its embryonic development and the various protective barrier layers it possesses. Broadly the two main regions of the eye can be categorized as the anterior chamber and the posterior chamber. Figure 2.1 below shows an overview of the anatomical structure of the eye detailing the various regions and the common routes of drug administration and drug elimination.

Figure 2.1: Anatomical Structure of the eye. In the anterior chamber, the primary physiologic blockage against installed drugs is the tear film. Transport of drugs to the anterior chamber is

mainly through the cornea. (I). For systemically administered drugs, the retinal pigment epithelium and the retinal capillary endothelium are the main barriers (II). Intravitreal injection is an invasive strategy to reach the vitreous (III). The administered drugs can be carried from the anterior chamber away either by venous blood flow after diffusing across the iris surface (1) or by the aqueous humor outflow (2). Drugs can be removed from the vitreous away through diffusion into the anterior chamber (3), or by the blood–retinal barrier (4). Image adapted from [1]

The anterior chamber of the eye comprises the area between the cornea and the lens. The posterior segment comprises the back two-thirds of the eyeball and is filled with the gel-like vitreous humour and bordered by the sclera. The posterior segment of the eye comprises the sclera, choroid, retinal pigment epithelium (RPE), neural retina and vitreous humour.

The sclera is the tough outer protective layer of the eye. It is a tough, collagenous tissue and consists of three main regions: the episclera, stroma, and the lamina fusca [2]. The sclera presents a large surface area, about 17 cm² in humans [3]. The choroid is the vascular layer which supplies blood and nutrients to the photoreceptors as well as other regions of the retina. The chorio-capillaries have fenestrated vascular walls with a relatively large luminal diameter. The Bruch's membrane is located between the RPE and the choroid and partly regulates the reciprocal exchange of biomolecules, nutrients, oxygen, fluids and metabolic waste products between the RPE and the choriocapillaries[4].

The retina is the light receiving nerve layer at the back of the eye. It is organized into eight different layers: Inner limiting membrane (ILM), the nerve fiber, ganglion cell layer (GCL), inner plexiform, inner nuclear, outer plexiform, outer nuclear, photoreceptors (rods and cones), and RPE. The macula is the retinal region containing the densest arrangement of photoreceptor cells (PR cells), hence it is the region of best visual acuity. A schematic of the various retinal layers is depicted in the illustration in Figure 2.2.

The RPE consists of tight-junction pigment cells which form the outer blood-retinal barrier. One of the main functions of RPE is the phagocytosis of shed photoreceptor outer segments (PR OS). The photoreceptor outer segments are digested, and essential substances are recycled and returned to photoreceptors to rebuild light-sensitive outer segments from the base of the photoreceptors [5].

With age, the RPE phagocytosis may not be as efficient, and photoreceptor debris or drusen may accumulate above and within the Bruch's membrane. Deposition of drusen can lead to inflammation. The presence of inflammation as well as the metabolic stress which induces hypoxia can cause the release of growth factors, such as Vascular Endothelial Growth Factor (VEGF) [6, 7]

Figure 2.2:Anatomy of the organization of the retinal layers and cells. From [8]

2.2 Age Related Macular Degeneration (AMD)

Diseases affecting the posterior ocular segment are the major cause of vision impairment, in developed nations. Some of the leading causes of blindness are age-related macular

degeneration (AMD), cataract and glaucoma, in addition to other diseases like diabetic retinopathy and retinal degeneration. Of these, AMD alone is responsible for a vast majority of severe vision loss in the developed world [9]. AMD causes progressive degeneration of the macula, the region of best vision in the eye. Figure 2.3 shows the picture of retina affected by AMD, and how it affects central vision. There are two kinds of AMD- Exudative or wet AMD and non-exudative or dry AMD. Even though dry AMD is the more common form of the disease, it is wet AMD which is more severe, and leading to blindness.

Figure 2.3: Picture of normal retina and retina affected by Macular degeneration and the corresponding effect on vision. Since macula is the region of best central vision, it is central vision that gets affected the most by AMD. The figures on the right show how vision is affected by AMD. Image from: <http://www.uniteforsight.org/eye-health-course/>

In both these conditions, there is accumulation of photoreceptor (PR) cell debris, commonly referred to as drusen. In the wet form of AMD, the drusen deposition disrupts the RPE structural integrity, which disrupts the connection between retina and choroidal capillaries. This leads to metabolic stress and hypoxia, which triggers secretion of VEGF, and compromises the structural integrity of the Bruch's membrane. This, in turn, leads to

proliferation of abnormal blood vessel growth from the choroidal capillaries to the RPE through the Bruch's membrane. This VEGF induced blood vessel growth is known as Choroidal Neovascularization (CNV) and is depicted in Figure 2.4. The new blood vessels are leaky, and causes fluid and blood accumulation in the macula. This can cause additional damage to the macular region and eventually may lead to permanent vision loss.

Figure 2.4: Image showing the anatomy of a healthy macula compared with macula affected by wet AMD. In the case of Wet AMD, abnormal blood vessels grow into the RPE from the choroid. From: www.lucentis.com.

2.3 Role of Vascular Endothelial Growth Factor (VEGF) in AMD

VEGF is a potent angiogenic growth factor, imperative in the creation of blood vessels in embryogenesis. VEGF is secreted in response to hypoxia. Over-expression of this protein can lead to vascular diseases in the retina and other parts of the body.

The VEGF family of growth factors consists of VEGFA, VEGFB, VEGFC, VEGFD and PlGF (Placental Growth Factor). Alternative splicing of the different VEGF entities lead to different isoforms with different biological activities. In humans, the isoforms are

VEGF-121, VEGF-145, VEGF-165, VEGF-189 and VEGF-206. Out of these isoforms, VEGF-165 is the most abundantly expressed isoform in humans.

VEGF mediates its angiogenic action by binding to the VEGF receptors. The three main receptors are VEGFR-1, VEGFR-2 and VEGFR-3, of which VEGFR-1 and VEGFR-2 are the receptors involved in angiogenesis [10, 11]. VEGFR-1 can bind VEGFA, VEGFB and placental growth factor (PIGF). VEGFR-1 is a key receptor in developmental angiogenesis (i.e. vessel formation during embryogenesis), but does not appear to be critical to pathogenic angiogenesis. VEGFR-2 binds to VEGFA, VEGFC and VEGFD and mediates most of the angiogenic effects of VEGF, and is also thought to stimulate angiogenesis on its own [10].

Anti-angiogenic treatment of wet AMD is designed to reduce the disease progression, by blocking the VEGF molecules from binding to the VEGF receptors, thus halting the choroidal neovascularization (CNV [12]. Several anti-VEGF monoclonal antibodies have been successfully used to treat AMD in recent times, such as bevacizumab/Avastin, ranibizumab/Lucentis [13-29], and aflibercept/Eylea [30, 31].

2.4 Current treatment and anti-VEGF therapy for AMD

Prior to the anti-VEGF therapies for AMD treatment, the primary approaches for AMD treatment were laser photocoagulation, photodynamic therapy and vitreoretinal surgery [32, 33]. Although these treatments are successful in halting the progression of vision loss, they are associated with complications such as non-selectivity and lack of improvement of visual acuity [33].

The rationale in paradigm shift of AMD treatment from the above destructive therapies to anti-angiogenic therapies was the finding that VEGF inhibition was found to impede the progression of CNV [34]. Pegaptanib was the first anti-VEGF drug initially licensed to treat AMD, in 2004. It is an aptamer, containing a sequence of oligonucleotides, fabricated to bind specifically to VEGF₁₆₅. Aptamers are synthetic oligonucleotides that

adopt a specific three-dimensional conformation that allows them to bind, with high specificity and affinity, to a wide range of therapeutic agents [33, 35]. Pegaptanib sodium is a 28-base ribonucleic acid (RNA) oligonucleotide with two branched 20 kDa polyethylene glycol (PEG) moieties attached in order to increase the half-life of the drug in the vitreous cavity.

Bevacizumab (Avastin) is a monoclonal antibody [Size: 148kDa] engineered to bind to the VEGF receptor. It was primarily used in the treatment of colon cancer [36] and not approved for treatment of AMD. However, it is still currently used off-label for the treatment of AMD.

Ranibizumab (Lucentis) is a fragment (Fab) antibody [48kDa] fabricated from the parent antibody bevacizumab and is licensed for AMD treatment. Due to its smaller size, it is expected to have better ocular tissue penetration and efficacy, as compared to bevacizumab. The binding of ranibizumab to VEGFA prevents the interaction of VEGFA with its receptors VEGFR-1 and VEGFR-2 on the surface of endothelial cells. However, to date there is no concurrence of opinion among physicians regarding the benefits and limitations of using ranibizumab over bevacizumab [19, 23, 27, 29, 37, 38].

Aflibercept (Eylea) is a relatively new player in the anti-VEGF field, approved by FDA in 2011, designed to bind to VEGFA, VEGFB & PlGF with higher affinity than to its natural receptors [30, 31].

Compared to bevacizumab, ranibizumab and aflibercept, pegaptanib is not as effective in combating the CNV. The inadequacy of pegaptanib was attributed to the fact that VEGF has two binding domains: a heparin binding domain and a receptor binding domain. Pegaptanib is engineered to bind to the heparin binding domain, and hence only has an effect in reducing the VEGF signal amplification, while the binding is still not affected. Bevacizumab, ranibizumab as well as aflibercept, on the other hand, bind to the receptor binding domain, inhibiting VEGF signaling [19].

2.5 Challenges to AMD drug delivery

Successful treatment modalities for AMD require maintenance of therapeutically effective drug concentrations at the sites of drug action, namely the vitreous, retina and/or choroid. However, drug delivery to the posterior segment is limited by several physiological barriers. Topical instillation, the most favored mode of ocular drug delivery is ineffective as there is considerable drug loss by tear fluid dilution, pre-corneal drainage, lacrimation, nasolacrimal drainage, and tear turnover [39]. There are some reports in the literature of antibody fragments passing to the vitreous via topical administration [22] [40], but the viability of this route of administration is questionable, due to the high losses involved [41]. There is also the problem associated with patient non-compliance to chronic eye drop administration, which is currently a challenge faced in treatment of glaucoma. Systemic administration is rendered ineffective by the blood-ocular barriers which restrict the diffusion of many therapeutic agents from the systemic circulation into the ocular chambers. Systemic administration also increases the possibility of adverse toxic effects from the drug, which includes cardiovascular toxicity. In the intra-vitreous mode of administration, the drug is directly injected into the vitreous humour. The anti-VEGF drugs are currently administered intravitreally, by monthly or bimonthly injections. The intravitreal route ensures sufficient drug concentrations in close proximity to the retina. The vitreous gel acts as a reservoir for the drug, and the drug diffuses slowly into the retinal and RPE tissues [15, 42-44]. However, intravitreal injections can pose several complications such as endophthalmitis, retinal detachment, and vitreous hemorrhage.

In recent years, periocular delivery approach has been gaining more attention as a viable route of drug delivery owing to the relatively large scleral surface area with favorable permeability characteristics, and to the less invasive nature of this route.

2.6 Various barriers to trans-scleral drug delivery to the posterior ocular segment

Periocular drug delivery approaches include subconjunctival, sub-tenon, retrobulbar, peribulbar, suprachoroidal and juxtasceral administration [45]. The various periocular routes are illustrated in Figure 2.5 below. This route offers quite a few advantages for drug delivery owing to the large scleral surface area, which can increase the rate and extent of absorption; high degree of hydration of the sclera, which is conducive to the diffusion of hydrophilic molecules, relatively high permeability to macromolecules [46, 47] and feasibility of administering controlled and sustained release dosage forms [48]. The drugs so administered may reach the posterior segment tissues by trans-scleral diffusion, systemic diffusion from the choroidal circulation or via the anterior route. Of the periocular routes, the subconjunctival route is considered the least invasive.

Figure 2.5: The different periocular routes for drug delivery. Adapted from [49]

However, this route faces three types of physiological barriers which may be broadly classified as static, dynamic and metabolic barriers [50]. The various characteristics of these barriers and their potential influence on trans-scleral drug transport are discussed below.

2.6.1 Static barriers

The sclera, choroid, and RPE together constitute the static or physical barrier to trans-scleral diffusion. The collagenous scleral matrix, the lipophilic choroid together with the underlying Bruch's membrane, and the tight junctions of the RPE restrict transport of solutes across them.

2.6.1.1 Sclera

It has been extensively reported in literature about transport of small molecules and proteins across the sclera. *Ex vivo* studies generally are performed using diffusion chambers such as Ussing chambers [51-67] and Franz type diffusion cells [24, 68-71]. Scleral transport has been studied and characterized extensively using explanted porcine [63, 65, 70], bovine [62, 63, 65, 72], rabbit [63, 73-75], and human scleral tissues [20, 28, 46, 52, 53, 55, 56, 76-79].

Sclera is found to be quite permeable to hydrophilic molecules and even macromolecules up to 150kDa [47]. The permeability is found to decrease exponentially with molecular radius, rather than with molecular weight. Globular proteins were found to be more permeable compared to linear dextrans, but this is possibly due to differential binding of the dextrans to the scleral matrix. While Rudnick et al. [75] had found that intraocular pressure decreases scleral permeability, Cruysberg et al. [57] explored the scleral permeability of higher molecular weight molecules, and found that the permeability is only a weak function of pressure. Scleral thickness also factors in the permeability-thinner scleral regions of the globe being more permeable.

The effects of lipophilicity and hydrophilicity on transport across sclera has also been characterized [65, 77, 80, 81] [62]. Sclera is found to be more permeable to hydrophilic compounds than hydrophobic/lipophilic molecules. Since the sclera is primarily acellular and comprising of 75% water [82, 83], the primary means of scleral transport is thought to be the aqueous pathways between the collagen fibers. Impermeability of some proteins has been attributed to differential binding to the collagen fibers [84]. Molecules with the same radius showed decreasing scleral permeability with increasing lipophilicity. The

effect of lipophilicity on transport across the sclera was characterized by Wen et al. [77]. Lag time is found to increase when the lipophilicity of the molecule is higher, possibly due to binding to the scleral collagen. Two major interactions are described here- one being the permeant lipophilicity and the other charge-charge interactions between permeant and sclera.

Sclera is more permeable to negatively charged molecules than positively charged molecules, because the negative charge of sclera causes positive molecules to bind to the sclera itself [65]. Also positively charged molecules exhibit a larger partition coefficient in the sclera compared to negatively charged molecules. The lag time and permeability coefficients observed may largely depend on the time duration of the experiment as well as the lipophilicity of the permeants.

In conclusion, predicting the transport of a molecule across the sclera requires taking many factors into consideration, including (but not limited to) charge, size and hydrophilicity.

Scleral permeability was found to be increased in presence of prostaglandins, such as latanoprost acid. It was found that anatomical changes to sclera by using collagenase caused an increase in permeability of sclera, implying that collagen density is an important parameter in determining scleral permeability [59].

Scleral transport was found to be enhanced by the method of trans scleral Iontophoresis [24, 68, 71, 72, 79, 85, 86] and osmotic pumps [87]. Also reported was a study using trans-scleral ultrasound to enhance delivery of protein loaded in silk fibroin nanoparticles[88].

The factors affecting scleral permeability, from various *ex vivo* studies are summarized in Table 2.1 below.

Table 2.1: Factors affecting Scleral Permeability.

Factor affecting Scleral permeability	Effect of factor on transport	Drug/Molecule tested	Type of tissue explant	Reference
Size	Exponential decrease with increasing radius	NaF, FITC–conjugated BSA, FITC–IgG, and FITC dextrans	Rabbit	[47]
Charge	Higher permeability for negatively charged molecules	NaF and R6G.	Bovine, Porcine	[65]
Lipophilicity	Increased Lipophilicity caused decreased transport	Celecoxib, Budesonide, NaF and R6G.	Bovine, Porcine	[65]
		8 different β -blockers	Human, bovine, porcine, rabbit	[63]
Age	No effect	5-fluorouracil Sucrose, dextran polymers, inulin tritiated (3H) dexamethasone	Human	[46]
Surgical thinning	Thinning increases scleral permeability	5-fluorouracil Sucrose, dextran polymers, inulin tritiated (3H) dexamethasone	Human	[46]
Cryotherapy	Found to have no effect on permeability	5-fluorouracil Sucrose, dextran polymers, inulin tritiated (3H) dexamethasone	Human	[46]
Exposure to Prostaglandins	Increase in permeability with prostaglandin exposure	Latanoprost acid pretreatment, transport of 10 kDa rhodamine-dextran	Human	[59]

2.6.1.2 Choroid

The choroid has binding sites for melanin, which has preferential affinity to bind lipophilic and cationic solutes. Certain molecules may preferentially bind to the choroidal melanin, and their transport might be affected [65]. However, the main challenge in choroidal drug delivery is the choroidal blood circulation. Choriocapillaries do an effective job of clearing the drugs away, such that, as per a simulation study conducted, only 4.3% to 17% of the total drug administered sub-conjunctivally passes the choroid [89]. Even if the drug were to pass through the sclera, the choroid would still present a formidable barrier to drug transport.

The effect of lipophilicity has been characterized for the sclera, choroid, RPE, Bruch's and optic nerve by Kadam et al., using different beta blockers to compare lipophilicities [81]. Increase in lipophilicity resulted in an increase in the melanin binding in the choroid and hence increased tissue partitioning [65]. Less lipophilic formulations may be chosen to reduce the non-productive binding to choroid, thus allowing greater trans-scleral delivery efficiency to the retina.

2.6.1.3 RPE and Neural Retina

RPE transport is more complicated due to the presence of tight junctions and active transport mechanisms [65].

Unlike the sclera and choroid, RPE possesses tight junctions between cells, and compounds with greater lipophilicity are found to be able to pass through better compared to less lipophilic compounds, indicating possibly a trans-cellular transport route [80]. Hydrophilic molecules on the other hand, move through the RPE-tight junctions. Lipophilic and higher molecular weight molecules have a higher lag time compared to hydrophilic and lower molecular weight molecules. This could be because the lipophilic molecules bind to melanin present in the RPE. RPE preferentially phagocytizes anionic particles [90], and smaller particles (~20 nm) are more rapidly phagocytized compared to larger particles (~2000 nm) [91] The permeability of RPE was

explored by Pitkanen et al. [80], for lipophilic and higher MW molecules. RPE limits the drug movement from choroid to retina (trans-scleral). Choroid to retina permeability was found to be about 2.4 times lower than retina to choroid permeability, and the permeability decreases exponentially with molecular mass. In comparison, scleral permeability is about 10 times greater than the choroid to retina permeability.

The ILM of the retina acts as an electrostatic as well as size barrier to movement of molecules from the vitreous side into the retina[44, 92] [93]. Of the retinal layers, VEGF activity was found mainly in the GCL and INL, and weakly in the choroid. This is the region where the anti-VEGF drugs need to actively target [17].

2.6.2 Dynamic barriers

In case of sub-conjunctival administration, the drug might also be cleared by episcleral blood vessels. The conjunctival lymphatic drainage also plays a role in clearing away the drugs [89].

The choriocapillaries does a very good job in effectively clearing the drugs by two possible routes: a) high plasma flow in choriocapillaries, b) fenestrated porous capillary structure. The effect of choroidal and conjunctival vasculature on drug clearance was studied by Kim *et al.* [74]. The authors found that when an episcleral implant loaded with MRI contrast agent was placed on the equator of the eye, the contrast agent was not visible at the vitreous, whereas it was detectable in enucleated eyes, demonstrating the effect of dynamic barriers in *in vivo* clearance [74]. For maximum bioavailability in vitreous, molecules should have maximum RPE permeability and minimum choroidal clearance [89].

Robinson *et al.* observed that by experimentally eliminating the choroidal blood circulation and lymphatic clearance, significant drug concentration of triamcinolone acetonide has been achieved in the vitreous trans-sclerally. A possible artifact of low drug delivery rate, but the choroidal flow was found to be less important compared to episcleral and conjunctival blood supply and clearance mechanisms [94].

2.6.3 Metabolic barriers

The eye possesses metabolizing enzyme systems which degrade foreign substances, which might otherwise harm ocular tissues. There is evidence of metabolic activity in the blood-ocular-barrier components, mainly the iris-ciliary body (blood aqueous barrier), choroid and RPE (blood retinal barrier), and to a lesser extent in the cornea, lens, aqueous humour and vitreous humour.

Both phase-I and phase-II metabolic enzymes are expressed in the eye; of which the cytochrome P450 family and lysosomal enzymes are the most predominant [95].

2.7 Sustained Ocular Drug Delivery

Several diseases affecting the posterior segment, such as AMD, diabetic retinopathy etc. are of chronic nature and currently require long term treatment and repeated injections. Repeated intravitreal injections are associated with several complications such as endophthalmitis, retinal detachment etc.[96] Sustained release formulations for these indications can not only reduce the frequency of these painful and invasive injections, but also reduce the burden of the expensive injections, to the patients. Existing drugs which are introduced by the intravitreal or periocular routes may be encapsulated in suitable micro/ nanocarriers for sustaining the release over longer durations. Various sustained-release formulations, including gels, microparticles, nanoparticles, and liposomes have been explored for this. Microparticles and nanoparticles introduced in the vitreous humour or periocular space are capable of sustaining the delivery of the encapsulated agents for longer durations compared to the drug alone [97-101]. Several researchers have studied liposomes for intravitreal and subconjunctival sustained delivery [13, 102-111]. For delivery of therapeutic agents for longer periods in a more controlled manner, solid implants or polymeric films have been studied. [112-120]. Intravitreal delivery of polymeric systems may cause clouding of vision, which is not the case for periocular administration. However, periocular sustained delivery, remains a relatively less explored field, particularly so in AMD.

Abrishami *et al.* formulated nanoliposomes of bevacizumab for studies in rabbit, injected as an intravitreal formulation [13]. These liposomes, made of EggPC-Cholesterol and DPPC-Cholesterol had encapsulation efficiencies of $45.5 \pm 5.6 \%$ and $37.7 \pm 4.2 \%$ respectively, and the Egg PC-Cholesterol formulation was used for the *in vivo* studies. At 42 days post injection, the vitreous levels of bevacizumab was found to be about 5 times higher than control injection with plain bevacizumab solution. This study shows that when administered intravitreally, liposomal bevacizumab is able to sustain the delivery for at least 6 weeks with therapeutic efficacy.

In order to extend the half- life of bevacizumab in the vitreous, other strategies have also been explored. In a study by Pan *et al.*, the effect of PLGA-encapsulated bevacizumab and PEGylated bevacizumab were evaluated in reducing the extent of experimentally induced choroidal neovascularization in a rat model. Intravitreal injection was used as the mode of administration. Although both the formulations, as well as free bevacizumab was shown to have significant reduction in CNV area with respect to controls, there was no significant difference between the groups themselves. [23]

Bevacizumab encapsulation in PLGA nanospheres was studied by Li *et al.* [21] and the *in vitro* release was found to be sustained for upto 90 days. The authors, however, measure the antibody concentrations by UV spectrometry, and thus there is no information on whether the bioactivity of the antibody was preserved. Varshochian *et al.* [121, 122] have further optimized the PLGA based delivery system using albumin as a protective agent, and have found that the bevacizumab vitreous concentration was maintained above 500 ng/ mL (which is the minimum inhibitory concentration of bevacizumab required to completely block VEGF-induced angiogenesis) for about 8 weeks compared with the controls.

Chitosan nanoparticles loaded with Bevacizumab was explored by Lu *et al.* [123] for expression of VEGF and VEGF mRNA in diabetic rats. In a comparison of saline control, plain bevacizumab, and bevacizumab in chitosan nanoparticles, the expression of VEGF

protein and VEGF mRNA was inhibited in the nanoparticle group for upto 8 weeks after injection. This study shows the potential of nanoparticles in providing sustained delivery and action of the antibody.

The anti-VEGF aptamer EYE001 encapsulated in PLGA microparticles was studied by Carasquillo *et al.* [124] Stability and bioactivity of the released aptamer were assayed by monitoring the RNA aptamer's ability to inhibit VEGF-induced cell proliferation in human umbilical vein endothelial cells (HUVECs). EYE001 co-lyophilized in the presence of trehalose prior to encapsulation in PLGA was found to have a better preservation of bioactivity and was found to have sustained release over a period of 20 days.

A different delivery route, the suprachoroidal space was explored for sustained delivery of bevacizumab from a light activated in situ gel system, developed by Tyagi *et al.* [125]. A polycaprolactone dimethacrylate (PCM)- hydroxyethyl methacrylate (HEMA) crosslinked gel was used to entrap bevacizumab. *In vitro*, the release of bevacizumab in was found sustained during a period of 4 months. In vivo delivery was assessed in Sprague Dawley (SD) rats by injecting Alexa Fluor 488 dye conjugated bevacizumab in the gel, and monitored noninvasively using a fundus camera and Fluorotron Master™. Delivery of Alexa Fluor- 488 dye conjugated bevacizumab was sustained for at least 60 days in the suprachoroidal space of SD rats. However, the limitation of the study is the lack of bioactivity testing of bevacizumab in the in vivo study, as well as the possibility of toxicity of the photoinitiator used during the in situ crosslinking procedure.

Bevacizumab loaded in hyaluronic acid/dextran-based in situ hydrogel were studied by Yu *et al.* for intravitreal injection in rabbit eye. A single intravitreal injection was able to sustain the delivery for up to 6 months and the retinal structure was not affected by implantation of the in situ gel [126]. This study shows the significant potential of hydrogels in sustained delivery of anti-VEGF.

A biodegradable, thermally-responsive hydrogel, based on poly(ethylene glycol)-poly-(serinol hexamethylene urethane) (ESHU) was developed to provide sustained release of bevacizumab in rabbit eyes. *In vivo*, the ESHU gel sustained the release of bevacizumab for over 9 weeks, with preserved bioactivity (as determined by ELISA)[127].

Intravitreal injection of a gel formulation of hydrophilic- hydrophobic block copolymers of methoxy-poly (ethylene glycol)-block-poly (lactic-co-glycolic acid) (mPEG-PLGA), cross-linked with 2, 2-bis (2-oxazoline) (BOX), was found to sustain bevacizumab for upto a month *in vivo* in a rabbit study. The bioactivity of the released bevacizumab was preserved, as evidenced by inhibited RF/6A and HUVEC cell proliferation [128].

A biodegradable and thermo-sensitive tri- block copolymer consisting of poly(2-ethyl-2-oxazoline)-b- poly(ϵ -caprolactone)-b-poly(2-ethyl-2-oxazoline) (PEOz-PCL-PEOz), that can sustain the release of bevacizumab *in vitro* was developed by Wang *et al.* The gel demonstrated good biocompatibility, as evidenced by preserved electrophysiology of the rabbit retina two months after intravitreal injection. The results show potential for sustained *in vivo* release for bevacizumab using this novel gel system Although this is primarily an *in vitro* study [129].

Unlike PLGA-based hydrogels, which generate acidic degradation by products, which may affect the drug bioactivity, silk hydrogels offer an alternative for maintaining bioactivity of protein therapeutics, for sustained delivery. Lovett *et al.* have developed silk hydrogels, incorporating bevacizumab [130]. These hydrogel formulations demonstrated sustained release of 3 months or greater both *in vitro* as well as *in vivo*, after intravitreal injection in Dutch-belted rabbits. Vitreous concentrations 3 months post administration were equivalent or greater than the levels for positive control at 1 month. The bioactivity of bevacizumab on release from the hydrogel was preserved, as observed by ELISA measurement. Terminal fundus photographs and ophthalmic examinations indicated no major signs of ocular tissue abnormalities, and at 3 months, biodegradation of the hydrogel was observed.

A composite “particle-in-particle” system was developed by Elsaid *et al.* with PLGA microparticles incorporating chitosan-based nanoparticles, for improved loading and sustained intravitreal delivery of ranibizumab.[131] Different kinds of chitosan-based nanoparticles composed of chitosan, CNAC (Chitosan-N-acetyl-L-cysteine) CNAC/tripolyphosphate (CNAC/TPP), chitosan/TPP (chit/TPP), or chit/TPP-hyaluronic acid (chit/TPP-HA) were incorporated in PLGA microparticles. The prepared nanoparticles had sizes between 17-350 nm and zeta potentials ranging from -1.4 to +12 mV. The entrapment efficiency of ranibizumab in these different formulations varied between 13 and 69%. Of the formulations compared, the PLGA microparticles containing CNAC nanoparticles showed significantly improved ranibizumab loading and release profile in the in vitro release study.

Another particle-in-particle formulation was developed by Yandrapu *et al.* using a supercritical carbon dioxide based technology(SC CO₂) [132]. The authors refer to this system as nanoparticle-in-porous microparticle or NPinPMP. This system utilizes the selective ability of supercritical carbon dioxide to expand PLGA matrix, and not PLA. Bevacizumab coated on PLA nanoparticles were encapsulated into a porous PLGA matrix, which was exposed to SC CO₂. This system showed sustained release of Bevacizumab for up to 4 months in vitro. In vivo delivery was assessed non-invasively using Alexa-conjugated Bevacizumab by Flurotron, in a rat model. Alexa-bevacizumab fluorescence from the NPinPMP system was detectable for up to 2 months, whereas the Alexa-bevacizumab solution fluorescence was at baseline levels by 2 weeks.

A composite drug delivery system consisting of ranibizumab or aflibercept, in PLGA microparticles, suspended in a thermoresponsive, injectable, PNIPAAm hydrogel were developed by Osswald *et al* [133]. The drug was loaded in PLGA (75:25) Microspheres which were then suspended in an injectable poly(N-isopropylacrylamide)-based thermoresponsive hydrogel to create a microsphere-hydrogel system. This system was found to sustain the release of ranibizumab or aflibercept for 196 days with a daily dose of 0.153 and 0.065 µg/day, respectively. The bioactivity of both the anti-VEGFs were retained throughout, as evidenced by significant inhibition of HUVEC proliferation by

the released drug from the system, compared against controls.

The different anti-VEGF sustained release techniques studied to date for have been summarized in Table 2.2.

2.8 Release and distribution of nanocarrier encapsulated drugs after intravitreal administration

The intravitreal route is currently the standard of treatment for most posterior segment disorders, and the drug can be administered in close proximity to the target tissues, and diffuse through the viscous gel-like vitreous humour to reach the target site of action. The diffusion is found to be dependent on several factors such as size, charge and surface properties of the molecules as well as on the disease condition [43, 64, 100, 134].

The effect of size of polymeric nanospheres on intravitreal kinetics have been studied by Sakurai et al.[134]. Particles of sizes 2 μm , 200 nm and 50 nm were used in this study and it was observed that the smaller 200 nm nanoparticles were localized in the retina 2 months post injection, while the micron sized particles were found distributed in the trabecular meshwork and vitreous cavity, possibly due to their sizes being bigger than the critical size to enter the retina. A charge based effect on intravitreal movement was observed by Kim *et al.* using differently charged human serum albumin (HSA) nanoparticles(NPs)[135]. Anionic nanoparticles used in the study with an average size 114 nm and an overall Z-potential of - 33.3 mV, were found to easily diffuse through the vitreal collagen fibrils and reach the retina, 5 hours after intravitreal injection, whereas the cationic nanoparticles, with an average diameter of 175.5 nm and a mean Z- potential of +11.7 mV, were found to remain aggregated in the vitreous.

Table 2. 2: The different Anti-VEGF sustained release techniques studied to date.

Sustained delivery vehicle	Drug studied	Type of study	Significant feature	Reference
Pegylated-bevacizumab and PLGA-encapsulated bevacizumab	Bevacizumab	<i>In vivo</i> ; Norway rats	Treatment of laser induced CNV found to be slightly improved with b-PEG compared to bevacizumab alone, not very significantly.	[23]
PLGA nanospheres	Bevacizumab	<i>In vitro</i>	Encapsulation efficiency claimed = >90%, but for 0.2-1 μm size particles, in the <i>in vitro</i> study	[21]
EggPC/DPPC liposomes with cholesterol	Bevacizumab	<i>In vivo</i> ; New Zealand albino rabbits	Sustained delivery in vitreous as shown by ELISA results over 42 days.	[13]
Albuminated PLGA nanoparticles	Bevacizumab	<i>In vivo</i> ; rabbits	Sustained release with antibody protection from aggregation and instability after intravitreal injection.	[121]
PLGA microspheres	Anti-VEGF aptamer EYE001	<i>Ex vivo</i> ; Rabbit sclera	In vitro release and successfully maintained bioactivity of aptamer	[124]
Light activated in situ forming gel	Bevacizumab	<i>In vivo</i> ; Sprague Dawley rats	Sustained release upto 60 days obtained in suprachoroidal space	[73]
Microspheres in a	Ranibizumab	Only <i>In vitro</i>	Sustained delivery of ranibizumab and	[133]

Thermosensitive PNIPAAm gel	and aflibercept	study reported	aflibercept observed for 196 days	
PLA nanoparticles in porous PLGA microparticles	Bevacizumab	<i>In vivo</i> ; rat	Supercritical carbon dioxide technology, sustained release for upto 2 months <i>in vivo</i> after intravitreal injection	[132]
Bevacizumab Chitosan nanoparticles	Bevacizumab	<i>In vivo</i> ; rat	intravitreal injection of bevacizumab - chitosan nanoparticles inhibit VEGF expression in retina upto 4 weeks	[123]
ESHU gel	Bevacizumab	<i>In vivo</i> ; rabbits	Release of bevacizumab was sustained for over 9 weeks, after intravitreal injection.	[127]
mPEG-PLGA gel	Bevacizumab	<i>In vivo</i> ; rabbits	Bevacizumab release sustained for upto a month after intravitreal injection.	[128]
PEOz-PCL-PEOz gel	Bevacizumab	<i>In vitro</i>	<i>In vitro</i> sustained release upto 20 days.	[129]
Silk hydrogels	Bevacizumab	<i>In vivo</i> ; Dutch-belted rabbits	Sustained release upto 3 months <i>in vitro</i> as well as <i>in vivo</i> after intravitreal injection.	[130]

Intravitreally injected polylactic acid (PLA) nanoparticles (size between 140-360 nm) were found to be preferentially localized in the RPE for up to 4 months after intravitreal injection, by Bourges et al. [136], pointing to a slow elimination, and ability to diffuse into the RPE from the vitreous humour. The elimination kinetics of intravitreally injected liposomes of different size and cholesterol composition were examined by Barza *et al.*, [32]. The authors observed that the rate of clearance was inversely related to the size, but not the cholesterol content. Smaller sized vesicles, of 60 nm size were cleared rapidly from the vitreous cavity compared to large unilamellar vesicles of mean diameter 400 nm. The cholesterol-containing formulation had enhanced sustained release of the encapsulated content in vitreous compared to non-cholesterol loaded formulation.

Table 2.3 below summarizes the nanoparticle properties which affect their vitreal transport and distribution.

Table 2.3: Summary of the nanoparticle properties which affect their vitreal transport and distribution.

Carrier property	Effect	Reference
Size	Smaller particles (50 nm-200 nm) found to be able to reach the retina post intravitreal injection. Vitreal clearance is also faster for smaller particles.	[110, 134]
Charge	Positively charged particles tend to remain clumped in the vitreous, without diffusing, while anionic particles are able to diffuse to the retina.	[135, 137]
PEG-ylation	PEGylated particles (even cationic) are able to diffuse to the retina.	[137]
Ocular Inflammatory conditions	Accelerated diffusion in, and clearance from vitreous in presence of inflammatory conditions or vitrectomy.	[138]

2.9 Release and distribution of nanocarrier encapsulated drugs after periocular administration

Trans-scleral delivery of drugs, proteins and nanocarriers have been studied extensively over the last few decades. The sclera is considered to be relatively less of a barrier to molecules, with passive diffusion facilitated by movement of molecules across the collagen fibrils. [2, 50, 60, 139]. *In vivo*, the circulatory and clearance systems may have a bigger role in limiting the transport to posterior segment tissues from the periocular space. By experimentally eliminating either choroidal blood circulation and lymphatic clearance, Robinson *et al.* observed that [94]. conjunctival lymphatics/blood vessels may be an important barrier to the transscleral delivery (triamcinolone acetonide in this case) compared with choroidal circulation. Another observation from this study was that by using a higher concentration of the plain drug, the depot was able to deliver vitreal concentrations, compared to a smaller starting concentration.

The same observation was reiterated using a Sodium fluorescein(NaF) loaded episcleral implant. Chan et al has shown that >99% of the implanted NaF was cleared by 3 hours, and by episcleral circulation. [118].When animals were euthanized immediately following the administration, the NaF was detectable, indicating that the elimination is occurring by means of the dynamic circulation.

In the absence of a sustained-release system, the drug-sclera contact times might be too brief to attain any significant therapeutic drug concentrations at target ocular tissues.

However, not many studies have looked at the properties of carrier molecules which make them suitable for sustained drug delivery from the periocular space. In this section, a summary of the studies existing on ocular tissue disposition and distribution of nanocarriers for sustained delivery from the periocular space are described, culminating in the rationale for this work.

Subconjunctival PLA microparticles of 3.6 μ m size and nanoparticles of 345 nm encapsulating budesonide [97] were found to demonstrate sustained release for upto 2 weeks from microparticles, and for 1 week from nanoparticles in Sprague Dawley rats. The encapsulation efficiency for the PLA nano and microparticles were 65% and 99%, respectively. The nanoparticles had a higher burst and lower encapsulation compared to microparticles. On Day 14 of the *in vivo* study, the microparticle group had higher concentrations in the retina and vitreous compared to the nanoparticle formulation or solution.

Subconjunctivally administered celecoxib-PLGA (lactide:glycolide 85:15) microparticles sustained retinal and other ocular tissue drug levels during the 14-day study in rats, for treatment of diabetes-induced retinal abnormalities in a rat model [99]. *In vitro*, the microparticles sustained celecoxib release for 49-days.

The size dependence on distribution of Fluorescent polycarbonate nanoparticles of size 2000 nm, 200 nm and 20 nm were studied by Amrite *et al.*[140] after subconjunctival injection. The smaller 20 nm particles were rapidly cleared away by circulation, while the 200 nm and 2000 nm particles were found to be retained at the subconjunctival space for upto 2 months post injection, Further follow up studies have elucidated the role of the episcleral circulation as a major route of clearance[64].

In a study of subconjunctival injection of carboxyfluorescein loaded liposomes in rabbit eyes, the liposome-encapsulated 6-CF was detectable for up to 7 days in cornea, sclera, choroid, retina and vitreous with prolonged detection mainly in the retina/choroid [141]. In order to further reduce the effect of clearance systems in drug elimination, a slightly invasive procedure using hollow microneedles was studied by Jiang *et al.* [142]. The authors studied distribution of micro/nanoacarrriers loaded with a fluorescent dye intrasclerally. The nanoparticle suspensions could be delivered into the sclera, whereas microparticles used in the study could only be delivered in the presence of collagenase or hyaluronidase spreading enzymes, which showed the scleral collagen fibers as rate limiting barriers to nanoparticle transport inside the sclera.

2.10 Challenges associated with protein encapsulation in sustained release formulations

Protein-based therapeutics have entered the mainstream medical therapeutic with the advent of human and humanized antibodies. Typically delivered intravenously, humanized antibody delivery often requires large quantities and multiple doses, which incur high drug costs as well as possible systemic side effects. One viable alternative is local delivery, and in that regard, posterior segment ocular therapy with monoclonal antibodies such as Ranibizumab and bevacizumab have achieved great success. [143, 144]. Local antibody delivery such as this provides high concentration at the target site with reduced systemic exposure and lower drug dose than would be required systemically. However, as with other protein drugs, there are stability and formulation challenges associated with antibody delivery, even when applied locally. Proteins also may undergo denaturation and subsequent inactivation easily, during formulation, storage or delivery. These limitations may be addressed in part by controlled release (CR) systems which may help sustain drug concentrations for longer periods. However, encapsulation and development of CR formulations for antibodies poses several challenges of its own. The encapsulation of protein molecules in nanocarriers for sustained delivery is challenging due to limitation on the solvents, environmental conditions and preparation methods which can be employed. Formulations often need to be also prepared in presence of special excipients such as buffers, preservatives or additives to preserve the native confirmation of the proteins. In a recent study involving bevacizumab encapsulation in PLGA, albumin was found to have a protective effect in increasing the stability of the protein post-encapsulation. [122]. Desirable properties in a carrier include the ability to encapsulate the protein without denaturation, the possibility of drug release by suitable amounts, and maintaining drug stability throughout release.

Among the potential nanocarriers, colloidal systems such as liposomes and nanoparticles have aroused considerable interest. Liposomes show versatile properties in terms of size, surface charge and lipid composition, and their ability to incorporate both hydrophobic

and hydrophilic drugs. Protein incorporation has been improved in liposomes by several techniques [145]. Compared to liposomes, encapsulation of proteins within the aqueous lumen of polymersomes can, in principle take advantage of the extended circulation kinetics and controlled release properties of polymersomes[146]. PLGA micro and nanocarriers possess excellent biocompatibility, and biodegradability, and hence is a widely used polymer for manufacture of nano- and microparticles. Modifying the typical encapsulation processes by addition of excipients and stabilizers such as trehalose, surfactants, albumin etc help to provide increased stability of encapsulated proteins. These carriers hold promise for encapsulation of Anti-VEGF antibodies such as Lucentis.

2.11 Rationale for this work

As described in Section 2.6, subconjunctival mode of drug delivery is a less invasive mode of drug delivery for targeting posterior segment ocular disorders. However, to achieve therapeutic benefits with more drugs reaching target tissues rather than getting cleared away by circulatory and lymphatic drainage systems, it is essential to develop sustained release formulations, which can enhance the duration of drug delivery, as well as provide protection against drug clearance and degradation. Of the carrier systems, liposomes are a particularly attractive system for subconjunctival drug delivery, owing to their biocompatibility, non-toxicity, and ability to entrap both hydrophobic and hydrophilic drugs [111]. Moreover, the size, charge and rigidity of the liposomes can be easily manipulated. By using a combination of different lipids, the properties and encapsulation efficiencies may also be modified. Drug release from the liposomes may occur either by diffusion across the intact bilayer membrane or by bilayer disintegration. [110]. The mechanism of transport of the drug loaded liposomes, also remains to be explored. Liposomes may either:

- a) Remain in the episcleral space, and release the drug alone, which may transport across the sclera
- b) Transport inside the sclera, and act as a depot and release the drug, which may then be transported across the sclera
- c) Transported intact, together with the drug, across the sclera and the intact system may

then release the drug on the choroidal side.

The aim of this thesis, is to determine the properties of sustained release platforms, which may be useful in delivery of therapeutic agents to the back of the eye, over prolonged duration. The distribution and localization of nanocarriers inside the sclera is of interest to elucidate which of the above mechanisms is actually followed by the nanocarriers. To this end, an *ex vivo* setup is developed in order to serve as a preliminary screening tool, which is then used in understanding the fundamental properties and characteristics of nanocarriers, which determine their trans-scleral transport properties. The understanding derived from this study is valuable in designing carrier systems for sustained delivery of different therapeutics, which may be dependent on the specific drug-carrier system as well.

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Chapter 3

Experimental Methodology

This chapter gives a detailed description of the experimental procedures used for assembling and optimizing the ex vivo setup, scleral tissue isolation and preparation for use in the ex vivo setup, nanoparticle fabrication, analysis, characterization and data analysis. The sclera was isolated from porcine eyeballs and used in an ex vivo setup to study transport characteristics of anti-VEGF antibodies, nanocarriers of different properties and anti-VEGF loaded nanocarriers. Also described in this chapter are the methods followed post-transport experiments to qualitatively and quantitatively determine the trans-scleral transport as a function of the various (nanocarrier and antibody) properties examined

3.1 Materials

The materials used in this study are tabulated in Table 3.1 below.

Table 3.1: Materials used in the study

Materials	Company
1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC)	NOF Corporation
1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC)	NOF Corporation
1,2-Dimyristoyl-sn-glycero-phosphocholine (DMPC)	NOF Corporation
1,2-Dipalmitoyl-sn-glycero-phosphocholine (DPPC)	NOF Corporation
1,2-dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP)	Avanti Polar Lipids
1,2-dipalmitoyl-3-trimethylammonium-propane (chloride salt) (DPTAP)	Avanti Polar Lipids
1,2-dimyristoyl-3-trimethylammonium-propane (chloride salt) (DMTAP)	Avanti Polar Lipids
1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt) (POPG)	Avanti Polar Lipids
1,2-dipalmitoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt) (DPPG)	NOF Corporation
1,2-dimyristoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt) (DMPG)	NOF Corporation
APTES	Sigma Aldrich
Bovine Serum Albumin	Sigma Aldrich

Carbonate Bicarbonate buffer	Sigma Aldrich
Chemiluminescent substrate for ELISA	Thermo Fisher
Chloroform	Tedia
Cholesterol, 94%	Sigma
Dialysis clips	Spectrum laboratories
Dialysis tubing's (100 kDa and 300 kDa)	Spectrum laboratories
FITC	Sigma Aldrich
Fluoresceinamine	Sigma Aldrich
Histidine-HCl	Sigma Aldrich
Hydroxyapatite nanoparticles	Sigma Aldrich
Jung's Optimal Cutting Compound	Leica Microsystems
Methanol	Tedia
MicroBCA kit	Thermo Fisher
Paraformaldehyde	Sigma Aldrich
PBS tablets	Sigma Aldrich
Phospholipid Assay	Abnova
PLGA	Purac Corbion
Polycarbonate filters (0.2 and 0.08 μm)	Whatman
Polysine slides	Thermo Fisher
Polystyrene Nanoparticles (FITC tagged)	NANOCS
Purified Egg Yolk Lecithin, > 95 Wt. %	NOF Corporation
Rhodamine B	Sigma Aldrich
Rhodamine-tagged lipids	Avanti Polar Lipids

Sodium Azide	Sigma Aldrich
Sucrose	Sigma Aldrich
D-(+)-Trehalose dihydrate	Sigma Aldrich
Triton X 100	Bio-Rad
Tween 20	Bio-Rad
Vectashield Mounting media	Vector Laboratories
Vascular Endothelial Growth Factor (VEGF)	RnD systems
Water with a resistivity of 18.2 MΩ cm	Millipore

Lucentis® (Ranibizumab, MW 48kDa, 10mg/ml, Genentech, South San Francisco, CA) and Eylea® (Aflibercept, MW 115kDa, 40 mg/ml, Regeneron, Tarrytown, NY), were obtained from eye surgeons of Tan Tock Seng Hospital (TTSH) and Singapore National Eye Centre (SNEC), Singapore and drug remnants from the injection vials were extracted for use in our study.

ELISA Assay components and special 96-well plates were purchased from Thermo Scientific, USA, and was used for the detection and quantification of active protein.

3.1.1 Scleral tissue for ex vivo experiments

The primary goal of this work is to study the trans-scleral route of ocular drug delivery and the possibility of sustained release for this mode of drug administration, using nanocarriers. In the intravitreal route of administration, the drug is introduced in the vitreous cavity, whereas in periocular routes of administration, the drug is introduced in the subconjunctival space, and the drug may diffuse through the tissues to reach the target site of action. In order to mimic the trans-scleral mode of drug administration, an *ex vivo* setup was designed using a suitable animal model. The scleral tissue from the eye is then mounted in the *ex vivo* transport flow cell setup. The drug or molecule of interest is

introduced in the episcleral side, and diffusion towards the choroidal side is studied.

Rabbit model is the most commonly used *in vivo* model in ocular drug studies, but rabbit sclera is about ten times thinner compared to human sclera[1], and the ocular vitreous volume is also not comparable with the human eye. Porcine sclera is known to be histologically similar to human sclera, even though it is about twice as thick as human sclera[2]. The collagen fibril organization is also similar between porcine and human sclera as shown in the SEM Image of collagen fibril organization of porcine and human sclera in Figure 3.1 (From Figure 2 in [2]). As such, porcine scleral tissue explants were chosen for use in this study.

Figure 3.1: SEM Image of the Collagen Fibril organization in Human and Porcine Sclera. Original magnification was 5,000X in panel A and 20,000X in panel B. From [2]

3.2 Ex vivo Setup design

3.2.1 Assembly and Optimization of ex vivo setup

An *ex vivo* setup was first designed for studying the transport of small molecules and carriers across the sclera, similar to the ones described in literature [3, 4]

The isolated porcine scleral tissues were mounted in modified Ussing chambers (Navicyle, Harvard Apparatus) using specially designed tissue mounting rings, such that the episcleral side was facing the donor chamber and the choroidal side facing the receiver chamber.

This was done to mimic the subconjunctival drug delivery mode (Drugs will be introduced in the episcleral side and they diffuse through the sclera to the vitreous). The receiver chamber was filled with 2.5 ml of buffer, phosphate buffered saline (PBS) (pH 7.4), with the drug on the donor side. The chambers were maintained at 37°C by means of a circulating water bath. The receiver chamber was perfused with PBS at a flow rate of 0.03ml/min throughout the experimental duration. During the experiment, the perfusate fractions were collected using a Gilson Fraction Collector.

The frozen scleral tissue was thawed for 30 minutes prior to each experiment. The peristaltic pump which is used to perfuse the receiver chamber was calibrated before each experiment. The thawed scleral tissue was cut into a circular section of 0.8 cm diameter, and mounted on the specially designed tissue mounting ring. The mounting ring was placed between the two chambers of the Ussing chamber, and the flow cell bubbles were eliminated and the system is allowed to equilibrate for about 30 minutes before start of transport experiments. The fraction collector time was adjusted and the receiver chamber was sampled at regular intervals by the fraction collector. The collected samples were analyzed for protein/drug content by appropriate techniques. The setup is schematically represented by Figure 3.2.

Figure 3.2: Schematic of the *ex vivo* transport system. The sclera is mounted on a specially designed tissue mounting ring between the donor and receiver chambers of the Ussing chamber. The circulator water bath maintains the temperature of the setup at 37°C. A peristaltic pump perfuses the receiver chamber with the buffer, and receiver chamber fractions are collected at predetermined intervals using the fraction collector.

The scleral tissue was tested for leakage prior to each experiment, by introducing buffer solution in the donor chamber with the sclera mounted and the setup was placed on a dry surface lined with Kim wipes. Any potential leakage due to problems in tissue mounting or cutting would result in buffer leaking onto the surface, and this was done for up to 30 minutes prior to experiment. Moreover, at the end of each experiment, the tissue was carefully observed for any leaks, and in addition during sectioning, the edges of the tissue where the tissue mounting ring ends, were carefully examined for any leak.

3.2.2 Preparation of Scleral tissue for *ex vivo* transport experiments

Porcine eyeballs were obtained from a local abattoir, with permission from the Agri-Food and Veterinary Association (AVA), Singapore. The adherent muscle tissue from the eye

was gently removed, and the eye was excised at the limbus. The anterior chamber was cut out, and the vitreous gel was completely removed. The choroid and retinal layers were gently peeled away with forceps and the sclera was washed with PBS. The scleral cup was then cut into four equal parts (Figure 3.3) and used either immediately or stored at -80°C until use.

Figure 3.3: Porcine scleral tissue after dissection and removal of the anterior segment tissues, vitreous humour, choroid and retinal tissues.

The freezing procedure is not expected to affect scleral histology [2] and, according to Olsen et al. [5], should not influence scleral permeability. The thickness of the scleral samples used for the ex-vivo experiments was measured with a digital caliper (Mitutoyo digital micrometer IP65 resolution 0.001 mm; Mitutoyo, Japan) and found to be 0.95 ± 0.25 mm.

3.3 Fabrication of nanocarriers for transport studies

To study the properties of nanocarriers affecting their ex vivo transport and scleral distribution, different types of nanocarriers were fabricated or purchased for the transport

studies. Details of the different carriers studied are described in detail in the following sections.

3.3.1 Liposomes

Liposomes were prepared by dissolution of all lipids in chloroform/methanol –mixture (3:2, vol/vol). For charged liposomes, appropriate amounts of the charged lipids were added into the lipid mixture. The solvent was evaporated using a rotary evaporator forming a thin lipid film on the round bottom flask. The film was then hydrated with PBS, pH 7.4 to generate multilamellar vesicles (MLVs). Further size reduction was achieved using a bench top extruder (Northern Lipids Inc, Canada), fitted with polycarbonate filters of different sizes, typically 0.2 μm , 0.1 μm and 0.08 μm to generate Large Unilamellar Vesicles (LUVs). For preparation of fluorescently tagged liposomes, the fluorescent lipids (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt)) were added in the organic solvent phase at a 0.1 mole % ratio, along with the base lipids, prior to the rotary evaporation phase.

The average particle size, polydispersity index (PDI) and zeta potential of the liposomes were monitored using a Malvern Zetasizer Nano ZS, UK. Cryo TEM was used to image the liposomes, and the procedure is described in Appendix A.

3.3.1.1 Characterization of Liposomes

The liposomes were characterized for their average size, size distribution and zeta potential by using Malvern Zetasizer Nano ZS (UK). The samples were diluted 100-times in deionized water, prior to the measurements. This device uses the principle of dynamic light scattering for the size measurement and the intensity of scattered light was measured at a fixed detector angle of 173° with a laser power intensity of 633 nm. The technique measures the size of particles based on their diffusion under Brownian motion in solution. The viscosity and refractive index of water at 25°C (0.8937 and 1.333 cP respectively)

were used for all measurements. The size of the liposomes was measured after fabrication, as well as upon storage.

The zeta (ζ)-potential is used to measure the intensity of the repulsive electrostatic interaction between charged colloidal particles. For charged particles, the zeta potential was measured using the Malvern Zetasizer Nano ZS (UK). A laser source is used to illuminate particles within the samples. When an electric field is applied to the measurement cell, any particles moving through the measurement volume will cause the intensity of light detected to fluctuate with a frequency proportional to the particle speed. This information is passed to a digital signal processor, and the electrophoretic mobility and hence the zeta potential information is calculated.

3.3.1.2. Liposome quantification by Phospholipid Assay

The liposomes quantification was attempted by using a Phospholipid Assay Kit, Abnova KA1635 purchased from Abnova Corporation, Taiwan and the assay was performed according to manufacturer's instructions. In this assay, phospholipids are enzymatically hydrolyzed to choline which is determined using choline oxidase and a H_2O_2 specific dye. The optical density of the pink colored product at 570 nm or fluorescence intensity (530/585 nm) is directly proportional to the phospholipid concentration in the sample.

The working reagent is prepared using the Assay Buffer, Enzyme Mix, PLD enzyme mix and dye reagent provided in the kit.

Standard calibration curves are also prepared using the 2mM lipid standard provided in the kit. 20 μ l of the sample is first pipetted onto the 96-well plate, followed by 80 μ l of the working reagent. The concentration of the unknown test sample was calculated by comparing it with the standard calibration curve prepared.

3.3.2 PLGA Micro and Nanoparticles

Fluorescently tagged PLGA micro and nanoparticles were prepared using a method adapted from Weiss et al. [6]. Briefly, PLGA (lactide:glycolide 50:50) and

fluoresceinamine were dissolved in acetonitrile with DMAP and incubated at room temperature for 24h under light protection and gentle stirring. The resulting FA-PLGA is then used for preparation of PLGA nano and microparticles. The procedure is explained schematically in Figure 3.4.

Figure 3.4: Schematic for fluorescent labeling procedure of PLGA with Fluoresceinamine

For the preparation of PLGA micro and nanoparticles, the following protocol was followed. A known amount of 50:50 (lactide:glycolide) PLGA-FA was weighed out and dissolved in DCM with sonication. The resulting mixture was added to 1% PVA, and homogenized for 2 hours at room temperature at 1000 rpm. The resulting solution was washed 3 times by suspending the formed particles in DI water, centrifuging at 10,000 rpm for 15 minutes. The particles are then freeze dried in the presence of 1% mannitol as cryoprotectant.

3.3.3 Polymersomes

Fluorescently labelled polymersomes for *ex vivo* transport studies were provided by colleagues from NTU-Northwestern Institute of Nanomedicine (NNIN). The preparation method followed is briefly described here. Polymersomes from PBD21: Polybutadiene-b-polyethylene oxide (PBD-b-PEO) were used in the study. The required amount of polymer was weighed out along with 0.1 mole % lissamine-rhodamine PE in chloroform.

The solvent was removed by blowing nitrogen gas into the test tube and by further drying in dessicator under vacuum for 24 hours. The film so formed was rehydrated with PBS and continuously stirred to obtain a homogenous solution, which was then extruded through a hand held mini-extruder to obtain polymersomes. The extruded formulations were left at room temperature for 24 hours to equilibrate.

3.3.4 Polystyrene nanoparticles

FITC-tagged polystyrene nanoparticles of sizes 100 nm were purchased from NANOCS Inc. The nanoparticles were provided as a 1% solution and were appropriately diluted for transport experiments. The manufacturer's report indicates that the dye does not leach out from these particles and the dye attachment is stable for upto 6 months of storage in the dark.

3.3.5 Gold nanoparticles

FITC-labelled gold Nanoparticles of an average size of ~10 nm were purchased from NANOCS. The particles were obtained as a 0.05% solution in aqueous media, and was diluted appropriately for transport experiments.

3.3.6 Gold- Spherical Nucleic Acid (SNA) Nanoparticles (from Northwestern University)

FITC and Cy5 tagged oligonucleotides and Au-SNAs were provided by Northwestern University as part of a Materials Transfer Agreement between NTU and Northwestern University. The fabrication procedure is explained in detail in [7]. The gold nanoparticles were not tagged fluorescently, and the oligonucleotide was tagged with either FITC or Cy5. The oligonucleotide used was a 20-mer DNA with the FITC/Cy5 in the middle and a concentration of 1.8 mM. For the FITC labelled SNAs, the gold nanoparticle concentration was 1.35 μM , and the oligomer concentration was 80 μM .

For the Cy5 labelled oligomers, the concentration was 481 μM . For the Cy5 labelled SNA particles, the gold NP concentration was 1 μM and the oligo concentration 40 μM . The SNAs were sonicated and vortexed prior to using in the transport experiments. All the particles were used as provided.

3.3.8 Liposomal- Spherical Nucleic Acid (SNA) Nanoparticles (from Northwestern University)

FITC tagged oligonucleotides and liposomal-SNAs were provided by Northwestern University as part of a Materials Transfer Agreement between NTU and Northwestern University. The fabrication procedure is explained in detail in [8]. The liposomes were made from DOPC and tagged with Rhodamine-PE. The total lipid concentration was 18 mM and the rhodamine-PE concentration was 0.4%, to account for any degradation during processing and shipping. The liposomal SNAs had a total lipid concentration of 18 mM lipid and 180 μM oligonucleotide. In this, the liposomes were rhodamine-PE tagged and the oligonucleotide was FITC-tagged.

3.4 Fabrication of Drug loaded Liposomes for transport experiments

There are several challenges associated with encapsulation of large hydrophilic molecules in liposomes with sufficiently high encapsulation efficiencies.

In order to theoretically calculate the amount of ranibizumab that can be encapsulated in the hydrophilic aqueous core of liposomes, the following calculation was performed. The hydrodynamic radius of ranibizumab has been found to be 4.1 nm [9] which corresponds to a volume occupied of 288.7 nm³. Assuming a diameter of the liposomes to be 100 nm, and bilayer thickness of 5 nm, the internal aqueous volume was calculated to be 3.81×10^5 nm³. The number of ranibizumab molecules that can theoretically fit into a liposome was then approximated. The theoretical molar lipid/drug ratio was then evaluated, assuming that the entire aqueous core volume is saturated. Encapsulation of 0.5 mg of ranibizumab (which is the drug dose of Lucentis in a single intravitreal injection) in the liposomes was then attempted, for a final molar ratio of lipid:drug of 5090. The final

lipid concentrations used were 17.8mM.

Two methods of encapsulation were attempted for encapsulation of anti-VEGF antibodies into liposomes. These methods are described in the following sections.

a) Passive loading

In this technique, the thin lipid film formed after rotary evaporation was hydrated with buffer solution containing the drug to passively entrap the drug during spontaneous formation of the vesicles during mixing. Liposomes so formed were further reduced in size by extrusion to obtain smaller vesicles. This method was not found to be as successful in giving high enough values of encapsulation efficiency for protein loading.

b) Dehydration Rehydration Method

Similar to the passive loading technique, the lipid film obtained after rotary evaporation was hydrated with buffer solution containing the drug. Vesicles thus formed were extruded, and freeze dried in the presence of trehalose to obtain lyophilized vesicles. The vesicles were prepared before use by reconstituting the lyophilized material in de-ionized water.

3.4.1 Calculation of Encapsulation efficiency of Liposomes

For calculating the encapsulation efficiency, the prepared liposome formulations were subject to ultracentrifugation using a Bench-top Ultracentrifuge (Thermo Sorvall MTX 150) at 155,000g for one hour at 4° C. Wallace *et al.* [10] have observed that when using ultracentrifugation for liposomes, a small % of the liposomes remained in the supernatant. Nevertheless, the technique is well reported in literature as a technique to separate the untrapped drug from the liposomal formulation, and to calculate the encapsulation efficiency. The centrifuge tubes and collection tubes used were coated with 1% BSA for 24 hours to prevent any protein loss by adsorption to the walls of the tube. The pellet obtained was reconstituted and broken using Triton-X 100 at a final concentration of 1% and analyzed using ELISA after sufficient dilution, to determine the

encapsulated protein content.

The Encapsulation Efficiency (EE) was calculated as follows:

$$EE\% = \frac{\text{Amount of protein in pellet}}{\text{Total amount of protein added}}$$

3.4.2 In vitro Release studies from anti-VEGF loaded Liposomes

In order to study the in vitro release of drug from the drug-loaded liposomes, a dialysis technique was used. Briefly, 1 ml of the drug loaded liposome solution was suspended in a dialysis bag of Molecular Weight Cut off (MWCO) 100kDa, of about 6 cm in length, clipped using dialysis clips at both ends and suspended in amber bottles containing 20 ml of PBS at pH 7.4, which maintained sink conditions. The bags were stirred in the release medium placed inside an orbital shaker incubator, maintained at 37°C and 50rpm shaking speed. At regular predetermined intervals, samples were withdrawn from the release medium, stored for analysis, and the entire medium was replaced with fresh buffer, in order to maintain sink conditions for release. At the end of the release study, the dialysis bag was opened and the liposome formulation was broken with Triton X-100 to quantify the amount of drug left behind.

3.5 Ex vivo trans-scleral transport experiments

This section describes the details of the different kinds of ex vivo experiments performed. The experiments were conducted using porcine scleral tissues, collected from a local slaughterhouse, and prepared as described in Section 3.2.2.

3.5.1 Ex vivo transport of bare anti-VEGF antibodies

In order to study the transport of the bare antibodies through the sclera, the drug molecules were introduced in the donor chamber and transport experiments were performed similar to described in Section 3.2.1. The receiver chamber solution was sampled at pre-determined intervals and was analyzed by ELISA as described in Section

3.2.4.1.1. Initial attempts were made to quantify the total protein using MicroBCA technique, but scleral proteins were found to leach out and interfere in the measurements hence this was not used. These measurements largely served as a benchmark for comparing with the transport of the loaded nanocarriers and the potential of the sclera to act as a depot to sustain the release of the therapeutic molecules. For transport experiments of bare anti-VEGF antibodies, the flow cells, tubing and collection tubes were coated with 1% BSA to prevent non-specific adsorption of protein to surfaces which may lead to a lower quantified value of the protein.

The concentrations of samples in the receiver chamber were then measured and the cumulative amount of protein which was transported was calculated. The permeability constant K_{trans} was determined as indicated in literature.

The Permeability constant (K_{trans}) is calculated as:

$$K_{trans} = \frac{R_{total}}{t * A} \times \frac{1}{D}$$

where,

R_{total} is the total amount of drug in the receiver chamber

t is the fraction collection time (in seconds).

A is the area of exposed sclera (in square centimeters).

This value— $R_{total}/(t * A)$ —is equal to the flux across the tissue.

D is the concentration of drug in the donor chamber.

The area of exposed sclera was 0.5 cm² for the setup used.

3.5.1.1 Quantification of proteins by MicroBCA

The Micro BCA Protein Assay kit determines the protein concentration of dilute samples. It is a bicinchoninic acid (BCA) formulation used for colorimetric detection and quantification of total protein in the range of 0.5-40 µg/ml [11].

MicroBCA reagents were purchased from Thermo Scientific. The MicroBCA kit consists of 3 reagents- Reagent A, B and C to be mixed in the ratio of 25:24:1. The samples are first introduced on the 96-well microplate, followed by an equal amount of the mixed MicroBCA reagent, and incubated at 37°C with gentle shaking for 2 hours. The absorbance is measured at 562 nm, using TECAN microplate reader (model: infinite 200). The concentration of unknown samples was determined by comparing their absorbance with a standard calibration curve, which was prepared using known concentrations of protein.

3.5.1.2 Quantification of antibodies by Enzyme Linked Immunosorbent Assay (ELISA)

Enzyme linked Immunosorbent Assay (ELISA) is a technique used to detect specific proteins. The technique is based on the property of proteins to be adsorbed on a surface. In the “Indirect ELISA” method, a capture antibody, directed against the protein, is linked to a solid support such as a plastic 96 well microtiter plate. If the antibody of interest is present, it will be captured by the bound antibody. The bound antibody is then detected by using a second antibody linked to an enzyme. A chromogenic molecule – one that is converted by the enzyme to an easily detectible product – is then added. There is signal amplification by the enzyme as a single catalytic enzyme molecule is capable of generating many product molecules. The signal generated thus is proportional to the amount of antigen absorbed specifically to the antibody coated in the first stage. Figure 3.5 depicts the above mentioned steps in an illustration.

ELISA based detection of antibodies was performed in a 96 well high-binding polystyrene plate. For detection of Lucentis, the Indirect ELISA technique described above was used. The plates are prepared by using VEGF, which was first incubated in the plates for 24 hours at 4°C. The unbound sites on the plate were then blocked with 2% BSA solution, and the plates were further incubated at 4°C and washed with wash buffer (0.05% Tween-20 in PBS buffer) before use. The samples, diluted in PBS were then

introduced on the plate, and incubated for two hours. The plates were further incubated with horseradish-peroxidase conjugated secondary antibody for 45 minutes. The final step is to trigger the chemiluminescent signal by using SuperSignal ELISA Pico chemiluminescent substrate over a timeframe of 0.1 seconds/well. The detection limit of the assay was approximately 5 ng/ml. Since there was found to be slight plate-to-plate variation in the ELISA standard curves, a standard calibration was done for every plate in each experiment. For Eylea analysis by ELISA, the same procedure was followed, except for use of a different secondary antibody.

Figure 3.5: Illustration of Indirect ELISA Technique, From Leinco Technologies, [12]. Step 1: Antigen (VEGF) is adsorbed in the well of the plate. Step 2: The non-binding sites are blocked by a blocking buffer containing a large protein such as BSA. Step 3: The primary antibody is added which binds to the antigen. Step 4: An enzyme (here, horseradish peroxidase) conjugated secondary antibody is used to bind to the primary antibody. Step 5: A substrate is added, which reacts with the enzyme to form a colored product, the absorbance of which can be measured.

3.5.2 Ex vivo transport of nanocarriers

In order to determine the localization and distribution of nanocarriers within the sclera, fluorescently tagged nanocarriers of different properties were introduced in the donor chamber to study the effect on transport. The carriers described in Section 3.3 were studied for their scleral transport and localization.

3.5.3 Ex vivo transport of anti-VEGF loaded nanocarriers

To study the transport of the antibodies from loaded nanocarriers, the ranibizumab-loaded nanocarriers were introduced in the donor chamber and drug transport experiments were performed similar to described in Section 3.2.1. The receiver chamber solution was sampled at pre-determined intervals and was analyzed by ELISA as described in Section 3.5.1.2. For transport experiments of anti-VEGF loaded carriers, the flow cells, tubing and collection tubes were coated with 1% BSA to prevent any non-specific adsorption of protein to surfaces which may lead to a lower quantified value of the protein. The antibodies were also stored in BSA-coated tubes to prevent losses by non-specific adsorption.

3.6 Post-transport tissue processing

Based on the type of ex vivo experiment conducted, the post-processing was conducted differently.

3.6.1 Ex vivo transport of bare anti-VEGF antibodies

After the transport experiments conducted with anti-VEGF antibodies, the left-over donor solution was removed, sclera was taken out of the setup, and effluxed in fresh buffer every 24 hours to release antibodies trapped inside the sclera. The receiver chamber solution which was collected at pre-determined intervals of time was analyzed by ELISA

to quantify the cumulative amount of drug that passed through the sclera in the given amount of time. The solution left in the donor chamber was also analyzed by ELISA to determine the antibody concentration.

3.6.2 Ex vivo transport of nanocarriers

After transport experiments with nanocarriers, left-over donor solution was removed, the sclera was carefully taken out of the setup and examined for any leakage, and fixed in 4 % paraformaldehyde for 24 hours. The tissue is then washed 3 times with 1x PBS and embedded in Jung's Tissue Freezing Media optimal cutting compound and frozen at -80°C for 24 hours prior to cryosectioning. Cryosections of 7µm thickness are made using a cryostat (Thermo HM550), onto poly-lysine coated slides. The 7µm thick tissue sections were imaged using a Carl Zeiss Axio Imager Z1 microscope (Zeiss, Germany). Bright-field images and fluorescence images at 528 nm–553 nm (red filter; Rh-PE) excitation wavelengths were acquired and overlaid to confirm and evaluate the extent of liposome penetration into the sclera. The Zeiss imaging software and Image J software were used to analyze the distance of penetration of the fluorescent liposomes into the sclera.

3.6.3 Ex vivo transport of anti-VEGF loaded nanocarriers

After the transport experiments conducted with anti-VEGF loaded nanocarriers, the left-over donor solution was removed, sclera was carefully taken out of the setup, and effluxed in fresh buffer every 24 hours to release antibodies trapped inside the sclera. The receiver chamber solution which was collected at pre-determined intervals of time was analyzed by ELISA to quantify the cumulative amount of drug that passed through the sclera in the given amount of time. The solution left in the donor chamber was also analyzed by ELISA to determine the antibody concentration.

3.7 Differential Scanning Calorimetry

The liposomes prepared using DPPC and DPPC: Cholesterol were evaluated by DSC to determine the effect of cholesterol addition on melting transition temperature of the lipid. MLVs of DPPC and DPPC- Cholesterol (80:20 m/m) were prepared as per the procedure followed in Section 3.3.1. The samples used for the DSC experiments were diluted with to final concentration of 1mM in PBS and degassed under vacuum for 15 min prior to being loaded into the calorimetric cell. DSC experiments were performed using a Nano-DSC (TA Instruments) under a constant external pressure of 3 atm in order to avoid bubble formation. Two thermal scans were performed over 20-60 °C, where the calorimeter cells were stabilized for 10 minutes before heating up the samples at a rate of 1 °C/min, and subsequently, cooling them down at 2 °C/min. Experimental data were corrected from small mismatches between the two cells by subtracting a buffer baseline prior to data analysis. The thermograms were defined by the onset and completion temperatures of the transition peaks obtained from heating scans.

3.8 Statistical Analysis

All experiments were performed in triplicates, unless otherwise specified. For all the ELISA and MicroBCA assays performed, a standard curve was included in each 96-well plate for maximum sensitivity and to eliminate plate-to-plate variations. Statistical analysis was performed using t-test, and a probability value of $p < 0.05$ was considered to indicate statistical significance.

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Chapter 4

Trans-scleral Transport of Bare anti-VEGF Antibodies

This chapter describes the trans-scleral transport studies performed using bare anti-VEGF antibody, Lucentis. The effect of buffers and pH on the transport is also studied. Lucentis was introduced in the donor chamber of the setup at a known concentration, and the amount of Lucentis transported through the sclera to the receiver chamber is quantified at different time points using ELISA. This study then serves as a baseline for comparison with the transport of Lucentis from Lucentis-loaded carriers, which is described in Chapter 6. A parallel study conducted with another anti-VEGF antibody, Eylea, is also included for comparison with the Lucentis transport studies.

4.1 Introduction

As described in detail in Section 2.6, the subconjunctival route of administration is significantly less invasive compared to the intravitreal route of injection for treatment of posterior ocular segment disorders. In the subconjunctival route of administration, the drug is administered in the space between the conjunctiva and the sclera. In order to mimic the subconjunctival route in an *ex vivo* setting, a setup is designed as described in Section 3.2.1. Isolated porcine scleral tissues are used as a tissue model, and the drug is administered in the episcleral region, and diffuses across the sclera to reach the choroidal side. In the experimental setup, the drug concentrations in the receiver chamber (the choroidal side of the tissue) are sampled and quantified at different time intervals by ELISA to measure the amount of drug transported.

Specific aims and objectives of this chapter are as follows:

- 1) Determination of total trans-scleral transport of Lucentis through porcine sclera, to serve as a benchmark for transport of Lucentis-loaded nanocarriers
- 2) Determination of the effect of buffers on trans-scleral transport of Lucentis
- 3) Determination of total trans-scleral transport for another Anti-VEGF agent, Eylea and comparison with transport of Lucentis.

4.2 Materials and Methods

Porcine eyeballs were obtained from a local slaughterhouse with permission from AVA, Singapore. The anterior segment tissues, vitreous humour and the choroid and retinal tissues were removed, and the sclera was isolated and stored after dissection as detailed in Section 3.2.2. The scleral tissue was stored at -80°C prior to use. Before start of each experiment, the tissue section for use was thawed at room temperature for 30 minutes. The sclera was dissected to fit into a specially designed tissue mounting ring and fixed between the donor and receiver chambers of an Ussing chamber diffusion setup. After mounting the tissue, it was tested for leakage for upto 30 minutes before starting the transport experiment. The receiver chamber was filled with Phosphate Buffered

Saline(PBS) at pH 7.4, with 0.05% sodium azide added. The chambers, the mounting rings, and tubings used in the experiment were coated with 1% BSA, for 24 hours prior to start of each experiment. This was done to prevent non-specific adsorption of the protein to the surfaces, which was previously found to result in a lower value of the protein quantified. The receiver chamber was perfused using a peristaltic pump at regular intervals, at a flow rate of 0.05 ml/minute. The collection tubes for storage of receiver chamber solution prior to analysis, were also coated with 1% BSA solution to avoid any non-specific adsorption. After the transport experiment, the setup was dismantled, the donor chamber solution was carefully removed, and the sclera was effluxed in PBS buffer which was replenished every 24 hours. The antibody concentration in the receiver chamber fractions was analysed using ELISA as described in Section 3.5.1.2. Each transport experiment was repeated in triplicates.

4.3 Results and Discussion

4.3.1 Trans scleral transport of Lucentis

Trans-scleral transport of bare Lucentis was performed using the *ex vivo* diffusion cell setup. Lucentis was introduced in the donor chamber at a concentration of 1 mg/ml, to maintain a starting amount of 0.5 mg Lucentis in the *ex vivo* setup and the acceptor chamber was sampled every 24 hours by perfusion at a flow rate of 0.5ml/min to ensure collection of the entire contents of the receiver chamber solution, and to replace with fresh buffer, to maintain sink conditions. Receiver chamber fractions were collected in BSA-coated tubes and quantified using ELISA.

The scleral permeability can be expressed by using the scleral permeability coefficient, expressed in cm/s, which is the ratio of steady state flux to the concentration gradient. The steady state flux is calculated as a function of the effective scleral diffusion area exposed to transport, the time over which the steady state transport is attained, and the initial concentration of the solution.

The permeability coefficient (of Lucentis through porcine sclera) was calculated as follows:

Permeability coefficient $K_{trans} = [R_{total}/(A \times t)] \times [1/C]$,

where

R_{total} equals the total moles passing through the sclera during time t ;

t (measured in seconds) is the period between the timepoints,

A is the surface area of the scleral sample (in square centimeters), and

C is the starting concentration of the solute in the donor chamber (moles per milliliter).

The surface area of exposed sclera was 0.5 cm^2 for all the experiments performed. The transport was conducted for a duration of 7 days.

The value of K_{trans} for Lucentis, for transport over the first 24 hours, at pH 7.4, when the transport was conducted in PBS was found to be $1.56 \pm 0.15 \times 10^{-6} \text{ cm/s}$.

These values can be used to infer the overall trans-scleral penetration of Lucentis when injected subconjunctivally. For example, assuming there is zero ocular clearance, with the permeability coefficient of $1.56 \times 10^{-6} \text{ cm/s}$ for Lucentis, an initial drug donor of 10 mg/mL , which is the currently administered dose, will be able to deliver approximately 1.3 mg of the drug across 1 cm^2 sclera in 1 day via the trans-scleral route. This amount is larger than the amount of Lucentis administered monthly via intravitreal injection in current neovascular therapy (0.5 mg).

Figure 4.1: a) Cumulative amount of Trans-scleral transport of Lucentis in PBS at pH 7.4 and b) %Cumulative amount of trans-scleral transport of Lucentis in PBS at pH 7.4

4.3.2 Trans scleral transport of Lucentis: effect of buffers, pH

Ranibizumab is stored and administered in a buffer consisting of 10 mM histidine-HCl, 10% α , α -trehalose dihydrate, 0.01% polysorbate 20, at a pH of 5.5. In order to determine the effect of different pH conditions on the trans-scleral transport of Lucentis, the transport was conducted in PBS at pH 7.4, and Intrinsic Buffer (IB) of Lucentis at pH 5.5. Figure 4.2 shows the trans-scleral transport of Lucentis in the Lucentis Intrinsic Buffer (IB) at a pH of 5.5.

Figure 4.2: a) Cumulative amount of Trans-scleral transport of Lucentis in IB at pH 5.5 and b) %Cumulative amount of trans-scleral transport of Lucentis in IB at pH 5.5

Compared with Figure 4.1, in which the trans-scleral transport was performed in PBS at pH 7.4, the total % cumulative transport in IB at pH 5.5 is about 2 times lower.

In addition, to check the effect of pH, the transport was also performed in phosphate buffer at pH 5.5. This was done to check the effect of pH on the trans-scleral transport of proteins in the *ex vivo* setup. It was observed that for the buffers at pH 5.5 (Phosphate buffer and IB), the transscleral transport was lower compared to the transport in PBS at pH 7.4. The results are depicted in Figure 4.3. No difference in cumulative transport was observed between the different buffers at pH 5.5. From the results obtained here, it was found that the Lucentis transport was significantly higher at pH 7.4 compared to pH 5.5.

A significant observation was the fact that using two different buffers at the same pH

(Intrinsic Buffer IB and Phosphate buffer, both at pH 5.5), there was no significant difference observed in the transport. This points to the role of pH in affecting the global charge of the protein, which in turn affects the transport of the drug through the sclera.

The excipients that Ranibizumab is stored and administered in, consists of 10 mM histidine-HCl, 10% α,α -trehalose dihydrate, 0.01% polysorbate 20, buffered at a pH of 5.5. All these excipients have a role in maintaining the stability of the antibody against aggregation and instability on long term storage. However, the ocular physiological conditions are more closely mimicked by PBS at pH 7.4, which is a commonly used buffer for release and in vitro studies.

Figure 4.3: Comparison of the % Cumulative transport of Lucentis in PBS at pH 7.4, in Phosphate buffer at pH 5.5 and in IB at pH 5.5.

Based on the observations from these set of experiments, PBS was used as a buffer for subsequent transport experiments involving the Lucentis loaded nanocarriers.

4.3.3 Trans-scleral transport of Eylea

Aflibercept (marketed under the trade name Eylea) is a 115kDa sized antibody, also approved by FDA in 2011 for treatment of AMD. Aflibercept acts as a "VEGF trap" by binding to the circulating VEGF molecules. It is capable of inhibit the activity of VEGF subtypes VEGF-A and VEGF-B, as well as to placental growth factor (PGF). Due to its higher binding affinity and longer duration of action, Aflibercept (Eylea) has theoretical advantages over Ranibizumab (Lucentis). Trans-scleral transport of Eylea was also studied using the *ex vivo* setup, similar to Lucentis studies described in Section 4.3.1 in order to compare with the transport of Lucentis.

The starting concentration of Eylea was maintained at 1 mg/ml, similar to the Lucentis transport experiment. The surface area of exposed sclera was 0.5 cm² for all the experiments performed. The scleral permeability coefficient was determined similar to the procedure described in Section 4.3.1

The value of K_{trans} for Eylea, in PBS at pH 7.4, was found to be $4.23 \pm 1.79 \times 10^{-7}$ cm/s. In comparison, the value of scleral permeability coefficient K_{trans} for Lucentis, for transport over the first 24 hours, in PBS at pH 7.4 was found to be $1.56 \pm 0.15 \times 10^{-6}$ cm/s.

The K_{trans} value for Aflibercept is found to be ~10 times lower than that of Ranibizumab. This result is expected, considering that the molecular weight of Aflibercept is 115 kDa, while that of Ranibizumab is 48 kDa. The trans-scleral diffusion is found to be inversely proportional to the molecular weight and molecular size of the permeants [1, 2].

Figure 4.4: Comparison of the % Cumulative trans-scleral transport of Lucentis and Eylea at pH 7.4

4.4 Summary

In this chapter, transport of the bare Lucentis across porcine sclera was studied, and the effect of different pH and buffer condition was characterized.

Lucentis is stored and administered in a buffer consisting of 10 mM histidine-HCl, 10% α,α -trehalose dihydrate, 0.01% polysorbate 20, buffered at a pH of 5.5. Sugars such as sucrose and trehalose are typically used as additives in protein buffers as they have the propensity to stabilize proteins by preferential hydration when water is in excess, and water replacement when the protein is in the solid state [3]. Non-ionic surfactants may also be used to reduce the non-specific adsorption, consequent aggregation and unfolding of proteins by preferentially adsorbing to surfaces in place of the protein and thus lowering the system's surface tension. Another strategy commonly used is the addition of proteins such as albumins and gelatins for protective purposes, but this obviates the use of certain analytical methods for protein quantification. For example, techniques such as MicroBCA, which are non-specific methods for protein quantification, cannot be used for quantifying the total protein content when BSA or HSA is used as a stabilizer, as these

protein amounts will also be added up in the total quantified amount. Amino acids such as histidine, arginine, lysine etc. are sometimes added to stabilize protein formulations. The amino acids are thought to bind transiently to the antibody side chains, altering the hydration property of the solvation shell, thus making unfolding and aggregation less energetically favourable [4]. In all the trans-scleral experiments involving Lucentis and Eylea, which were performed without the use of excipients, the transport flow cells, collection tubes and the tubings were coated with 1% BSA to prevent loss of protein by non-specific adsorption.

From the observations in this chapter, it was observed that the Lucentis transport was significantly higher at pH 7.4 compared to pH 5.5 ($p < 0.05$). A significant observation was the fact that using two different buffers at the same pH (Intrinsic Buffer IB and Phosphate buffer, both at pH 5.5), there was no significant difference ($p > 0.05$) observed in the transport, pointing to the role of the global charge of the proteins in affecting scleral transport.

The sclera is largely acellular and is composed of collagen fibrils arranged in a random fashion, and this imparts a net negative charge to the sclera[5]. Lucentis is known to have an isoelectric point(pI) around 8.8[6]. This means that at pH 5.5, the Lucentis is relatively more positively charged, compared to pH 7.4. Due to the scleral negative charge, it is likely that the Lucentis at pH 5.5 has a stronger affinity to the sclera, resulting in less protein being transported across the sclera.

Since ELISA is a highly specific technique for protein quantification, only the active protein which has unblocked binding sites and is able to bind to VEGF would be quantified by this technique.

For hydrophilic molecules, the molecular size is a dominant factor which affects diffusion through the sclera. A previous study has demonstrated the hydrodynamic radius of ranibizumab to be 2.76 ± 0.04 nm and, 3.70 ± 0.03 nm for aflibercept [7]. Using these values, the aqueous diffusion coefficient for ranibizumab was found to be 8.2×10^{-11} cm/s

and that for aflibercept was found to be 6.11×10^{-11} cm/s at 37°C. As for the scleral diffusion, a trend of higher scleral diffusion was observed for molecules of larger molecular weight, compared to small molecular weight compounds, from previous studies[1, 8]. In this study, a similar trend was observed, with slower diffusional transport observed for the larger antibody Eylea compared to Lucentis. In a previous study using human sclera for determination of transport characteristics of Lucentis and Avastin, it was observed that Lucentis demonstrated higher permeability and shorter transport lag time compared to Avastin. In our study, it was observed that there was a similar trend of molecular size- dependent permeation. In addition to molecular weight, the molecular charge of a macromolecule can affect its trans-scleral transport and permeability [9, 10]. At physiological pH of 7.4, human sclera is negatively charged. Bevacizumab and Ranibizumab have a slight positive charge at physiological pH[6]. This could result in charge-charge interactions between the molecules and sclera, which can affect the transport by possible binding to the scleral tissue. The positively charged molecules demonstrate greater transport lag time compared to negatively charged molecules, with regards to trans-scleral transport. This might be a possible explanation for the lower transport of the Anti-VEGF molecules observed at pH 5.5 compared to pH 7.4.

Currently, the FDA approved anti-VEGF drugs Lucentis and Eylea are administered intravitreally for treatment of AMD. Trans-scleral delivery route is a safer alternative, and this study shows that the antibody demonstrates trans-scleral transport, and hence the possibility of this route as an alternative to intravitreal delivery. A few other studies on trans-scleral permeation of anti-VEGF molecules (particularly bevacizumab) have focused on the use of invasive techniques such as iontophoresis, macroesis etc to deliver the agents to the posterior segment [11-14]. Wen et al have demonstrated in a similar study, the trans-scleral permeability of bevacizumab and ranibizumab, in addition to dextrans and ficolls, using human sclera in an *ex vivo* setup. [8]. Ranibizumab has a molecular weight of 48 kDa, which is smaller than both bevacizumab, 149 kDa and aflibercept 115 kDa. Hence, similar to the results obtained by Wen et al., it would be expected that aflibercept would show a slower transscleral permeability compared to ranibizumab, but higher than that obtained from studies with bevacizumab.

This study is significant in showing the possibility that the anti-VEGF agents, primarily ranibizumab can be effectively transported across the sclera, and the possibility of administration via the trans-scleral route, through subconjunctival injection or episcleral implants. However, *in vivo*, the smaller size of ranibizumab may make it more susceptible for rapid clearance by the episcleral circulation and the lymphatic drainage systems, compared to bevacizumab or aflibercept. In similar studies of subcutaneous antibody administration, lymphatic clearance was found to be a more predominant factor in clearance of antibodies after administration[15]. Studies by Ranta *et al.* have examined the barriers to periocular drug delivery using computational models in combination with existing *ex vivo* and *in vivo* data[16]. The main implications of this study were that local clearance by the blood flow and lymphatics removes most of the drug dose injected subconjunctivally. *In vivo* studies are warranted to determine the pharmacokinetics and clearance profiles of ranibizumab and aflibercept introduced subconjunctivally. This brings us to an important point, the need for development of sustained release mechanisms which can not only prolong the release of ranibizumab, but also protect the bare Ranibizumab from faster clearance. In this particular case, the sclera can act as an additional reservoir for the drug, instead of acting as a barrier to the transport.

When using sustained release mechanisms for delivering ranibizumab transsclerally, there will be two barriers to the transport of drugs to the target tissues: one being the carrier, and the other being the scleral tissue itself. The results obtained in this chapter addresses the latter, as it can be observed from the release profiles that the barrier action of the sclera is able to provide a depot effect by slowing down the release of the drug from the episcleral space. If the main barrier to delivery by the periocular route is the sclera, this simple ranibizumab delivery route will be able to deliver effective therapeutic agents to the posterior segment tissues. However, dynamic barriers such as circulatory systems and lymphatic drainage systems are more important barriers, preventing the drugs from reaching high enough concentrations in the posterior ocular tissues [17]. Hence, sustained delivery systems developed to encapsulate the drug in sufficiently high amounts would be

a better drug delivery system accounting for the drug clearance. These studies form the basis of the research described in Chapter 5 and Chapter 6.

Overall, this study would be a useful baseline for comparison with trans-scleral transport studies of Lucentis-loaded nanocarriers (Chapter 6).

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Chapter 5

Trans-scleral Transport of Nanocarriers

The results and findings from transport experiments conducted with various kinds of nanocarriers is explained in detail, in this chapter. In order to study the properties affecting transport, an array of particles with different properties were studied for transport, such as liposomes, polymersomes, PLGA micro and nanoparticles, polystyrene nanoparticles, gold nanoparticles, and Spherical Nucleic Acid (SNA) Nanoparticles. The particles chosen for transport studies were such that properties such as size, charge, flexibility and hydrophobicity and their effect on the trans-scleral transport could be explored for the different types of carrier systems.

5.1 Introduction

As described in Section 2.6, trans-scleral transport properties of small molecules, proteins as well as some macromolecules have been described in great detail in literature. However, there are no systematic studies conducted to date on transport of nanocarriers with different properties through the trans-scleral route. Studies by Kim *et al.* [1] suggest that the drug can penetrate trans-sclerally but the studies indirectly conclude that nanocarriers do not make their way through the sclera. Also, in a study by Amrite *et al.* [2], the fate of rigid polycarbonate nanoparticles (fluorescently labelled) of sizes 20 nm, 200 nm and 2000 nm were studied after subconjunctival injection. It was observed that even for sizes as small as 20 nm, the transport into the sclera was less than 0.5 %.

Liposomes are polymeric nanocarriers widely used for drug delivery, and particularly gaining prominence in ocular drug delivery. However, liposomes have not been studied to date for trans-scleral transport. Previously, Natarajan *et al.* have observed that a single subconjunctival injection of liposome-encapsulated Latanoprost was able to provide sustained IOP reduction in rabbits for upto 5 months [3, 4]. The mechanism by which this was possible, or the fate and distribution of the carriers was not elucidated. The sustained action of the drug seems to be from its action as a depot in the subconjunctival region. As already proven from the results in Chapter 4, Anti-VEGF antibody Lucentis is able to transport across the sclera in the ex vivo transport studies. Hence there is potential that the drug encapsulated in nanocarriers, and administered in the subconjunctival space may provide sustained delivery targeting the posterior segment as well. No systematic studies have also been conducted to determine the trans-scleral penetration and disposition of the nanocarriers themselves, post subconjunctival injection. It was hypothesized that nanocarriers such as liposomes are able to act as a depot, either episclerally or intrasclerally and release the drug, which can then traverse transsclerally to be delivered to the target tissues. To test this hypothesis, this section investigates several carrier formulations with different properties, and the effect of each of these on the transport is studied. Liposomes were used as a preliminary tool to study the transport, by fabricating formulations of different sizes, charges, and acyl chain composition. Further studies were

performed with Polymersomes, PLGA Micro and Nanoparticles, Polystyrene nanoparticles, Gold nanoparticles and Spherical Nucleic Acid (SNA) nanoparticles to elucidate other factors affecting the transport, as well as to understand the transport phenomena.

5.2 Materials and Methods

In order to study the localization and transport of nanocarriers in the sclera, fluorescently tagged nanocarriers were used in the study. Preparation of the carriers are described in Section 3.3. After the completion of each *ex vivo* trans-scleral transport, the remaining donor chamber solution was removed, and the sclera was taken out, fixed in 4% PFA for 24 hours. The tissue was then washed 3 times with 1x PBS and fixed in Jung's OCT compound at -80°C for 24 hours. Cryosections of 7µm thickness were made and transferred to polysine glass slides and imaged using epifluorescence microscopy. The procedure is explained in detail in Section 3.6

5.3 Results and Discussion

5.3.1 Liposomes

Liposomes are versatile polymeric nanocarriers in that their size, charge and lipid composition can be varied easily and they are able to encapsulate both hydrophobic as well as hydrophilic drugs. For the trans-scleral transport, liposomes were chosen as a preliminary model carrier molecule for transport studies. Phospholipids, which are the building blocks of liposomes exhibit a temperature dependent ordering behavior. Lipids exhibit a phase transition temperature, which is affected by factors such as hydrocarbon length, unsaturation, charge, and headgroup species. Also, presence of a cis double bond into the acyl group puts a kink in the chain which requires much lower temperatures to induce an ordered packing arrangement. At temperatures below the melting transition temperature (T_m) the acyl chains are more tightly packed due to increased van der Waal's forces and interaction between the chains. This ordered packing leads to constrained motion of the chains, and decreased permeability of drugs and solutes across the bilayer. At temperatures above the transition temperature, the van der Waals and hydrophobic

forces of interaction become weaker leading to disordered acyl chain packing and increased flexibility of the bilayer. The T_m is characteristic of the lipids and saturated lipids have higher T_m and unsaturated lipids have lower T_m .

Figure 5.1: Schematic representation of lipid bilayer arrangement for a) POPC liposomes and b) DPPC liposomes. POPC is an unsaturated lipid type, and has a T_m of -2°C , and hence a more leaky/fluidic bilayer while DPPC is a saturated lipid type, with a T_m of 41°C which has a more rigid bilayer structure.

The acyl chain composition of POPC with a saturated chain in the sn-1 position and unsaturated chain in the sn-2 position makes the lipid bilayer of POPC have a leaky structure and has a lower transition temperature. Hence, POPC, an unsaturated lipid type was selected for preliminary studies.

5.3.1.1 Trans-scleral transport as a function of time

Trans-scleral transport using *ex vivo* setups have been studied for several drugs, small molecules and even proteins, for time points of typically 24 hours[5, 6]. The initial transport experiments were thus designed for 24 hours. The protocol used is explained in Section 3.5.2. Transport experiments were conducted for a few formulations at first at different time points to determine which time point to consider for further transport studies.

Formulations preliminarily compared were POPC LUV of ~ 90 nm and ~ 70 nm, POPC-DOTAP LUV (positively charged) and POPC-POPG LUV (negatively charged). This transport study of some representative carriers over 1-day and later extended to 4 days was performed in order to determine the time duration best suited for transport studies in Figure 5.2 to Figure 5.5.

Figure 5.2 shows the transport of POPC ~90 nm liposomes, over 1-day (a) and 4-days (b). It can be seen that over time, there is a larger diffusion into the sclera.

Figure 5.2: Representative Epifluorescence micrographs of a) ~90 nm POPC after 1-day transport and b) ~90 nm POPC after 4-day transport. The edges of the tissue have been marked for clarity. The liposomes are tagged with Rhodamine-PE (red).

Figure 5.3: Representative Epifluorescence micrographs of a) ~70 nm POPC after 1-day transport and b) ~70 nm POPC after 4-day transport. The edges of the tissue have been marked for clarity. The liposomes are tagged with Rhodamine-PE (red).

Figure 5.3 shows the transport of POPC 70 nm liposomes, over 1-day (a) and 4-days (b). It can be seen that compared to the POPC 90 nm liposomes, there is a larger degree of transport into the sclera at the 1-day time point, and over time, there is a greater diffusion into the sclera at 4 days compared to the POPC 90 nm liposomes.

Figure 5.4 shows the transport of positively charged POPC DOTAP LUV liposomes, over 1-day (a) and 4-days (b). It can be seen that compared to this set of liposomes, there is more of an episcleral distribution rather than an intrascleral diffusion, which does not change with time. Even at the 4-day time point, the diffusion is similar to what is observed at 1-day, without intrascleral diffusion. This points to a charge-charge interaction of the carriers with the sclera.

Figure 5.4: Representative Epifluorescence micrographs of a) POPC DOTAP LUV after 1-day transport and b) POPC DOTAP LUV after 4-day transport. The edges of the tissue have been marked for clarity. The liposomes are tagged with Rhodamine-PE (red).

Figure 5.5 shows the transport of negatively charged POPC POPG liposomes, over (a) 1-day and (b) 4-days. It can be seen that over 4 days, there is a certain degree of diffusion into the sclera, as compared to the positively charged POPC DOTAP liposomes, but less than the neutral POPC 90 nm liposomes.

Figure 5.5: Representative Epifluorescence micrographs of a) POPC POPG LUV after 1-day transport and b) POPC POPG LUV after 4-day transport. The edges of the tissue have been marked for clarity. The liposomes are tagged with Rhodamine-PE (red).

Qualitatively, it is visible that at Day 4, there is a higher diffusion of the liposomes into the sclera compared to Day 1. Comparing the charged formulations depicted in Figure 5.4 and Figure 5.5 a) and b) it is seen that at Day 1, the transport behavioral differences are not obvious between the formulations. However, at Day 4, the differences are noticeable, for example the positively charged liposomes clearly depict an episcleral localization, possibly due to charge-charge interactions with the sclera, compared to the neutral liposomes, which show an intrascleral diffusion and localization. Therefore, after considering the 1 day and 4-day transport behavior of the different liposomal formulations, the 4-day time point was chosen for all further experiments, as by this time point, marked differences in transport were visible.

5.3.1.2 Effect of size on trans-scleral transport

The effect of size of liposomes on the transport properties was explored by using Multilamellar Vesicles (MLVs) and Large Unilamellar Vesicles (LUVs) of different sizes. For preparation of MLVs, the lipid film was prepared as described in Section 3.3.1, with addition of fluorescent Lissamine-Rhodamine PE, and the hydrated film was used as such without extrusion. For LUVs of 90 nm size, the hydrated film was subject to extrusion through polycarbonate filters of 0.2 μm size for 5 times and 0.08 μm size for 7 times.

Further size reduction was achieved by extruding the MLVs through 0.2 μm filters 5 times, 0.08 μm sized filters 7 times and 0.05 μm sized filters 7 times. The properties and stability for the liposomes are tabulated in Table 5.1

Table 5.1: Z-average size and PDI of the liposomes used in the size-dependent transport study

Formulation	Z average Size (d nm)	PDI
POPC MLV	1471	0.22
POPC LUV	94.9	0.09
POPC LUV	73.2	0.07

Figure 5.6: Representative Epifluorescence micrographs of a) POPC MLV b) POPC LUV ~ 90 nm and c) POPC LUV ~ 70 nm after 4-day transport. The edges of the tissue have been marked for clarity. The liposomes are tagged with Rhodamine-PE (red).

As seen from the epifluorescent micrographs in Figure 5.6, the POPC MLVs which were of size ~ 1.5 μm were found to be localized in the episcleral region, and not show appreciable intrascleral diffusion. On the other hand, liposomes of average size 94 nm were found to show some intrascleral diffusion. Smaller liposomes of average size 73 nm size were found to show even further diffusion into the sclera, compared to the 94 nm sized liposomes**.

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5.3.1.3 Effect of charge on trans-scleral transport

The effect of charge of the particles on ex vivo transport was investigated by the use of negatively charged and positively charged nanoparticles for transport studies. Similar studies were performed to elucidate intravitreal diffusion of nanoparticles previously[7-10], but not for their effect on trans-scleral transport.

In order to study the effect of charge of the nanocarriers on transport, liposomes were prepared using charged lipids in defined ratios, in addition to using the base lipid. For positively charged liposome transport studies, POPC and DOTAP were used in 80:20 molar ratio, along with 0.1 mole % of fluorescent Rhodamine PE. For negatively charged liposome transport studies, POPC and POPG were used in 80:20 molar ratio, along with fluorescent Rhodamine PE. In order to further verify if this effect is due to charge of liposome, and to elucidate any interplay between liposome saturation and charge, one other set of charged liposomes were studied:

- a) DPPC-DPTAP of 80:20 molar ratio which was positively charged and DPPC-DPPG of 80:20 molar ratio which was negatively charged.

The size and Zeta potential of the formulations used in the study are tabulated in Table 5.2 below:

Table 5.2: Z-average size and PDI of the liposomes used in the charge-dependent transport study

Formulation	Z average Size (d nm)	PDI	Zeta potential (mV)
POPC DOTAP	87.5	0.08	+59.2 ±15.3
POPC POPG	90.4	0.07	-61.1 ± 16.3
DPPC DPTAP	96.0	0.11	+38.5 ± 11.0
DPPC DPPG	110.0	0.098	-44.7 ±19.3

Figure 5.7 b) and c) shows the transport of POPC-DOTAP LUVs and POPC-POPG LUVs respectively at 4 days as compared with POPC LUVs in a). It can be seen that the

positively charged liposomal formulation sticks to the episcleral surface without demonstrating any transport into the sclera, whereas comparatively, the negatively charged POPC-POPG shows some intrascleral transport. This could be explained based on the charge-charge interactions of the particles with the sclera. The positively charged liposomes exhibit charge-interaction with the negatively charged sclera, and adhere to the surface without diffusing in trans-sclerally.

Figure 5.7: Representative Epifluorescence micrographs of a) POPC 90 nm vs b) POPC-DOTAP c) POPC-POPG after 4-day transport. The edges of the tissue have been marked for clarity. The liposomes are tagged with Rhodamine-PE (red).

Figure 5.8: Representative Epifluorescence micrographs of a) DPPC vs b) DPPC-DPTAP c) DPPC-DPPG after 4-day transport. The edges of the tissue have been marked for clarity. The liposomes are tagged with Rhodamine-PE (red).

The same observations were confirmed by studying different pairs of charged lipids, using DPPC as base lipid (Figure 5.8).

From these two sets of studies, a clear effect of the charge of liposomes on transport could be observed. The positively charged liposomes made from POPC-DOTAP and DPPC-DPTAP are all localized preferentially at the episcleral space, and show no appreciable transport into the sclera. This can be attributed to charge-charge interactions between the negatively charged sclera and the positively charged carrier. Negatively charged liposomes made of POPC-POPG and DPPC-DPPG on the other hand are found to show some episcleral penetration and transport. This could be due to absence of any attractive interaction with the sclera, resulting in some transport in the ex vivo setup. This has previously been observed for trans-scleral transport of charged molecules [11, 12] where negatively charged molecules were found to transport better in the sclera compared to positively charged molecules. Similarly, in a study of charged nanoparticle transport and diffusion in the vitreous humor (which is also a gel-like network of collagen fibers) negatively charged nanoparticles were found to be able to diffuse in the vitreous while positively charged nanoparticles remained stuck at the vitreous humor without showing appreciable transport[9].

5.3.1.4 Effect of lipid saturation (flexibility) on trans-scleral transport

To study the effect of the backbone chain on the transport, liposomes of different saturation properties were prepared and evaluated for transport. Liposomes of different melting transition temperature (T_m) were selected for this purpose. As explained in Section 5.3.1, the melting transition temperature represents the temperature below which the liposomes exist in an ordered packed bilayer state, and above which the membrane is more fluid. The T_m of lipids is affected by several factors such as fatty acid acyl chains, addition of cholesterol, head groups, pH and ionic strength. Saturated lipids have higher T_m and consequently lower membrane flexibility at physiological temperature compared to unsaturated lipids which have lower T_m . The size and saturation of the formulations used in the study are tabulated in Table 5.3 below:

Table 5.3: Z-average size and saturation of the liposomes used in the lipid-saturation dependent study

Formulation	Z average Size (d nm)	Saturation	T _m
POPC LUV	94.9 (0.09)	16:0,18:1	-2°C
DPPC	87.3 (0.05)	16:0,16:0	41°C
DOPC	91.6 (0.03)	18:1, 18:1	-18°C

Figure 5.9: Representative epifluorescence micrographs of a) POPC LUV b) DPPC LUV and c) DOPC LUV. The edges of the tissue have been marked for clarity. The liposomes are tagged with Rhodamine-PE (red).

As can be observed from the epifluorescence images in Figure 5.9, the DPPC liposomes, which have a transition temperature of 41°C demonstrate less degree of intrascleral diffusion compared to the POPC and DOPC liposomes which have T_m of -2°C and -18°C respectively. The structures of the lipids a) POPC, b) DPPC, and c) DOPC are shown in Figure 5.10. DPPC liposomes have a more rigid bilayer structure, compared to POPC or DOPC, being a saturated lipid type.

The melting transition temperature of the saturated lipid type DPPC is 41°C compared to that of unsaturated lipid type POPC, which is -2°C. Therefore, at the experimental temperature which is 37°C, POPC exists in the gel phase with increased membrane fluidity whereas the packed rigid bilayer structure of DPPC is still maintained. This

relationship between membrane fluidity and transition temperature is schematically illustrated in Figure 5.11.

This is also observed in case of DOPC, which has double unsaturation in the acylic chains, which reflects in an even lower transition temperature of -18°C .

Figure 5.10: Representative structural representation of acyl chain and saturation of a) POPC b) DPPC and c) DOPC. The location of the unsaturation on the acyl chains are marked.

Figure 5.11: Schematic illustration of the lipid phases and the effect of transition temperature on the phase transition

5.3.1.5 Effect of cholesterol on trans-scleral transport

Cholesterol, a hydrophobic molecule is typically added to liposomal formulations to alter/adjust the fluidity of the bilayers. The addition of cholesterol to unsaturated lipid types have been found to increase the bilayer flexibility which consequently has been found to slow down the release of hydrophilic drugs loaded in the liposomal core [13-16]. The effect of addition of cholesterol on transport properties was studied with both saturated and unsaturated lipid types, POPC and POPC- Cholesterol (Figure 5.12) as well as DPPC and DPPC-Cholesterol (Figure 5.13). The sizes of the formulations used in the study are tabulated in Table 5.4 below:

Table 5.4: Z-average sizes of Cholesterol containing liposomes used in the transport study

Formulation	Z average Size (d nm)	PDI
POPC	94.9	0.09
POPC-Chol	100.6	0.04
DPPC	87.3	0.05
DPPC-Chol	116.0	0.07

Figure 5.12: Representative epifluorescence micrographs of a) POPC and b) POPC-Cholesterol (80:20) after 4-day transport. The edges of the tissue are marked for clarity. The liposomes are tagged with Rhodamine-PE (red).

Figure 5.13: Representative epifluorescence micrographs of a) DPPC and b) DPPC-Cholesterol (80:20) after 4-day transport. The edges of the tissue are marked for clarity. The liposomes are tagged with Rhodamine-PE (red).

The addition of cholesterol affects the rigidity of the bilayer membrane. POPC has a double bond in one of the acyl chains, which leads to a leaky bilayer structure. In case of unsaturated lipids, the kink in the acyclic chain of the phospholipid is filled by the cholesterol, which thus decreases the flexibility of the chain. [13, 14, 16, 17]. Owing to the dense steroid rings of cholesterol, the density of the hydrophobic section of the lipid bilayer is increased. This increases the mechanical rigidity of the fluid bilayers and decreases their lateral diffusion. In contrast, the addition of cholesterol to saturated lipids disrupts local packing orders and increases the diffusion coefficient and decreases the elastic modulus. This is schematically illustrated in Figure 5.14. Addition of cholesterol to DPPC is thus expected to increase the chain flexibility and increase the bilayer leakiness. This was also evident by DSC results of DPPC when compared to DPPC-Cholesterol of 80:20 ratio. This decrease in bilayer fluidity was also evident in case of drug loaded liposomes, which causes faster release of the encapsulated hydrophilic contents (Refer to Chapter 6, Section 6.3.3).

Figure 5.14: Effect of temperature and Cholesterol on phospholipid bilayer permeability. Cholesterol eliminates the transition phase of the lipid bilayer and increases the lipid bilayer permeability in the gel phase and decreases it in the fluid phase. Adapted from [18]

5.3.2 PLGA Micro and Nanoparticles

PLGA microparticles and nanoparticles are an attractive ocular drug delivery nanocarrier, and have been explored by several research groups [19-22]. PLGA is an FDA approved polymer with good biocompatibility and biodegradability properties. Several ocular drug delivery formulations have been developed using PLGA as a sustained release vehicle, both for intraocular as well as subconjunctival applications. [19-25]. Previous studies have demonstrated the encapsulation of anti-VEGF RNA aptamer (EYE001) in PLGA microspheres and sustained release for a period of 20 days without losing bioactivity. This shows the potential for using PLGA as a nano/micro carrier for release of Anti-VEGF antibodies. Hence, there is an interest in studying the transport and distribution of these particles upon periocular administration. Similar to distribution studies with liposomes, fluorescently tagged PLGA Micro and Nanoparticles were prepared and introduced in the donor chamber of the ex vivo setup, and their localization and trans-

scleral transport studied by the techniques described in Section 3.3.2. The particles had a Zeta Potential of -2 mV.

The properties of the particles used in the study are summarized in Table 5.5. The particles used in the experiment had a concentration of 25 mg/ml in the experiment.

Table 5.5: Properties of PLGA micro and nanoparticles used in the transport study

Formulation	Z average Size (d nm)	Zeta potential
PLGA microparticles	75 ± 1.2 µm	-2 mV
PLGA nanoparticles	236 nm (0.12)	-2 mV

Figure 5.15: Representative epifluorescence micrographs of a) PLGA microparticles of 75µm size vs b) PLGA nanoparticles of 236nm size after 4-day transport. The edges of the tissue have been marked for clarity. The PLGA particles are tagged with Fluoresceinamine (green).

From the *ex vivo* transport experiment results shown in Figure 5.15 it was observed that the PLGA microparticles of ~75 µm size did not preferentially remain on the episcleral surface and could not be visualized by fluorescence microscopy. On the other hand, PLGA nanoparticles, of approximately 200 nm, remain preferentially adsorbed on the episcleral surface without showing intrascleral diffusion. The particles have a slight negative charge, which however, is not significantly large. *In vivo*, the microparticles

might also be retained at the site of administration for longer periods of time without being cleared away by circulatory and lymphatic systems [2], due to the larger size.

5.3.3 Polymersomes

Polymersomes are hollow shell nanoparticles, which have unique properties which have applications in drug delivery. Polymersomes are comprised of two layers of synthetic polymers enclosing an aqueous core. Compared to phospholipids which constitute liposomes, the individual amphiphilic-block copolymers which are the building blocks of polymersomes are considerably larger. They have several advantages over liposomes, including enhanced stability, longer circulation times, mechanical robustness, and the ability to carry large quantities of hydrophobic and hydrophilic drug molecules. Since the polymers have high molecular weights, the bilayer membranes of polymersomes are thicker compared to that of liposomes, which impart higher stability to polymersomes compared to conventional liposomes. In addition, the hydrophilic block of the copolymers is usually Poly Ethylene Glycol (PEG), which helps to make the polymersomes have longer circulation times. These advantages could make them good candidates for

A PBD-b-PEO diblock type of polymersomes of average size 206.2 nm were obtained from collaborators at the NTU-Northwestern Institute of Nanotechnology (NNIN).

The PBD-b-PEO diblock polymersomes is a diblock type of polymersome and the Tg of the block copolymers is -69°C. The trans-scleral transport demonstrated by the PBD-b-PEO polymersomes is indicated by the epifluorescence micrographs in Figure 5.16. In comparison with PLGA nanoparticles of similar size, these PBD-b-PEO polymersomes were found to demonstrate intrascleral diffusion. It was hypothesized that this observed difference in transport could be due to the larger rigidity of the PLGA, which has a Tg of 44-55°C, compared to the polymersomes which are more flexible [23]. This again points to the role of rigidity of nanoparticles on the transport through sclera.

Figure 5.16: Representative epifluorescence micrographs of PBD-b-PEO polymersomes after 4-day transport. The edges of the tissue have been marked for clarity. The polymersomes are labelled with Rhodamine-PE (red).

5.3.4 Polystyrene nanoparticles

From the transport studies performed for liposomes of different sizes and charges described in Section 5.3.1, it was observed that negatively charged liposomes of approximately 100 nm in size were found to demonstrate some amount of intrascleral transport, compared to positively charged liposomes. In order to decipher whether this effect is also applicable to negatively charged, rigid nanoparticles such as polystyrene, transport experiments were performed using fluorescently labelled Polystyrene nanoparticles (FITC-labelled) of 100 nm purchased from NANOCS. The particles had a Z-average hydrodynamic diameter of 104 nm and an average Zeta potential of -24.8 mV.

The particles were diluted to a concentration of xx mg/ml prior to the transport experiment.

Figure 5.17: Representative epifluorescence micrographs of trans-scleral transport of a) FITC-tagged Polystyrene nanoparticles of 100 nm size (PS-100) over 4-days compared with transscleral transport of b) POPC liposomes of 90 nm size over 4-days. The liposomes in Figure b) have been labeled with Rhodamine-PE (red). The edges of the tissue are marked for clarity.

From the epifluorescence micrographs in Figure 5.17, it can be seen that the polystyrene nanoparticles remain episclerally localized, compared to negatively charged liposomes of similar size. Polystyrene has a glass transition temperature of 100°C. [26], and is hence rigid and non-deformable at room temperature. In nanoparticle rigidity studies conducted by Nakano *et al.* [27], polystyrene particles were used as a reference for particle rigidity, and the original study looked at rigidity of various liposomal formulations. Particle rigidity was calculated as H/D where H is the height of the particle from AFM measurements and D is the diameter measured by DLS. The rigidity of polystyrene particle was calculated to be 0.94 ± 0.001 . This indicates that polystyrene particles exhibit considerable rigidity and was not significantly distorted during the AFM measurements. This could explain the limited penetration of these particles, similar to what was studied for rigid polycarbonate particles prepared by Amrite *et al* [2].

5.3.5 Gold nanoparticles

Gold nanoparticles of an average size of 10nm were purchased from NANOCS. The charge of these particles was 0.85 mV

Figure 5.18: Representative epifluorescence micrographs of FITC-tagged Gold nanoparticles.

The FITC-labelled gold nanoparticles, had average size of 10 nm. There was trans-scleral transport observed for these particles, as seen in Figure 5.18. Even though the gold nanoparticles are rigid particles, their smaller size likely allows for permeation between the collagen fibrils and facilitates intrascleral diffusion. A similar observation made for 20 nm rigid polycarbonate nanoparticles, also further supports this hypothesis[2].

5.3.6 Spherical Nucleic Acid (SNAs) Nanoparticles

Spherical nucleic acids, or SNAs, are three-dimensional nanostructures, typically consisting of densely functionalized and highly oriented nucleic acids covalently attached to the surfaces of spherical nanoparticle cores. These particles serve important biological functions, and they have properties very different from regular nucleic acids. The core of the SNA may be made of inorganic nanoparticles, polymers and micelles or even proteins.

The core imparts the SNA conjugate with novel chemical and physical properties and acts as a scaffold on which the oligonucleotides can be assembled and oriented.

FITC tagged Oligonucleotides targeting MMP-9 sequence, and the oligonucleotide sequence attached to Gold Nanoparticles were provided by collaborators from Northwestern University, Illinois. The size and Zeta potential of the formulations used in the study are tabulated in Table 5.6.

Figure 5.19 a) shows the transport of FITC-tagged oligo in 4-days. The fluorescence seen uniformly distributed throughout the sclera suggests that the oligomer is able to diffuse in and transport through the sclera. The Oligonucleotide is a 20-mer with a molecular weight of 8202 Da. The oligomer has a net charge of -26.7 mV, as measured by Zeta potential measurements. Trans-scleral transport for small molecules such as these are reported in literature [5, 28].

Figure 5.19: Representative epifluorescence micrographs of a) FITC oligo and b) FITC-SNA after 4-day transport. The edges of the tissue are marked for clarity.

In contrast, Figure 5.19 b) shows the trans-scleral transport when the same FITC-tagged oligonucleotide is covalently conjugated to Gold Nanoparticles. The zeta potential of the conjugate is -29.7 mV. As visible by the fluorescence gradient, it can be seen that the transport is hindered in this case and the oligomer attached to the gold nanoparticles

shows a slowed-down diffusion into the sclera. This observation shows the effect of the carrier in providing a depot effect and slowing down the transport of the oligomer, when it is attached to the gold nanoparticle.

Another class of SNAs are liposomal SNAs which have a core consisting of liposomes. [29] These molecules comprise of a 30 nm liposomal cores stabilized with a dense shell of oligonucleotides with a hydrophobic tail that can intercalate between the phospholipids that define the liposomal structure (Figure 5.20). Similar to conventional SNAs, these have the capability to enter multiple cell lines and show effective gene knockdown via their antisense properties. The oligonucleotide cargo in this case is arranged on the surface of the liposomes, and not loaded inside the liposomes. The 30 nm-sized liposomes were prepared using DOPC as a monomer.

These liposomal SNA structures have been found to be remarkably stable compared to the native 30 nm liposome constructs from which they derive. In addition, the DNA corona on the surface acts as a protecting layer for the liposomal core preventing degradation by serum proteins *in vivo*.

Figure 5.20: Schematic showing the Assembly of Liposomal SNAs from DOPC SUVs and Tocopherol-Modified DNA

The transport experiments performed with the liposomal-SNA conjugate for 4 days, is depicted in Figure 5.21. The liposomes were tagged with Rhodamine PE (similar to the bare liposomes described in Section 5.3.1 and the oligomer was labelled with FITC, as in the case of Gold-SNA NPs. Figure 5.21 a) depicts the fluorescent image from the

Rhodamine channel, and shows localization of the liposomes close to the episcleral region. Figure 5.17 b) shows the FITC fluorescence from the oligomer, and it can be observed that the FITC-oligomer released from the liposome is able to transport trans-sclerally. Some FITC still remains attached to the liposomes, which results in yellow fluorescence observed in the overlay image (Co-localization of the red liposome fluorescence and the green FITC fluorescence).

The size and Zeta potential of the formulations used in the study are tabulated in Table 5.6 below:

Table 5.6: Z-average size and Zeta potential of liposomal SNAs used in the transport study

Formulation	Size (nm) (PDI)	Zeta potential (mV)
FITC-oligo		-26.7 +/- 4.73
SNA-FITC-oligo		-29.7mV +/-6.65
Liposomal SNA	85.6 (0.25)	-42.7 +/- 4.39

The liposomes had an overall negative charge, and were found to have aggregated during the shipping, with a large size distribution. This could explain why the liposomes, even though considered to have a size comparable to those used in our studies, could not demonstrate higher permeability into the sclera.

Figure 5.21: Representative epifluorescence micrographs of a) Liposomal SNA encapsulating FITC-oligo a) Liposome distribution b) FITC-distribution c) Overlay after 4-day transport. The liposomes are labeled with Rhodamine-PE (red) and the oligo with FITC (green) and the sclera is stained with DAPI (blue). The edges of the tissue have been marked for clarity.

5.4 Summary

Development of sustained drug delivery systems for subconjunctival administration is desirable to extend the duration of action of drug molecules targeting the retina. The intravitreal distribution and transport of nanoparticles with a variety of different properties have been previously studied by several research groups. [7, 9, 10, 30-33]. Properties such as size, charge as well as surface properties of the nanoparticles have been shown to influence the intravitreal transport and distribution. However, intravitreal injection of suspended particles may cause vitreous clouding, thus interfering with vision. The subconjunctival route, does not cause interference with vision, in addition to being less invasive, and hence can be a potentially useful route for sustained delivery using nanocarriers.

The sclera is a largely acellular collagenous tissue. It can be considered as a porous membrane containing water-filled channels. The mechanism of scleral transport could thus be passive diffusion. Active transport is unlikely due to the absence of cellular barriers in the sclera. The results described in this chapter with various nanoparticles show a time-dependent distribution and localization of the nanoparticle systems into the sclera depending on their properties, as depicted by the results from epifluorescence micrographs taken post-transport experiments.

Previous scleral *ex vivo* experiments performed for long periods such as 6 days using rabbit sclera have indicated that under the experimental conditions, some swelling of collagen fibrils is expected compared to fresh sclera, as indicated by TEM micrographs but the ultrastructure is preserved [34]. Moreover, any disintegration of the collagen fibril organization would have led to a sudden increase in permeability, or a higher transport, which was not observed in our case. This observation was also consistent with results from other research groups who have conducted similar *ex vivo* experiments for trans-scleral diffusion of solutes, in short and long term studies [6, 35-37]. This justifies the time duration chosen for trans-scleral transport studies in this work.

The following sections summarize the trans-scleral transport results.

Liposomes

From this study, a size dependent penetration and diffusion of the liposomes into the scleral tissue was observed. The larger sized POPC MLVs were found to transport only to a very small distance in the sclera compared to the POPC Large Unilamellar Vesicles (LUVs). The smallest POPC vesicles used in this study, of ~70 nm size, demonstrated the largest transport distance into the sclera, among the liposomal formulations in the study.

The encapsulation efficiency for large, hydrophilic drug molecules such as Lucentis may be larger with larger vesicles such as MLVs. Hence, the larger sized MLVs may be used for sustained delivery if no trans-scleral diffusion is desired, as the carriers may remain at the episcleral site and slowly release the encapsulated drug alone which may transport across the sclera to reach the desired site of action. The smaller sized liposomes, which also do not completely transport across the sclera may, on the other hand be available as a depot inside the sclera. However, smaller the size, smaller will be the capacity for drug cargo in the core of the liposome, which may then necessitate a trade-off between transport and encapsulation.

Among the charged liposomes studied, the positively charged liposomes (POPC-DOTAP) demonstrated the least transport, of less than 2 % of the scleral thickness from the episcleral surface. The negatively charged liposomes used in the study (POPC-POPG) demonstrated higher transport than the positively charged liposomes, but much less than the neutral liposomes. This could be due to the interaction of positively charged carriers with the negatively charged sclera[38]. In comparison, repulsion of the slightly negatively charged liposomes may be the cause for the superior scleral transport of negatively charged liposomes. For the charged liposomal formulations, the positively charged liposomes may act as an episcleral depot by attraction to the negatively charged scleral

surface whereas the negatively charged liposomes show some penetration owing to the absence of clearance systems.

The effect of fluidity of the liposomes on the transport was also studied, and it was observed that the unsaturated lipid type POPC, (which has higher fluidity due to its lower T_m of -2°C) used in the study shows higher transport into the sclera compared to DPPC which is a saturated lipid type. The unsaturated liposomes POPC is probably able to diffuse better between the collagen fibrils owing to its fluidity and show better transport into the sclera. By the use of these unsaturated, neutral lipid types, it may be possible to increase the scleral residence time if the liposomes can act as a depot inside the sclera, without being cleared rapidly by the circulatory and lymphatic systems. With regards to clearance of particles, a previous *in vivo* study on transport and clearance of (rigid) polycarbonate nanoparticles by Amrite *et al.* [2] showed that the 20 nm particles were cleared fast from episcleral surface, and were transported across the sclera to some extent. In comparison, 200 nm particles were found to be retained at the site of action for more than a month post injection (due to slower clearance of the particles compared to the smaller 20 nm particles). Based on this, the particles used in our study are of sizes such that they should be retained at the site of application and not subject to rapid clearance *in vivo*. The observations here regarding the diffusion of nanocarriers into the sclera is different from that of Amrite *et al.* in that only rigid polycarbonate nanoparticles were used in their study. The membrane fluidity of the liposomes used in this study may have an effect on the transport properties observed.

For liposomes, the results of properties affecting the trans-scleral transport are summarized as given in Table 5.7

Table 5.7: Summary of the properties affecting trans-scleral liposome transport and their implications

Property	Effect	Explanation	Implication
Size	Smaller the size, higher the transport	Smaller sized carriers are better able to diffuse between the scleral collagen fibrils	Smaller carriers only can hold a smaller amount of drug cargo
Positive Charge	Remain as an episcleral depot	Adherence to the surface of the negatively charged sclera and not favorable for transport	The positively charged carriers maybe useful as an episcleral depot
Negative Charge	Show some degree of intrascleral transport	Possibly repelled by the sclera	Have potential as an intrascleral depot
Lipid type	Unsaturated lipids transport better	Saturated liposomes less flexible hence may transport less	Need trade-offs between encapsulation efficiency and transport

PLGA micro/nanoparticles

PLGA 50:50 is reported to have a glass transition temperature of between 44-55°C. In this study, trans-scleral transport of PLGA microparticles, of approximately 75 μm size, and PLGA nanoparticles of approximately 200 nm size was studied. It was observed that, compared to flexible nanocarriers such as liposomes, the PLGA nanoparticles showed preferential episcleral localization and did not show trans-scleral transport. PLGA microparticles, on the other hand showed no preferential attachment to the sclera and were not visible in the epifluorescence micrographs as they were possibly washed from the episcleral surface during the scleral tissue post-processing and fixing in PFA. *In vivo* however, the microparticles as well as the nanoparticles of PLGA could be expected to remain episclerally localized due to their sizes. However, it remains to be seen whether sizes as large as 75 μm of the PLGA microparticles may be feasible for patient comfort

after subconjunctival injections. PLGA micro/nanoparticles with sufficient loading of the drug have a possibility to provide sustained release by their episcleral depot action. Biodegradable micro/nanoparticles of PLGA, when injected subconjunctivally, would most likely be retained in the periocular spaces until they reach a much smaller dimension that is suitable for clearance. PLGA microparticles have previously been shown to have higher encapsulation efficiency, lower burst and thus a longer sustained release compared to PLGA nanoparticles [21, 39].

Polymersomes

Polymersomes are a class of artificial vesicles made from synthetic amphiphilic block copolymers, [40] which were studied for trans-scleral transport. Similar to liposomes, they consist of an aqueous core, surrounded by a membrane bilayer. Hydrophilic drugs may be encapsulated in the core, while hydrophobic drugs can be entrapped in the bilayer [41]. The polymersomes used in this study, the PBD-PEO polymer has a The PBD-PEO block is reported in literature to have a Tg of -69°C. Notably, compared with PLGA nanoparticles of similar sizes, differences in transport were observed for the polymersomes. Polymersomes made of PBD-PEO, of 200 nm in size showed appreciable intrascleral transport, whereas PLGA nanoparticles of similar sizes were found to remain episclerally localized. This again points to the effect of the particle flexibility, in intrascleral transport. If polymersomes are able to show sufficient encapsulation of drugs, they have the potential to act as intrascleral sustained delivery vehicles.

Polystyrene nanoparticles

Polystyrene, which was used as a model of a rigid nanoparticles, to study trans-scleral transport, has a glass transition temperature of 100°C [26]. As seen with rigid PLGA nanoparticles, Polystyrene nanoparticles were also found to remain episclerally localized. This was also observed with 200 nm sized polycarbonate particles in vivo[2]. The particles remained localized at the site of administration for upto 2 months post injection.

Gold nanoparticles and SNAs

The gold nanoparticles studied for trans-scleral transport had an average size of 10 nm. There was trans-scleral transport observed for these particles, as seen in Figure 5.18. It is likely that the small size of the particles allowed for permeation between the collagen fibrils and facilitates intrascleral diffusion. The spacing of collagen fiber bundles in the sclera is considered to be such that it allows internal diffusion of particles less than 300 nm [42, 43]. A similar observation made for 20 nm rigid polycarbonate nanoparticles, also further supports this hypothesis[2].

Gold-SNA tagged oligonucleotides used in the transport studies were found to have a hindered transport into the sclera compared to the bare oligonucleotides. In this case, the oligonucleotide is covalently attached to the gold nanoparticle and the complex transports intact. In contrast, from the liposomal SNA studies, the oligonucleotide is encapsulated in the liposome, and the released oligonucleotide is able to transport across the sclera in the similar way as the bare oligonucleotide does. This result is of particular significance in emphasizing the episcleral depot concept and mode of action that trans-scleral carrier formulations are able to provide. In this case, the liposomal carriers act as a depot in the episcleral space, and release their cargo, which in this case is the oligomer. The oligomer is then able to transport through the sclera to reach the target site of action. The molecular target for oligonucleotide therapy is inside the cell (cytoplasm). So, the drug in this case needs to enter the cells, which may be better assisted by the use of the nanocarriers. Hence, rather than release of bare oligonucleotides from the intrascleral depot, a carrier which may transport trans-sclerally together with the drug (such as the Gold-SNA, but with properties tuned for enhanced trans-scleral transport) may be the best strategy to get the desired action. Also based on the intrinsic property of the SNAs to be taken up by different types of cells, they can reach inside the cells and the drug could reach the molecular target.

The average distances of diffusion into the sclera, for the different formulations studied in this chapter are shown in Figure 5.22.

Figure 5.22: Scleral Penetration distance (in μm) after 4-days of trans-scleral transport for the different formulations tested.

Attempts to quantify the transport of nanocarriers

Quantitative measures of fluorescence were attempted for the different liposomal formulations tested, but were not successful. The first obstacle was that some of the fluorescent molecules from the donor chamber were found to adhere to the scleral tissue mounting ring and couldn't be recovered for analysis. Another attempt was made for quantification by performing an efflux of the carriers from the sclera. The formulations tested were POPC MLV, POPC LUV of 90 nm, POPC LUV of 70 nm, POPC DOTAP LUV, POPC POPG LUV, DPPC MLV and DPPC LUV of 90nm size. The sclera was dissected to have the same size for all specimens tested, with an area of 1 cm^2 and the tissue was immersed completely in the fluorescently labeled liposomal formulation contained in Eppendorf tubes. For each formulation, five different tissue samples were used in separate experiments ($n=5$). Incubation with the liposomal formulations was conducted for 4 days, followed by immersing the tissue into fresh buffer every 24 hours to efflux out the carriers. The buffer solution was then analyzed for fluorescence and compared with a fluorescence standard curve. This was continued until 10 days when

there was no fluorescence detectable in the buffer solution anymore. It was observed that complete efflux or recovery of the fluorescent liposomes and molecules from the sclera also was not possible. Figure 5.23 shows the % of the total liposomal solution left in the initial equilibration solution, as quantified by fluorescence. and Figure 5.24 represents the % Cumulative efflux released in 10 days into fresh buffer, with the buffer changed every 24 hours. Only less than 10% of the fluorescence could be recovered in total, after 10 days of efflux, indicating about 10-20% unrecovered from the total liposomes in the starting formulation. Even upon homogenization of the sclera, the remaining fluorescence could not be recovered into the supernatant, due to possible adherence of the carrier molecules to the sclera, which resulted in incomplete recovery. Hence this method of quantification was unsuccessful in quantifying the fluorescence intensity.

Figure 5.23: Graph representing the % of the total liposomal solution left after equilibration

Figure 5.24: Graph representing the % Cumulative efflux released in 10 days in fresh buffer

Conclusions

From our studies on trans-scleral transport of nanocarriers, it can be concluded that the size, charge, as well as flexibility of the nanocarriers plays a role in the distribution of nanocarriers after a subconjunctival introduction. The various nanocarriers used in this study showed either episcleral or intrascleral localization. Intrascleral transport might provide better retention and sustained action of delivery for the drugs when compared to carriers remaining on the episcleral surface.

Within the framework of this study, it was observed that even though the liposomes used in this study were not able to cross the sclera completely in the time of observation (4 days), a marked difference in the extent of penetration was observed for the various formulations. Among the liposomes, size, charge and membrane fluidity were found to be significant parameters influencing the transport characteristics and depot-forming abilities. The type of liposome best suited for any particular application may need further consideration such as the drug release profile for the clinical need and the interaction of drug and liposome, partitioning effects, degree of encapsulation, etc. Encapsulation of the

anti-VEGF Lucentis was attempted in liposomes of different properties, and the encapsulation efficiency and release and trans-scleral transport properties of the encapsulated Lucentis are dealt with in Chapter 6.

It should be pointed out that the present study was an ex vivo study and may not be predictive of actual in vivo conditions owing to the absence of vasculature and clearance mechanisms. Also, this study only addresses the static barrier to transport, which is the sclera, and dynamic barriers to transport may play an even higher role in trans-scleral drug delivery by clearance.

Collectively, the results from this chapter support the hypothesis that nanocarrier formulations can be used as an episcleral or intrascleral depot system which can then provide sustained ocular delivery provided sufficient quantities of the drug maybe loaded into the carriers. The bottleneck then becomes the encapsulation/release profiles of the nanocarriers. The major significance of this study is in the fact that the factors affecting distribution of carriers has been elucidated, which can be applied to development and optimization of sustained delivery systems targeting the posterior ocular segment, depending on the drug, the application and drug-carrier interactions.

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Chapter 6

Trans-scleral Transport of anti-VEGF Loaded Nanocarriers

This chapter describes the results of encapsulation and release studies on Lucentis loaded in Liposomes. In vitro release studies were performed with the different loaded formulations. In order to study the effect of carrier properties, and the depot effect offered by the different carrier systems, different carrier formulations were tested for ex vivo transport, and the release of free/released protein was quantified by ELISA. Comparisons were drawn between the depot effect of the various carriers, compared to the release of bare drug, as explained in Chapter 4.

6.1 Introduction

The results obtained in Chapter 4 and Chapter 5, provide valuable information about the transport of bare ranibizumab through the sclera, and the disposition of several nanocarriers (based on their properties) when applied subconjunctivally.

Although protein drugs exhibit high specificity and tight binding to their targets, their high hydrophilicity and large molecular weight, along with structural fragility are obstacles to their use in drug delivery systems. Proteins also may undergo denaturation and subsequent inactivation easily, during formulation, storage or delivery. Thus far, attempts to improve the protein bioavailability and targeting have ranged from tailoring the physicochemical properties of peptide molecules to the inclusion of functional excipients into specially adapted drug delivery systems.

Antibody delivery is in particular, challenging due to the high hydrophilicity, large size and relative ease of denaturation of these molecules, which influence strongly the in vivo pharmacokinetic and pharmacodynamic behavior. Aptly designed sustained release mechanisms can offer several advantages to protein delivery, compared to classical administration routes. Desirable properties in a carrier include the ability to encapsulate the protein without denaturation, the possibility of drug release by suitable amounts, and maintaining drug stability throughout-release. However, the encapsulation of these molecules in suitable nanocarriers for sustained delivery is challenging due to limitation on the solvents, environmental conditions and preparation methods which can be employed. Formulations often need to be also prepared in presence of special excipients such as buffers, preservatives or additives to preserve the native confirmation of the proteins. As an example, in a recent study involving bevacizumab encapsulation in PLGA, albumin was found to have a protective effect in increasing the stability of the protein post-encapsulation. [1].

From our studies on trans-scleral transport of nanocarriers, it can be concluded that the

size, charge, as well as rigidity of the nanocarriers plays a role in the distribution of nanocarriers after a subconjunctival introduction. (Refer chapter 5, Section 5.3.1). In particular, comparing PLGA micro and nanoparticles with liposomes, it could be seen that the liposomes exhibit better scleral penetration and transport, compared to PLGA particles, which is hypothesized to be due to the higher bilayer fluidity of the liposomes which facilitate better intrascleral diffusion. Intrascleral transport might provide better retention and sustained action of delivery for the drugs when compared to delivery from the episcleral surface. Moreover, for liposomes, used as drug reservoirs, parameters such as size, charge and lipid composition can be easily tuned. Also, methods of liposome preparation have not been found to affect the protein stability during encapsulation[2].

Lucentis (the commercial form of ranibizumab) encapsulation has been recently attempted by several researchers, using PLGA microparticles as well as composite systems[3, 4]. The researchers have focused mainly on *in vitro* release studies involving the drug-loaded carrier systems. However, liposomal encapsulation has not been explored for ranibizumab, possibly due to low encapsulation and/or fast release which could result in fast clearance of the drug. A liposomal formulation, developed for bevacizumab [5] has only been explored for intravitreal route of administration. Natarajan et al., [6] on the other hand have observed sustained clinical effects for an anterior segment disorder, glaucoma, from a liposomal formulation of latanoprost for upto 6 months after a single subconjunctival injection in rabbits. Carrier-mediated subconjunctival delivery of anti-VEGF agents from a non-invasive depot system such as the one described in this work, has not been previously considered.

The initial efforts were involved in the optimization of suitable carriers for trans-scleral delivery of ranibizumab. From chapter 5, the liposomes which form episcleral/intrascleral depots to different degrees, have been identified. In this chapter, the encapsulation and release of Lucentis from various liposomal compositions, which were selected based on the observations from Chapter 5, have been evaluated and compared. Specifically, the effects of liposome types and addition of cholesterol and the effect of liposome charge on the *in vitro* and subsequently *ex vivo* release are evaluated. The results obtained in *in vitro*

release as well as *ex vivo* transport are discussed in detail in the following sub sections.

6.2 Materials and Methods

Liposomes encapsulating Lucentis were prepared as described in Section 3.4 and in the *in vitro* and *ex vivo* release was monitored.

6.3 Results and Discussion

6.3.1 Preparation of Lucentis loaded liposomes

The method followed for preparation of the liposomes is described in Section 3.4.

Liposomes were prepared using POPC, POPC-Cholesterol (80:20 m/m ratio), DPPC, DPPC-Cholesterol (80:20 m/m ratio), DPPC-DPTAP (80:20 m/m ratio) and DPPC-DPPG (80:20 m/m ratio). The formulations were chosen based on the transport experiment results obtained from Chapter 5.

POPC liposomes were chosen based on the observation that these liposomes had better intrascleral diffusion and transport. POPC-Cholesterol liposomes have higher rigidity, hence these liposomes are expected to have better encapsulation of their hydrophilic contents and slower release. DPPC liposomes, on account of their higher transition temperature have higher rigidity, hence a higher encapsulation is expected compared to POPC liposomes. DPPC-Cholesterol liposomes were shown to have better intrascleral transport compared to DPPC liposomes, and were also studied. Positively charged DPPC-DPTAP liposomes and negatively charged DPPC-DPPG liposomes were explored to study the loading, release and drug transport from charged liposomes.

The size and zeta potential of the liposomes, are indicated in Table 6.1. The encapsulation efficiencies of DPPC, DPPC-Cholesterol, DPPC-DPTAP and DPPC-DPPG liposomes are depicted in Figure 6.1. For POPC and POPC-Cholesterol liposomes, the encapsulation efficiencies couldn't be determined by ultracentrifugation as these liposomes couldn't

form a pellet upon centrifugation. The size of liposomes was measured after formation by extrusion, as well as after reconstitution. For size stability analysis, the size of liposomes was measured after 2 weeks of storage at 4°C.

Liposomes (LUVs) of mean particle size $Z_{avg} \sim 100$ nm with a low polydispersity index (PDI) were successfully prepared by extrusion.

Table 6.1: Size and Zeta potential of the liposomal formulations used. Size measurement reported here is the average mean size from a single batch of liposomes.

Formulation	Size after extrusion Z-Avg. d,nm(PDI)	Zeta potential (mV)	Size after reconstitution Z- Avg d, nm (PDI)
POPC	104.6 (0.05)	-	107.1 (0.12)
POPC-Cholesterol	101.6 (0.07)	-	115.2 (0.19)
DPPC	89.28 (0.11)	-	105.8 (0.20)
DPPC- Cholesterol	134.0 (0.19)	-	180.5 (0.20)
DPPC-DPTAP	108.8 (0.19)	+39.1 (12.6)	234.6 (0.81)
DPPC-DPPG	105.5 (0.06)	-38.9 (10.2)	105.2 (0.16)

Figure 6.1: Encapsulation efficiency as calculated by ultracentrifugation for the liposomal formulations DPPC, DPPC-Cholesterol, DPPC-DPTAP and DPPC-DPPG

The size stability of the liposomes as indicated by the sizes upon storage at 4°C for 2 weeks are represented in Figure 6.2. The DPPC-DPTAP is found to be visibly aggregated upon storage, and the size is found to increase to micron range from the initial size of 110 nm. All the other formulations exhibited size stability upon storage at 4°C for 2 weeks.

Figure 6.2: Size of the liposomal formulations, after extrusion, and upon storage at 4°C for 2 weeks. Size measurement reported here is the average mean size from a single batch of liposomes.

6.3.2 In vitro release profiles of the Lucentis loaded liposomes

As described in Section 3.4.2, the in vitro release was performed for the liposomal formulations described. A control experiment with the same starting concentration of plain Lucentis was also performed to rule out any permeability barrier effect due to the molecular weight cut off of the dialysis bags (100kDa) used. All experiments were performed in triplicates.

For estimation of encapsulation efficiency, a 1 ml sample was taken from the liposomal formulation and ultracentrifugation was performed as described in Section 3.4.1 to estimate the encapsulation efficiency.

The initial *in vitro* release was performed in PBS at pH 7.4, without the presence of any

surfactant in the release medium (Figure 6.3). From quantification of the release samples, it was observed that the total starting amount of Lucentis couldn't be recovered even from the control formulation of bare Lucentis in the dialysis bag. This could be due to non-specific adsorption of the antibody to the walls of the dialysis bottle, or to the dialysis clips. Proteins and antibodies are known to exhibit non-specific adsorption to surfaces [7] and special care should be exercised when designing the experimental conditions for recovery and quantification.

Figure 6.3: In vitro release profile of the Lucentis from liposomal formulations in PBS at pH 7.4. No surfactant was added to the release buffer in this release experiment. However, the total starting amount of Lucentis couldn't be recovered from the formulations after release. Hence, 0.05% Tween 20 was added to the subsequent *in vitro* release studies.

Keeping this result in mind, Further release experiments were then conducted in release medium containing 0.01% of Tween 20 as a surfactant to prevent non-specific adsorption to the dialysis setup and ~95% of Lucentis could be recovered from the control solution, indicating that the loss in the previous case was indeed due to non-specific adsorption. Table 6.2 shows the percentages of drug recovered in total from the formulations when the release was performed with and without surfactant Tween 20. In addition, the tubings and the collection tubes used for release were coated with 1% BSA during the *in vitro* release procedure to avoid loss of quantifiable amounts of protein due to the non-specific

adsorption.

Table 6.2: Percentage recovery of the Lucentis from the different formulations with and without addition of Tween 20 in the release medium

Formulation	Lucentis	POPC	POPC-Chol	DPPC	DPPC-Chol	DPPC - DPTAP	DPPC-DPPG
% total recovered without Tween 20	66.83%	70.83%	71.74%	74.86%	54.14%	77.99%	56.56%
% recovered with Tween 20	95.59%	97.28%	89.04%	83.93%	98.92%	92.22%	76.32%

Further *in vitro* release studies were performed with addition of Tween 20 in the release medium as well as BSA coating of the tubings and collection tubes used for the dialysis.

From Chapter 5, liposomes composed of the unsaturated lipid POPC were found to exhibit better scleral penetration compared to the other liposomal formulations tested. Lucentis loaded POPC liposomes were studied for the *in vitro* release profile and compared with the control solution of Lucentis at the same starting concentration. In addition, POPC-Cholesterol liposomes with an 80:20 molar ratio was also formulated and compared against POPC for *in vitro* release. For the POPC and POPC-cholesterol formulations, the encapsulation efficiency couldn't be determined by Ultracentrifugation method, as the drug pellet couldn't be formed. The free drug was not separated from the total drug for the *in vitro* release studies. Figure 6.4 shows the *in vitro* release comparison of the drug release from POPC liposomes and POPC-Cholesterol liposomes. The *in vitro* release profile from POPC liposomes is not seen to be different from the bare Lucentis pointing to an insufficient encapsulation and/or fast release from the more fluid bilayer structure of POPC. Addition of cholesterol to the POPC liposomes is shown to slow down the release. With addition of cholesterol to POPC liposomes, the lipid ordering increases, and the liposomes become more rigid, (as reported in literature [8-11]). This

could lead to a slowing down of the drug release caused by the more rigid bilayer.

Figure 6.4: In vitro release of Lucentis from POPC liposomes and POPC-Cholesterol(80:20) liposomes, compared against the control formulation of Lucentis.

Since POPC liposomes were found to have a fast release profile, DPPC liposomes were studied for the in vitro release. From the results of Chapter 5, DPPC liposomes, owing to their increased rigidity when compared to POPC liposomes, exhibit lesser degree of transport intra sclerally compared to POPC liposomes. Slower release was obtained for the DPPC liposomes compared to the POPC liposomes. (Figure 6.6). The structural arrangement of the acyl chains is different between POPC and DPPC, with POPC being an unsaturated lipid type while DPPC is a saturated lipid type. The double bond in the acyl chain of POPC leads to a leaky bilayer structure, which is also evident from the peak melting temperature (T_m) values of POPC (-2°C) being lower when compared to DPPC (41°C). At the release conditions (37°C), POPC liposomes would remain in the disordered bilayer state, leading to a faster drug release from the leaky bilayer whereas the DPPC liposomes would exist in the more ordered bilayer state thus leading to a slower drug release.

Figure 6.5: In vitro release of Lucentis from POPC liposomes and DPPC liposomes, compared against the control formulation of Lucentis.

The effect of cholesterol addition on the in vitro release from DPPC liposomes was studied, and the results are shown in Figure 6.6. It was observed that the DPPC-cholesterol formulation showed a release not significantly different from that of the control Lucentis formulation. Generally, the trend observed with addition of cholesterol is the increase of rigidity of the bilayer, in the case of unsaturated lipids. However, this is not the case for saturated lipids such as DPPC. On addition of cholesterol to saturated lipids, the membrane rigidity has been found to decrease, which causes an increase in solute permeability in and out of the membrane. This has also been recently proved by AFM studies of the rigidity of saturated liposomes by Takechi-Haraya et al. [12]. In order to further validate this, DSC measurements were performed using NanoDSC, for DPPC and compared with that of DPPC-Cholesterol (80:20 m/m) and a higher cholesterol ratio (60:40 m/m) in order to elucidate the effect of cholesterol addition on the melting transition temperature. In the absence of cholesterol, DPPC heating scan shows two sharp endothermic peaks at 34 °C and 41.2°C. The results are shown in Figure 6.7. The first peak corresponds to the pretransition ($L_{\beta'}/P_{\beta'}$) and the second one to the main phase transition ($P_{\beta'}/L_{\alpha}$) (1). On addition of cholesterol, the pretransition disappeared. Furthermore, the main phase transition was significantly broadened in presence of 20 mol%

Figure 6.6: In vitro release of Lucentis from DPPC liposomes and DPPC-Cholesterol (80:20) liposomes, compared against the control formulation of Lucentis.

Figure 6.7: Nano DSC micrographs of DPPC compared with DPPC-Cholesterol (80:20 m/m) and DPPC-Cholesterol (60:40 m/m).

cholesterol, and at 40 mol% cholesterol. These results show that both tested cholesterol concentrations reduced or eliminated both the enthalpy and the cooperative hydrocarbon chain-melting phase transition. Cholesterol, disrupting the gel-state organization of DPPC,

is then able to cause faster release of the encapsulated contents. This could explain the faster Lucentis release from liposomes made of DPPC-Cholesterol (80:20) compared to liposomes made of bare DPPC.

The effect of charge of the liposomes on the encapsulation of Lucentis was explored by using positively and negatively charged liposomes with the base lipid DPPC. DPPC-DPTAP liposomes of 80:20 molar ratio and DPPC-DPPG liposomes of 80:20 molar ratio were studied as positive and negatively charged liposomes respectively (Figure 6.8). Encapsulation efficiency was found to be the highest for DPPC-DPPG (80:20 molar ratio) among all the six liposomal formulations studied. Lucentis, with an isoelectric point of 8.8, has a slightly positive charge at physiological pH. However, in the Intrinsic buffer of Ranibizumab in which it is prepared, Lucentis is more positively charged, and this could be the reason for the higher encapsulation efficiency of the antibody in the negatively charged liposome DPPC-DPPG compared to the other formulations. The release from the charged formulations is compared with that of DPPC and the control Lucentis formulation, in Figure 6.10. Since the release of Lucentis from DPPC-DPPG liposomes was quantifiable beyond 7 days (which was the *in vitro* release study time point for other formulations), the release was continued upto 14 days.

The sizes of the liposomes after the *in vitro* release were measured after removing the samples from the dialysis bag. The sizes after release are tabulated in Table 6.3 and compared with the sizes immediately after extrusion. Except for DPPC-cholesterol and DPPC-DPTAP, the other formulations were not found to have changes in size. The DPPC-Cholesterol and DPPC-DPTAP formulations also showed visible aggregation in the dialysis bag, on completion of release.

Figure 6.8: *In vitro* release of Lucentis from DPPC liposomes vs positively charged DPPC-DPTAP (80:20) liposomes vs negatively charged DPPC-DPPG liposomes, compared against the control formulation of Lucentis.

Table 6.3: Comparison of liposome sizes after extrusion and after the *in vitro* release

Formulation	Size after extrusion Z-Avg.(d, nm)	Size after release Z-Avg (d, nm)
POPC	104.6 (0.05)	101.8 (0.08)
POPC Cholesterol	101.6 (0.07)	106.2 (0.10)
DPPC	89.28 (0.11)	82.4 (0.51)
DPPC Cholesterol	134 (0.19)	442.5 (0.68)
DPPC DPTAP	108.8 (0.19)	5004.3 (0.42)
DPPC DPPG	105.5 (0.06)	111.3 (0.32)

6.3.3 Ex vivo transport studies of the Lucentis loaded liposomes

For the formulations which were tested for encapsulation and *in vitro* release, *ex vivo* transport studies were performed using porcine sclera, similar to the bare Lucentis experiments described in Chapter 4, Section 4.3.1. The liposomal loaded Lucentis was introduced in the donor chamber of the *ex vivo* setup and the amount of Lucentis

transported into the receiver chamber across the sclera was quantified using ELISA at different time points (Methods described in Chapter 3, Section 3.5.3). The *ex vivo* experiments were conducted for a period of 7 days. The free drug was not separated from the total drug for the *ex vivo* transport studies.

Previous *ex vivo* experiments performed for long periods such as 6 days *in vitro* have indicated that under the experimental conditions, some swelling of collagen fibrils is expected compared to fresh sclera, as indicated by TEM micrographs but the ultrastructure is preserved. [13]. Moreover, any disintegration of the collagen fibril organization would have led to a sudden increase in permeability, or a higher burst which would be visible in the daily release, which was not observed in our case. This observation was also consistent with results from other research groups who have conducted similar *in vitro* experiments for trans-scleral diffusion of solutes, in short and long term studies [14].

The cumulative *ex vivo* transport of all the six formulations described in this study, compared with the transport of bare Lucentis as described in Chapter 4, are depicted in Figure 6.9.

The *ex vivo* trans-scleral transport was performed for POPC, POPC-Cholesterol, DPPC, DPPC-Cholesterol, DPPC-DPTAP and DPPC-DPPG liposomes. The POPC liposomes, which demonstrated intra-scleral penetration showed a slower transport of released Lucentis compared with bare Lucentis alone ($p=0.008$; statistically significant difference). The POPC-Cholesterol formulation, which has a higher bilayer rigidity when compared to POPC, and demonstrated lower intra scleral penetration compared to POPC, had a statistically significant lower release compared to POPC ($p<0.05$). The release was also significantly lower compared to bare Lucentis ($p<0.05$). For release of Lucentis from an encapsulated liposomal formulation, either POPC and POPC-cholesterol may be useful depending on the trade-off between intrascleral transport (which could lead to lower episcleral clearance *in vivo*) and encapsulation efficiency/release rate.

Figure 6.9: *Ex vivo* % Cumulative trans-scleral transport of the different liposomal formulations compared with *ex vivo* trans-scleral transport of Lucentis. Each transport experiment was performed in triplicate.

Comparing DPPC and DPPC-Cholesterol formulations, the DPPC-Cholesterol owing to its leakier bilayer compared to DPPC, shows higher release of encapsulated contents in the *in vitro* study, as well as larger *ex vivo* transport. For the DPPC-Cholesterol formulation, there was no significant difference observed in the *ex vivo* transport compared to bare Lucentis *ex vivo*. The positively charged DPPC-DPTAP and negatively charged DPPC-DPPG formulations also show statistically significant difference from the DPPC or the bare Lucentis formulations. The positively charged liposomes themselves localize episclerally and may act as an episcleral depot from which the Lucentis is released. On the other hand, DPPC-DPPG liposomes had a higher degree of encapsulation, and possibly a charge-interaction of the Lucentis with the liposome, which has resulted in the slower *ex vivo* release observed, among all the formulations tested.

Comparing the *ex vivo* transport of different formulations against the transport of

Lucentis, all the formulations except DPPC-Cholesterol (p value of Lucentis *ex vivo* vs DPPC-Cholesterol *ex vivo* = 0.12) were found to be statistically significant ($p < 0.05$).

Figure 6.10 compares the Lucentis release *in vitro* and the transport *ex vivo*. *Ex vivo* transport of Lucentis was studied in Chapter 4. Over the 7 days, in the *ex vivo* setup, only about 50% of the drug was transported and quantifiable in the receiver chamber, as transported across the sclera from the donor chamber. This shows the prospect of the sclera in acting as a depot and capable of providing sustained release from the subconjunctival/episcleral space.

The comparison of *in vitro* release from all the six liposomal formulations compared with the *ex vivo* release, is depicted in Figure 6.11. Since the same batch of liposomes were used for both in vitro as well as *ex vivo* experiments, the starting amounts of drug is the same for all the formulations. The graphs show that the *ex vivo* cumulative transport shows a zero order profile, and the maximum amount which has been released in vitro has not been transported *ex vivo* during the 7-day *ex vivo* study. The *ex vivo* studies were not carried out for any longer time periods due to the limitations of scleral tissue viability.

Figure 6.10: Comparison of in vitro release and ex vivo transport of the bare Lucentis. Each experiment (in vitro and ex vivo) was repeated in triplicates.

The comparison of *in vitro* release and *ex vivo* transport as observed in these experiments points to the significant role of the sclera in acting as a depot for providing sustained release using the liposomal formulations. The negative charge of the sclera may be a further advantage to the transscleral transport in this case, with Lucentis exhibiting a slight positive charge, which may help in attractive interactions with the sclera.

Figure 6.11: Comparison of *in vitro* release and *ex vivo* transport of the different liposomal formulations. Each experiment (*in vitro* and *ex vivo*) was repeated in triplicates (n=3).

These *ex vivo* results were extrapolated to the total amount of drugs released in the *in vitro* scenario, to represent the ideal scenario that the *ex vivo* release can predict *in vivo* conditions, in the absence of any drug clearance, could be sustained for upto 39 days with the DPPC-DPPG liposomes. This linear extrapolation of *ex vivo* transport to *in vitro* release is shown in Figure 6.12. Table 6.4 shows the details of the *ex vivo* – *in vitro* extrapolation.

Figure 6.12: *Ex vivo* release results extrapolated to the total amount of drugs released in the *in vitro* scenario.

Table 6.4: Extrapolation of the *ex vivo* linear release curves to the maximum amount released in the *in vitro* release.

Formulation Type	Extrapolated to total In vitro
POPC	15days
POPC-Cholesterol	26 days
DPPC	17 days
DPPC-Cholesterol	12 days
DPPC-DPTAP	35 days
DPPC-DPPG	39 days

It is to be noted that the *ex vivo* transport experiments were conducted for only 7 days, in line with previous reports on stability of scleral tissue in *ex vivo* experiments. Extrapolation was performed for the results of the *ex vivo* transport, to the total amount present inside the liposomes as evidenced by the *in vitro* release, to determine approximately how long the Lucentis might continue to be released, if the same transport trend is followed. It should, however, be borne in mind that in the current *ex vivo* scenario, there is only a unidirectional movement from a closed compartment, whereas *in vivo* there is three-dimensional diffusion with a high propensity of episcleral clearance. The observations from this study are being further pursued in screening and considering carriers which might be best suited for *in vivo* studies.

6.4 Summary

This chapter builds upon the results from Chapter 4 on trans-scleral transport of ranibizumab alone, and the results from Chapter 5 on trans-scleral transport of nanocarriers of different properties. In this chapter, the trans-scleral transport of Lucentis from liposomal formulations of various properties are studied, on the basis of their encapsulation, release and trans-scleral transport properties.

This study explored the encapsulation of Lucentis in different liposomal formulations, which were chosen based on their intrascleral distribution and transport into the sclera. The encapsulation efficiency was found to be different for the different formulations, as well as the *in vitro* release profiles. Encapsulation efficiencies obtained for the liposomes used ranged from 14% to 57%, the highest being for negatively charged DPPC-DPPG liposomes. The *in vitro* release obtained was also most sustained for DPPC-DPPG liposomes compared to the other formulations. In a similar study of encapsulation of Bevacizumab in liposomes, encapsulation efficiencies obtained were 37 ± 4 % for DPPC liposome and 11 ± 2 % for DPPC-Cholesterol liposomes. [5]

The average sizes of the liposomes were around 100 nm with a narrow PDI, and the sizes of the formulations (except DPPC-cholesterol and the positively charged DPPC-DPTAP) were found to be stable during storage and release. Comparing POPC and POPC-Cholesterol liposomes, a slower release and slower *ex vivo* transport were observed from Cholesterol containing liposomes. The *ex vivo* transport of Lucentis from POPC liposomes was found to be significantly different from that of bare Lucentis. ($p < 0.05$). The *ex vivo* transport of Lucentis from POPC-Cholesterol liposomes was found to be significantly different from that of POPC liposomes, as well as from bare Lucentis ($p < 0.05$). The addition of cholesterol to POPC formulations, is expected to increase the bilayer rigidity of the liposomes. This is likely reflected in the lesser degree of *ex vivo* transport, as observed in Chapter 5, Section 5.3.1.5, as well as the slower *in vitro* release from POPC-Cholesterol liposomes compared to POPC liposomes. In comparison, release was more controlled and sustained for DPPC, compared to DPPC-Cholesterol liposomes. For Lucentis transport from DPPC-Cholesterol liposomes, compared to the bare Lucentis formulation, there was no statistically significant difference ($p = 0.12$, $p > 0.05$). This could likely be due to the instability introduced in the bilayer of saturated lipids by addition of Cholesterol. Among the charged liposome formulations, the negatively charged DPPC-DPPG formulation had the highest encapsulation, slowest *in vitro* release and *ex vivo* transport.

Overall, it can be concluded that there are a few factors influencing the *ex vivo* transport

of drug from nanocarriers:

- 1) Drug-carrier interaction and encapsulation efficiency
- 2) Carrier localization in the sclera
- 3) Drug release rate from the carrier
- 4) Drug-sclera interactions

By manipulation of the size/charge/rigidity properties of the liposomes, it is possible to get formulations of different encapsulation efficiencies or retention abilities at the subconjunctival space. This could be a very interesting and novel application for a non-invasive depot forming system which can then deliver the encapsulated cargo from the periocular space, thus avoiding the invasive intravitreal route. *In vivo* studies, based on these *ex vivo* observations can elucidate better, the effect of circulatory systems in clearance of the unencapsulated drug, and the overall sustained release duration. Anatomical features of the sclera, particularly the hypocellularity and large surface, as well as the scleral tolerance of foreign bodies overlying its surface (e.g., scleral buckles) makes trans-scleral diffusion from non-invasive depots such as these, clinically feasible.

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Chapter 7

Conclusions and Future Work

This chapter summarizes and ties together, the results described in Chapters 4,5 and 6, along with the implications and impact of this thesis. The focus of this dissertation was to examine the possibility of sustained delivery of Anti-VEGF antibodies for treatment of AMD. In Chapter 4, transport of bare Anti-VEGF antibody across sclera was successfully performed, which then served as a baseline for comparison against the transport of antibody loaded in nanocarriers (Chapter 6). From the studies on nanocarrier transport, described in Chapter 5, size, charge and flexibility of the nanocarriers were found to be parameters affecting the scleral localization and transport. Lucentis loaded in liposomes of different properties were studied in Chapter 6, and some liposomal formulations were found to have characteristics which make them favourable as subconjunctival depots for drug delivery. This novel study, thus opens up a new strategy for anterior and posterior segment delivery based on a scleral depot concept, which could be either an episcleral depot or an intrascleral depot. Based on these studies, further in vivo studies need to be conducted to evaluate the pharmacokinetics of the drug loaded liposomes and its drug distribution upon subconjunctival injection to obtain ex vivo-in vivo correlation.

7.1 Overview of Research Findings

Age Related Macular degeneration, is the leading cause of blindness in the world, caused by progressive degeneration of the macular region of the retina. The area of the macula, comprises only about 2.1% of the retina, but, of the visual field, almost half of the visual cortex is devoted to processing macular information. The loss of central vision, thus has a devastating effect on visual functioning and the nature of vision loss is such that it affects normal functioning and physical and mental health of the geriatric population and their families. The pathogenesis of AMD results from the imbalance between production and accumulation of damaged Photoreceptor cells and their phagocytosis by the RPE. Hypoxia resulting from this causes production of VEGF, leading to abnormal blood vessel growth from the choroid into the retina, which can leak exudate and fluid and also cause hemorrhaging.

The current gold standard of treatment for AMD involves monthly (painful and invasive) intravitreal injections of Anti-VEGF antibodies such as ranibizumab (marketed as Lucentis) or aflibercept (marketed as Eylea). These Anti-VEGF antibodies are capable of binding to the VEGF, preventing the VEGF from acting, thus halting the neovascularization process. These monthly injections also place a huge economic burden on the patients, in addition to the physical trauma of the intravitreal injections. In contrast, a subconjunctival injection is much less painful and involves less possibility of complications, as it does not involve puncturing and thus destroying the integrity of the eyeball. If a subconjunctival injection is effective in delivering the Anti-VEGF antibody for treatment of AMD and if the frequency of these injections can be reduced by sustained release action, it could be highly beneficial to the patients suffering from AMD. This is the aim that was set out to achieve at the start of this work.

Surveying the literature, it was found that some sustained release formulations for the antibody bevacizumab (marketed as Avastin) which is used off-label for AMD, have been tested *in vivo* intravitreally and found to be capable of sustaining the release. [1-15]. Avastin and Lucentis have been found to have the ability to be trans-sclerally transported

in an *ex vivo* setup [16]. Also, sustained *ex vivo* trans-scleral transport has been previously demonstrated for anti-VEGF aptamer EYE001[17]. Closer to home, researchers from our research group have demonstrated that after a single subconjunctival injection of a liposomal formulation of Latanoprost, sustained IOP reduction effects were observed for about 5 months in a preliminary human trial[18, 19]. Building on all this knowledge, it was hypothesized that subconjunctivally applied sustained release of Lucentis could be applied to improve the current AMD treatment regimen.

In order to mimic the subconjunctival mode of drug delivery, and to have a model system to test the drugs and nanocarriers, an *ex vivo* setup was developed first. For the animal tissue model, surveying the various types of animal tissue models commonly used *ex vivo* for their properties, it was found that porcine scleral tissues to be the most similar anatomically to human scleral tissues. An Ussing chamber system was used for the transport, with the scleral tissue being mounted between the 2 chambers of the setup. The scleral tissue was mounted such that the episcleral side of the tissue faces the donor side of the Ussing chamber, and the choroidal side faces the receiver chamber. This way, the subconjunctival mode of delivery could be mimicked, whereby the drug is introduced in the episcleral region, and has to diffuse across the sclera in order to reach the retinal tissues. Drugs and nanocarriers used were introduced in the donor chamber and the receiver chamber solution was sampled for transport, during the *ex vivo* experiments conducted.

Preliminarily, bare ranibizumab was studied for transport. This was so, because if the bare ranibizumab would be unable to show any trans-scleral transport, the future efforts with loaded nanocarriers wouldn't be meaningful. Moreover, establishing the transport profile of bare ranibizumab, it was then possible to compare the transport with that of ranibizumab loaded in nanocarriers to evaluate the benefit offered by the different sustained release formulations and compare them. The transport of the antibody aflibercept was also attempted across the sclera, and it was found that the trans-scleral transport was slower for aflibercept compared to ranibizumab.

To determine what properties of nanocarriers make them suitable candidates for trans-scleral transport, and to see if some carriers do better than others, trans-scleral transport studies with nanocarriers were then performed. Liposomes were chosen as a preliminary nanocarriers, due to their ability to encapsulate both hydrophobic and hydrophilic molecules, as well as their versatility of properties which can be easily tuned. By selection of the different types of lipids, liposomes of different charges and saturation may be obtained. By tuning the method of liposome formation, the size of liposomes may also be manipulated to some degree. The liposomes were fluorescently labelled, and introduced in the donor chamber of this transport setup. After a few optimization studies, a 4-day time point was selected for diffusional transport experiments. Post-transport, the scleral tissue was examined to study the extent of localization of nanocarriers in the sclera. Our results from liposome transport experiments indicate that unsaturated liposomes (which have a lower transition temperature and thus a higher degree of bilayer fluidity) show a higher degree of intra-scleral diffusion compared to liposomes made of saturated lipids. Of the unsaturated liposomes, Multilamellar vesicles, which have sizes in the range of a few microns, and a larger size distribution, have lesser degree of intrascleral penetration in comparison with large unilamellar vesicles of about 90 nm in size. In comparison, liposomes of 70 nm showed even more diffusion into the sclera. In addition, comparing charged liposomal formulations, it was observed that positively charged liposomes demonstrate an episcleral localization, possibly stuck to the negatively charged scleral surface, instead of showing intrascleral diffusion. In contrast, negatively charged liposomes were able to diffuse intrasclerally.

In order to examine if these results apply to other nanocarriers (which may also be considered in future for anti-VEGF sustained release), polymersomes, PLGA micro/nanoparticles and gold nanoparticles were also studied for their scleral disposition. Similar to liposomal transport studies, the carriers were fluorescently labelled for studying their scleral distribution. The PLGA micro and nanoparticles were found to be episclerally localized. Polymersomes of comparable size to PLGA nanoparticles were found to show intrascleral distribution, thus indicating the role of particle flexibility in facilitating intrascleral diffusion. Gold nanoparticles owing to their small size showed

trans-scleral transport all the way across the sclera. Spherical Nucleic Acids, (from Northwestern University) which are useful carriers for oligonucleotides, and hence promising for gene therapies, were also tried out for their trans-scleral transport. Two types of SNAs were studied: Gold particle conjugated oligomers and liposomal Oligomers. In case of the gold-SNAs, the Gold nanoparticle was covalently conjugated with the oligomer, and from scleral transport studies, it was seen that the conjugate possibly travels intact, with the diffusion hindered by the conjugation with nanoparticle, compared to bare oligomer transport. In comparison, the liposomal formulation has the oligomer encapsulated. It was observed that the oligomer alone can detach and transport trans-sclerally while the liposomes remain episclerally (and partially intrasclerally) localized. This final result is a very interesting and important observation. Carriers such as liposomes are able to remain either in the episcleral space or intrasclerally, as a depot and release their encapsulated contents, which can then diffuse trans-sclerally to reach the site of therapeutic action.

The main novelty of this work lies in the fact that properties of nanocarriers such as size, charge and fluidity have been studied for their effect on trans-scleral transport, which have not been studied previously. These factors and their effects are summarized in Table 7.1 below:

Table 7.1: Summary of the properties of nanocarriers affecting their trans-scleral transport.

Property of nanocarrier	Effect on trans-scleral transport
Size	Smaller the size of the particle, higher is the extent of trans-scleral penetration and transport
Charge	Positively charged nanoparticles are found to be episclerally localized; whereas negatively charged nanocarriers show some degree of intrascleral penetration
Rigidity of nanoparticle	Nanoparticles with higher fluidity are found to exhibit a higher degree of intrascleral diffusion when compared to rigid nanoparticles.

Armed with the knowledge on nanocarriers, encapsulation of Lucentis was attempted in liposomes. Based on the observations on transport, an array of unsaturated liposomes (POPC and POPC-Cholesterol), saturated liposomes (DPPC and DPPC-Cholesterol) and charged liposomes (DPPC-DPTAP (positive) and DPPC-DPPG(negative)) were tested for their Lucentis encapsulation, release profiles and finally their trans-scleral *ex vivo* transport. The *ex vivo* transport of these formulations were compared with that of bare Lucentis. The liposomal formulations were introduced in the donor chamber of the Ussing chamber and the Lucentis which gets released from the carriers, was quantified in the receiver chamber samples after being transported trans-sclerally. Among the formulations, the negatively charged DPPC-DPPG was found to have the highest encapsulation efficiency and the slowest and sustained release profile. In addition, the *ex vivo* transport was slower for the DPPC-DPPG formulation and significantly different from the transport of bare-ranibizumab. If this transport profile can be replicated *in vivo*, this carrier formulation could prove to be a promising candidate for Lucentis sustained release as it combines the properties of acting as an intrascleral depot, high encapsulation and slow and sustained release.

One main limitation in this study is the constraint on the time duration of the *ex vivo* transport experiments which was possible to be performed. Previous *ex vivo* experiments have been conducted for 6-days using rabbit sclera [17] and the scleral ultrastructure was found to be preserved. Further extension of the *ex vivo* transport experiments beyond 1-week is not recommended considering the limited viability of the tissue explant. Hence, *in vivo* experiments are warranted to follow up on long term sustained release studies, preselected on the basis of the preliminary *ex vivo* studies. *In vivo* studies can also provide information on the effect of circulation and clearance systems on the drug and carrier distribution.

7.2 Implications of research findings

The hypothesis that certain formulations can help achieve sustained delivery of drugs after subconjunctival administration was explored in this dissertation. This was done using an *ex vivo* setup using porcine scleral tissue with which initial studies of bare drug and bare carriers were conducted followed by studies with drug loaded carriers. Within the time period of the study, we showed a marked difference in scleral penetration of the various carrier formulations depending on their specific properties. The carriers adhered episclerally or showed varying degrees of intrascleral diffusion. After encapsulating ranibizumab in liposomes, *in vitro* release studies and *ex vivo* scleral transport studies were performed using various formulations. For *ex vivo* carrier mediated transport studies, the amount of drug transport was quantified across the sclera. It was possible to show that certain formulations have promising characteristics: intrascleral diffusion thus forming a depot, high encapsulation efficiency thus being able to hold larger drug cargo, and slow, sustained release of drug in quantifiable amounts which then traversed across sclera.

Ex vivo setups such as the one used in this study are extremely useful screening tools before embarking on costly, *in vivo* work. In that regard, a successful system has been developed, which can be used in future for testing a spectrum of drugs, nanocarriers, or other molecules. The type of sclera used for the work in this thesis was porcine sclera due to its anatomical similarity to human sclera. The setup may be easily customized to use any other animal tissue models such as rabbit or human sclera.

Trans-scleral transport of various nanocarriers were studied in order to study the nanocarriers properties affecting scleral localization and transport of the carriers. The nanocarriers studied include liposomes, polymersomes, PLGA micro and nanoparticles and Gold nanoparticles. Size, charge, and fluidity of the liposomes were modulated and the effect of these properties on transport were studied. It was observed that small sized, neutral liposomes made of unsaturated lipids were able to distribute intrasclerally more compared to larger sized neutral liposomes. Positively charged liposomes adhered

preferentially on the episcleral surface without showing intrascleral diffusion, compared to negatively charged liposomes which had a small degree of intrascleral transport. Saturated liposomes which had high transition temperatures were also found to show a lower degree of intrascleral diffusion compared to unsaturated liposomes which have more fluid bilayers. Rigid particles such as PLGA, on the other hand demonstrated episcleral localization without appreciable intrascleral diffusion.

By the use of these unsaturated, neutral lipid types, we may be able to increase the scleral residence time if the liposomes can act as a depot inside the sclera, without being cleared rapidly by the circulatory and lymphatic systems. Moreover, by manipulation of the size/charge/rigidity properties of the liposomes, it is possible to get formulations of different encapsulation efficiencies or retention abilities at the subconjunctival space. This could be a very interesting and novel application for a non-invasive depot forming system which can then deliver the encapsulated cargo from the periocular space, thus avoiding the invasive intravitreal route.

A major bottleneck for providing sufficient therapeutic amounts in a sustained fashion is the degree of encapsulation possible in the carrier system. The encapsulation efficiency for large, hydrophilic drug molecules may be larger with larger vesicles such as MLVs or microparticles. Hence, the larger sized MLVs or microparticles may be used for sustained delivery, as the carriers may remain at the episcleral site and slowly release the encapsulated drug alone which may transport across the sclera to reach the desired site of action. The nano-sized carriers which also do not completely transport across the sclera may, on the other hand be available as a depot inside the sclera. However, smaller the size, smaller will be the capacity for drug cargo in the core of the liposome, which may then necessitate a trade-off between transport and encapsulation. In case of charged liposomes, a charge-interaction such as the one observed in case of DPPC-DPPG liposomes towards Lucentis, might prove to be useful in providing a high degree of encapsulation, as well as slow release. The type of liposome best suited for any particular application may need further consideration such as the drug release profile for the clinical need and the interaction of drug and liposome, partitioning effects, degree of

encapsulation, etc.

Overall, it can be concluded that there are a few factors influencing the *ex vivo* transport of drug from nanocarriers:

- 1) Drug-carrier interaction and encapsulation efficiency
- 2) Carrier localization in the sclera
- 3) Drug release rate from the carrier
- 4) Drug-sclera interactions

With the prototype formulations discussed in this work, we have shown that Anti-VEGF antibodies can be reliably delivered trans-sclerally with retention of bioactivity, using this *ex vivo* model. However, in order to test the efficacy of these formulations in an animal model, *in vivo* studies are warranted. The formulations may be applied subconjunctivally, and the pharmacokinetics and drug distribution may be studied. Based on a review of the existing literature on barriers to trans-scleral drug delivery, it can be seen that dynamic barriers play a major role in drug clearance *in vivo*. [20]. Episcleral clearance and conjunctival lymphatics maybe responsible for rapid clearance of drugs introduced subconjunctivally [21-23]. Hence, intrasclerally localized depots might provide better retention and sustained action of delivery for the drugs when compared to carriers remaining on the episcleral surface

Potentially, the results gained from this study can be used and extrapolated in designing and tailoring suitable sustained release carriers for several posterior ocular segment disorders. The outcomes from this study are significant and point to improved benefits for AMD treatment via the subconjunctival route

Some unanswered questions remain that need to be addressed, for which recommendations are made in the following section and highlighted with suitable methods for future studies.

7.3 Recommendations and Future Work

The research presented here represents some of the first studies of its kind to examine the properties of nanocarriers for trans-scleral delivery. This work can predominantly serve as a screening tool to understand and study the distribution of nanocarriers inside the sclera from an episcleral depot system. This being the first study of its kind on trans-scleral nanocarrier transport, could open up avenues for further developments in this field.

Some unanswered questions/interesting follow on work that could be addressed in this section.

- 1) There is a need for future work focusing on understanding the *in vivo* intraocular pharmacokinetics of Anti-VEGF drugs after subconjunctival injections. Experiments should be designed to understand how different drug formulations and properties of nanocarriers affect their pharmacokinetics in the eye, following this route. Understanding and determining the concentration of drugs in the intraocular tissues will help determine the potential of these injections have to treat posterior ocular segment diseases.
- 2) This work has resulted in the development of an *ex vivo* system which may be used to screen different types of drug molecules or nanocarriers across the sclera. There is scope for using the setup to expanding the repertoire of carrier molecules which can be tested for trans-scleral transport.
- 3) *In vivo* studies with the bare liposomes and loaded liposomes
It would be interesting to evaluate the pharmacokinetics of the drug loaded liposomes and its drug distribution within various ocular tissues on subconjunctival injection to obtain *ex vivo-in vivo* correlation. The liposomes and drug may either be fluorescently labelled or radiolabeled, for the *in vivo* studies. Studies on rabbits or mice as a suitable animal model can help to determine its future clinical potential.

4) Studies with Eylea and other Anti-VEGF drugs

It would be interesting to evaluate if drugs such as Avastin, Eylea, which are also Anti-VEGF antibodies, would behave similarly for sustained release *ex vivo*, and the studies could be extended *in vivo*. This would help us to gain insight into how the molecular sizes of drug can affect transport and loading in liposomes and different release behaviors.

5) Loading liposomes in a hydrogel

In addition to using liposomes themselves as a carrier, a combination of liposomes and biopolymer-based hydrogels could offer important advantages by improving drug formulation stabilities and extending the drug release profile. Preliminary work on incorporating drug-loaded liposomes into a hydrogel have been conducted by Bora et al. [24, 25]

6) Exploring the encapsulation and delivery of anti-VEGF using carriers such as polymersomes, Layer by Layer (LbL) type of carriers, chitosan nanoparticles, etc also could be interesting work which can be further explored. Liposomes may also be coated with suitable polyelectrolytes to enhance episcleral/intrascleral localization depending on the clinical need for particular disease models.

7) The depot idea of subconjunctival drug delivery, is also applicable for treatment of anterior segment disorders which require extended therapy, such as glaucoma or post-surgical inflammation management for cataract. Deeper scleral tissue penetration may not be a requirement in this mode of treatment and episcleral localization might suffice to deliver the drugs anteriorly, in a sustained fashion.

In the context of ocular drug delivery, this work offers an exciting opportunity to explore a new strategy to provided minimally invasive and sustained drug delivery from a subconjunctival depot formulation. The setup by itself can serve as a valuable research tool that can lead to the screening and choice of several drugs and nanocarriers

formulation for trans-scleral studies. It could lead to development of optimal formulations for applications in the subconjunctival drug delivery. We hope this work will pave the way for new and exciting research to explore and develop better therapies for the treatment of ocular diseases.

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APPENDIX

A. Cryo TEM imaging of POPC liposomes

Cryo TEM studies were carried out using a Carl Zeiss Libra 120 Plus TEM (Germany), at 120kV using a TRS Sharp Eye slow scan dual speed CCD camera (Troendle, Mooren Wies, Germany) and the ITEM software (Olympus-SIS, Soft Image System, Munster, Germany) was used for image acquisition. 1 μ L of the POPC liposome was deposited on the lacey Formvar grid, which was post-treated with 30s glow discharge using JEOL HDT-400 in order to obtain a hydrophilic surface. Excess sample was blotted away and using a Gatan Cryoplunge 3 (Cp3), the samples were quickly vitrified inside liquid ethane. The grids were quickly transferred to liquid nitrogen and stored in dewar flask until imaging was performed. Special care has to be exercised while transferring samples to prevent formation of ice crystals.

Figure A.1 Cryo-TEM image of POPC liposomes.

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Technology disclosure/Provisional patent application

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