Factors affecting trans-scleral transport of poly (butadiene-b-ethylene oxide) polymersomes

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**FACTORS AFFECTING TRANS-SCLERAL TRANSPORT
OF POLY (BUTADIENE-*b*-ETHYLENE OXIDE)
POLYMERSOMES**

VINCENT ANITA

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

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OF POLY (BUTADIENE-*b*-ETHYLENE OXIDE)
POLYMERSOMES**

VINCENT ANITA

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Master of Engineering

2019

Statement of Originality

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

8th August 2019

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8th August 2019

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Prof. Subramanian Venkatraman

Authorship Attribution Statement

This thesis includes content where I received support from my supervisor and collaborators as follows:

- Professor Subbu Venkatraman provided the initial project direction and edited the thesis.
- I optimized the formulation protocols along with Dr. Alexandra Alvarez Fernandez.
- Dr. Rini Rachel Joseph designed the initial experimental setup which was optimized by me with assistance from Ms. Ramya Bhuthalingam.
- Dr. Xiong Minru Gordan obtained scanning electron microscopy images of sclera.

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Abstract

Polymersomes are synthetic analogues of liposomes formed by the self-assembly of amphiphilic block copolymers as a result of hydrophobic-hydrophilic interactions with the surrounding solvent. These particles retain the drug loading versatility of liposomes while exhibiting enhanced membrane toughness and reduced permeability. As a relatively 'young' nanoparticle system, most work in the field of polymersomes focus on designing unique polymersome assemblies via synthesis of new polymers, new formulation techniques, particle functionalization etc. There is then a need to develop polymersome technology towards practical applications.

In this light, this research work examines the suitability of polymersomes for ocular drug delivery via periocular route of drug administration. Periocular drug delivery presents an alternative treatment strategy for posterior segment eye disease (PSEDs) by administering drugs into the periocular space surrounding the eye orbit; the drugs then diffuse from this space across the sclera and choroid into the posterior segment of the eye, allowing for improved bioavailability and therapeutic effect. Drug loaded polymersomes could further enhance this treatment strategy by forming periocular or intrascleral drug depots capable of releasing a sustained dose of drug over extended time periods.

In order to study the potential of polymersomes for periocular drug delivery via the formation of intrascleral drug depots, poly (butadiene – b- ethylene oxide) polymersomes with varying particle properties (hydrodynamic size, bilayer bending rigidity, surface charge and hydrophilicity) were formulated. All formulations were meticulously characterized in order to understand the effect of polymersome properties on trans-scleral transport behaviour as observed by *ex-vivo* passive transport experiments across porcine sclera.

Polymersomes were found to form periocular depots in the case of most formulations with several formulations forming intrascleral depots, thus proving their viability as

nanoparticles for periocular drug delivery. Further, the observed trans-scleral transport behaviours of the different polymersome formulations were found to be affected by particle properties such as hydrodynamic size, bilayer bending rigidity, surface charge and hydrophilicity. In general, small hydrophilic polymersomes with flexible bilayers and low negative surface charge were found to have maximum permeation into the sclera.

In conclusion, this research study has proven the ability of poly (butadiene – b – ethylene oxide) polymersomes to form periocular and intrascleral depots which indicates the possibility of using the nanoparticle system for periocular drug delivery. Future work focused on optimizing drug loading and release of these particles would determine the eventual viability of polymersomes for periocular drug delivery.

Lay Summary

As people grow older, they often face health issues related to vision which can greatly affect their quality of life. Most of these health issues occur at the back of the eye which makes treatment quite difficult, as the drugs used to treat these medical conditions have trouble reaching the problem areas. One way around this is to deliver the drug into the space behind the eyeball without puncturing the eyeball itself, thus reducing discomfort and side effects. Drugs in the space behind the eye move across the tissue layers of the eye and into the eyeball itself, reaching the problem areas quickly to treat the existing condition.

While delivering the drug into the space behind the eyeball can improve treatment of back-of-the-eye medical conditions, natural biological processes can remove the drug from this space before it can move into the eyeball. Further, while the procedures associated with this method of treatment is less invasive, it still requires visits to a clinician as it cannot be done without training. One way to improve on these aspects of the treatment is to use nanosized particles; a nanosized particle can hold the drug within itself, thus protecting it from being removed prematurely and can release it slowly over a long time period, reducing the need to frequent clinical visits.

Polymersomes are one such type of nanoparticles and are made from polymer chains that arrange themselves in water to form bubble-like structures. In this study, these polymersome particles were studied in terms of their ability to move into the sclera which is the main tissue present at the back of the eye. The particles either sit on the surface of the sclera or move into the sclera and are embedded in the tissue to form a drug depot. From these depots, any loaded drug can then be released slowly, thus providing long term treatment of back-of-the-eye medical conditions.

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Abbreviations

PSED	Posterior Segment Eye Diseases
P _c	Packing factor
AMD	Age-related Macular Degeneration
WHO	World Health Organization
Bd-EO	Poly (butadiene-b-ethylene oxide)
Bd-AA	Poly (butadiene-b-acrylic acid)
AA	Acrylic acid
Rho	Rhodamine
PBS	Phosphate-Buffered Saline
DLS	Dynamic Light Scattering
PDI	Polydispersity Index
SLS	Static Light Scattering
Cryo-TEM	Cryogenic Transmission Electron Microscopy
AVA	Agri-food and Veterinary Authority of Singapore
DAPI	4', 6-diamidino-2-phenylindole
DSC	Differential Scanning Calorimetry
T _g	Glass transition temperature
T _m	Melting temperature

Chapter 1

Introduction

This chapter describes the background and hypothesis of this research work followed by a precise definition of its objectives and scope of study. In brief, this research work hypothesizes that polymersomes can act as drug delivery systems for periocular drug delivery by forming intrascleral drug depots and aims to identify the specific particle parameters affecting trans-scleral transport. In addition, a concise overview of the dissertation summarizing all constituent chapters is provided, along with some insight into the novel outcomes of the work.

1.1 Background and Hypothesis

Polymersomes refer to bilayered vesicles formed by the self-assembly of amphiphilic block co-polymers. This self-assembly is driven by various intrinsic and extrinsic factors and leads to a versatile particle that can be loaded with hydrophobic (in the bilayer) or hydrophilic (in the core) moieties; it is worthwhile then to study the viability for these particles as drug carriers for specific therapeutic applications.

The success of any drug carrier can be considered to depend on two factors: 1) its interaction with the drug under study which in turn determines drug loading and release profiles, and 2) its behavior in the specific biological environment of its therapeutic application. Most research work in the field of polymersomes focus on the former, studying diverse polymer-drug combinations. In contrast, this thesis aims to understand the behaviour polymersomes at a potential site of action - the periocular space.

Periocular drug delivery presents a potential treatment method for posterior segment eye diseases (PSEDs) with improved efficiency and reduced side effects [1]. The main barrier for drug delivery via this route is the sclera, a disordered fiber matrix mainly composed of collagen (Type-I) and elastin [2] with widely dispersed fibroblasts and proteoglycans [3]. This specific architecture of the sclera results in a porous barrier with a net negative charge [4]; these scleral characteristics determine the transport of material (drugs, nanoparticles etc.) across the sclera.

Keeping the properties of the sclera in mind, it is then necessary to understand the trans-scleral transport behavior of polymersomes in order to evaluate their potential for trans-scleral drug delivery, possibly by forming an intrascleral drug depot within the sclera. In particular, this study focuses on the properties of the particle itself such as hydrodynamic size, surface charge and membrane fluidity on trans-scleral transport.

To this end, the hypothesis of this thesis is as follows:

“There are certain structural features of polymersomes that facilitate their permeation into the sclera to form an intrascleral drug depot for periocular drug delivery.”

The formation of trans-scleral depots by polymersomes would open up the possibility of using these particles for the treatment of posterior segment eye diseases (PSEDs) via sustained release of ocular drugs.

1.2 Objectives and Scope

In order to test the hypothesis of this work, trans-scleral experiments would have to be conducted using polymersomes with varying particle properties. The formulations developed would need to have comparable vesicle morphologies and acceptable particle size dispersity in order to reliably identify the crucial parameters affecting transport. The trans-scleral experiment itself would require the development of a methodology that would adequately model in-vivo conditions, via the use of porcine sclera modelling the human sclera due to its similar architecture and permeability [5].

The objective of this work is then *to identify the main particle parameters (hydrodynamic size, block copolymer properties such as molar masses of the two blocks, surface charge) affecting trans-scleral transport and to understand the mechanisms involved.*

The scope of this work is then as follows:

1. Formulation and characterization of polymersomes with varying particle properties
2. Development of a robust and reproducible trans-scleral transport methodology
3. Study of trans-scleral transport of polymersomes of varying properties
4. Identification of specific mechanisms leading to observed trans-scleral transport

The knowledge obtained from this study could in turn lead to greater understanding of the properties of the sclera itself which could potentially aid in the design of more nanocarriers for trans-scleral drug delivery.

1.3 Dissertation Overview

The thesis consists of five chapters, the contents of which are described as follows:

Chapter 1 provides background information supporting the rationale of this research work and clearly defines the project hypothesis, objectives and scope. A brief summary of the novel outcomes of the work is also provided.

Chapter 2 summaries the existing literature relevant to the current state of research in the field of polymersomes and their potential application in the treatment of posterior segment eye diseases (PSEDs) via periocular route of administration.

Chapter 3 describes the rationale for selection of materials used to study trans-scleral transport of polymersomes. Polymersome formulation and characterization techniques are explained as well as trans-scleral transport experimental methodology.

Chapter 4 presents the results of trans-scleral transport experiments of various polymersome formulations and explains the observed behaviours in terms of polymersome properties.

Chapter 5 summaries the overall project and addresses the implications of the outcomes with reference to the initial hypothesis. Limitations of the work and recommendations for future improvements are also provided.

1.4 Novel Outcomes

This research has resulted in several novel outcomes by:

1. Proving the ability of polymersomes to form minimally invasive trans-scleral depots to treat posterior segment eye diseases (PSEDs)
2. Correlating polymersome properties to observed trans-scleral transport behaviour, providing a framework for design of polymersomes for periocular drug delivery
3. Achieving enhanced trans-scleral transport by forming blended polymersomes that exploit polymersome-sclera interactions to improve particle permeation

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Chapter 2

Literature Review

This chapter summaries the existing understanding of the polymersome system and identifies its potentials in the field of drug delivery. It also provides a detailed review of the challenges of conventional ocular drug delivery techniques and proposes periocular drug delivery as a potential alternative. The challenges of trans-scleral drug delivery are also defined and previous trans-scleral transport studies of drug molecule and/or nanoparticles are analyzed as the starting point for this research project.

2.1 Polymersomes

Vesicles formed by self-assembly of diblock copolymers or ‘polymersomes’ were first demonstrated by Discher and coworkers in 1999 [1] using poly(ethylene oxide-ethylethylene) as the building block (Fig.2.1). Discher built upon prior studies on the self-assembly of natural phospholipids in the cell membrane as well as synthetic systems like liposomes to develop these ‘polymer-based liposomes’ that retained the drug loading versatility of liposomes while exhibited enhanced membrane toughness and reduced permeability [1]. These unique properties along with the wide scope of block copolymer chemistry has led to great interest in understanding polymersome synthesis and properties (Fig.2.2).

Figure 2. 1: Schematic representation of a polymersomes and its possible loaded moieties.

Figure 2. 2: Number of papers published on the topic of polymersomes from 1999 to 2018 (PubMed database); steady increase in publications indicate growing interest in polymersome systems for a wide range of applications including drug delivery, nano-reactors etc.

While a significant amount of work is being done on developing and understanding novel polymersome systems, there is a need to develop the concept towards a useful application. Polymersomes have been proposed as possible candidates for application in a wide range of fields ranging from drug delivery to nano-reactors to biosensors; understanding the specific advantages and challenges of polymersome formulation and function could aid in exploiting the system to significantly improve current technology.

2.1.1 Mechanism of formation

The formation of polymersomes is a self-assembly process driven by hydrophobic-hydrophilic interactions with the surrounding solvent. Self-assembly can be described as an aggregation process when the final structures are ordered and can be reproduced; it is necessary then, that the components undergoing self-assembly have some kind of affinity to each other. In the case of amphiphilic block copolymers, this affinity is the relative hydrophobicity/hydrophilicity of the different blocks – the hydrophobic block of a polymer chain associates with that of another chain. This association is the beginning of

the polymersome self-assembly process (Fig. 2.3) and is driven by increase in block copolymer concentration in the solution; the block copolymers are now close enough to self-assemble into micelles that shield the hydrophobic blocks away from the aqueous solvent. At this point, the formation mechanism diverges along one of two possible pathways: A) the micelles grow into rod-like bilayers that bend into itself to reduce surface tension, thus forming vesicles [2], or B) the micelles swell and rearrange themselves to form semi-vesicles that then develop into vesicles [3]. It is then necessary to determine the concentration at which these vesicles are formed in order to develop stable formulations. The exact mechanism pathway may depend on the specific block copolymer as well as environment conditions such as solvent interactions, presence of salts etc.

Figure 2. 3: Mechanism of formation of polymersomes as a function of increasing polymer concentration; the process may follow one of two pathways A) micelles form rod-like bilayers that bend to form vesicles or B) micelles swell into vesicles.

The final structures formed by the self-assembly of block copolymers also depend on thermodynamic considerations that aim to minimize the free energy of the system. The specific geometry of these structures can be largely predicted based on the critical packing parameter P_c of the block copolymer. Critical packing parameter P_c was first proposed by Israelachvili and coworkers to study the self-assembly of amphiphilic

molecules [4]. P_c links the geometric properties of a single amphiphile to the potential morphology of its self-assembly [4] (Table 2.1) and is defined as:

$$P_c = \frac{v_A}{a_B \times L_{total}}$$

where v_A is the volume of hydrophobic block A, a_B is the area of hydrophilic block B and L_{total} is the total length of amphiphile; in essence, P_c relates to the space occupied by the hydrophilic block of an amphiphile to that of its hydrophobic block. An easier way of evaluating this ratio is to look at the weight fraction of hydrophilic block ($f_{hydrophilic}$) of a given block co-polymer. In general, $f_{hydrophilic}$ for block co-polymers forming vesicles tends to be lesser than 0.45 [5]; for a specific family of block copolymers (e.g. poly (butadiene-b-ethylene oxide)), this value can be narrowed down further (~ 0.35) [6].

Table 2.1: Critical packing parameter associated with self-assembly of amphiphilic molecules.

Geometry of single chain	Range of P_c value	Self-assembled morphology
$P_c < \frac{1}{3}$	Micelles
$\frac{1}{2} > P_c > \frac{1}{3}$	Rod-like micelles
$1 > P_c > \frac{1}{2}$	Vesicles

2.1.2 Polymersomes for drug delivery

Much of the interest in polymersome systems has been focused on its potential as a drug delivery carrier. As a synthetic analogue of liposomes, it retains the drug loading capabilities of its lipid-based cousins while introducing enhanced physiochemical stability and chemical versatility [7]. The enhanced properties for polymersomes mainly arise from the properties of its amphiphilic building blocks; exploiting the diversity of polymer chemistry could then be used to achieve polymersomes with the specific properties required for each drug delivery application.

2.1.2.1 Characteristics of drug delivering polymersomes

Self-assembled polymersomes can be characterized in terms of size profile, surface chemistry and bilayer properties (thickness, rigidity etc.). These particle characteristics are a consequence of specific properties of their block copolymers (polymer chain length, chain flexibility, microstructure etc.) and in turn, determine the suitability of polymersomes for a given application. The exact properties required of a given polymersome formulation greatly depend on its intended application; for example, 200nm sized polymersomes have been shown to accumulate in murine peritoneal tumours when administered into the peritoneal space [8] but would be rapidly eliminated from the blood stream if administered systemically [9].

Polymersomes as drug delivery systems require uniform, reproducible size profiles (<200nm) with narrow size distribution (PDI) which allows for greater control of formulation properties (e.g. drug loading) and behaviour (e.g. permeation across biological barriers). This can be achieved by modifying 1) intrinsic properties of polymers chains (molecular weight, microstructure, chain fluidity etc.) and 2) formulation conditions (temperature, extrusion etc.). Polymersome particle size tends to decrease with decrease in molecular weight of a given polymer chain structure [10]. Changes in microstructure such as the presence of branching can modify the polymersome formation mechanism, allowing for size control [11]. Polymersomes formed by more fluid polymer chains (characterized by low glass transition temperatures) allow to greater size control using formulation techniques such as extrusion due to their

highly fluid bilayers [12].

Aside from size, the in-vivo fate of polymersomes can also be affected by surface chemistry (surface charge, hydrophilicity etc.) or bilayer rigidity. For example, negatively charged polymersomes have been shown to have a significantly different biodistribution compared to neutral polymersomes in mice (systemic administration) [13]. Hydrophilicity (arising from polymer chain chemistry) and bilayer rigidity (arising from polymer chain fluidity and self-assembly) becomes particularly relevant in applications that involved permeation across a tissue barrier such as skin, sclera etc. [14].

2.1.2.2 Current work on polymersomes for drug delivery

Much of the work done on developing polymersomes for drug delivery focuses in studying the effect of block copolymer properties on polymersome performance. A wide range of block co-polymers have been studied so far, often with poly (ethylene oxide) as the hydrophilic block in order to achieve enhanced circulation time [15]. Some of the block copolymers first studied include poly (butadiene-b-ethylene oxide), poly (lactic acid-b-ethylene oxide) and poly (caprolactone-b- ethylene oxide) [16-18]. These systems show loading of hydrophobic molecules (doxorubicin/paclitaxel) comparable to liposomes with release occurring either by passive diffusion through the bilayer or by hydrolytic degradation of the particle itself. Other studies focus on developing novel block copolymers that responded to pH, temperature or even magnetic field for triggered release their drug cargo [19-21]. Another area of focus is developing new formulations techniques as well as to optimize existing methods to cater to the diverse properties of the drug molecules under study; for example, blending a homopolymer with a diblock copolymer with gradual addition of aqueous buffer was found to enhance the loading of hydrophilic biomolecules [22].

Aside from drug delivery, polymersome systems have also been studied as potential bioimaging probes, nanoreactors, synthetic cell mimics, etc. [7, 23, 24]. However, much of the work being done in these areas is still at a fundamental research level; there is then a need for application-based work that could lead to scale-up and a viable product.

2.2 Ocular drug delivery

Humans are visual creatures. The evolution of trichromatic vision along with a highly adaptable brain [25] capable of processing entire images in as little as 13 milliseconds [26] means that we interact with the environment predominantly via visual cues. This in turn has led to a social order where blindness is a severe handicap. Blindness and visual impairment presents a significant health burden (Fig. 2.4) that looks poised to increase with the increase in ageing populations worldwide [27]. In particular, age-related ocular diseases such as age-related macular degeneration (AMD), glaucoma etc. that affect the posterior segment of the eye are expected to becoming leading causes of visual impairment and blindness [28]. It is then necessary to understand the challenges of treating posterior segment eye diseases (PSED) and to develop effective treatment plans to mitigate them.

Figure 2. 4: Leading causes of A) visual impairment and B) blindness according to WHO as of 2010; PSEDs are a significant cause and would need effective treatment plans to mitigate.

Challenges to ocular drug delivery arise from the unique physiology to the eye. Specific barriers to drug delivery depends on the route of administration i.e. topical administration, localized injection or systemic administration [29]. The barriers can be physical barriers such as ocular tissue (cornea, sclera, choroid etc.) that inhibit transport of drug to the required target or processes such as tear turnover, conjunctival blood circulation etc. that clear the drug away from the point of administration [29]. Very often, a combination of physical barriers and clearance processes work together to limit the amount of functional drug reaching the target area (bioavailability).

For example consider a posterior segment eye disease (PSED) such as age-related macular regeneration. Conventional routes of administration of drugs targeting PSEDs present a variety of challenges that limit bioavailability and subsequently compromise the effectiveness of the treatment (Table 2.2).

Table 2.2: Advantages and disadvantages of common routes of drug administration for posterior segment eye diseases (PSEDs)

Route of administration	Advantages	Disadvantages
Topical	Non- invasive High patient compliance	Increased diffusion path and large number of barriers to cross to reach posterior of the eye [30] Loss of drug due to processes like tear production, conjunctival blood circulation etc. [29] Bioavailability of topical drug < 5% [31]
Intravitreal injection	High drug conc. at injected site Little to no clearance by blood or lymphatic circulation	Invasive method => lesser patient compliance Potential side effects such as retinal detachment, hemorrhage etc. [32]
Systemic (oral)	Non- invasive High patient compliance	Low drug conc. at target site to due to: <ul style="list-style-type: none"> dilution/degradation during systemic circulation blood-retinal barrier Accumulation of drug in non-ocular tissue leading of adverse side effects Bioavailability of systemic drug < 2%

There are then two possible strategies to enhance bioavailability of drugs for PSEDs, 1) develop therapeutic agents that can pass through the barriers or 2) find a new route of administration. Therapeutic agents can be designed to overcome the problem of bioavailability by exploiting charge interactions, hydrophobic-hydrophilic affinity, size etc. in order for pass through ocular tissue without being cleared away while alternative routes of administration such as injection into the periocular space might open up new avenues for treatment. Most probably, a more effective treatment plan would require focus on both strategies in order to significantly improve the existing treatment.

2.3 Periocular drug delivery

Periocular route of ocular drug delivery includes a variety of drug administration techniques such as subconjunctival, sub-tenon, retrobulbar, peribulbar and posterior juxtасcleral routes (Fig. 2.5) [33]. Drugs for PSEDs administered via these routes have shorter diffusion paths through the sclera, choroid and retinal pigment epithelium to the retina and even the vitreous humor; they bypass the cornea and conjunctiva while avoiding clearance by tear production, conjunctival circulation etc. [33]. Periocular techniques has been used extensively for administering ocular anesthesia due to which there is sufficient data about the effectiveness of these methods and the associated side effects [34]. In general, while risks such as hemorrhaging and chemosis have been observed, the prevalence is low and less traumatic compared the side effects of intravitreal injections [35]. Further improvements in technology and personnel training can reduce risk involved in periocular drug delivery [36].

Figure 2. 5: Periocular routes of ocular drug delivery. Adapted from [33]

Drugs administered in the periocular space move into the posterior of the eye through three identified pathways: trans-scleral diffusion, systemic circulation (via the choroid) and trans-conjunctival diffusion into the anterior segment [37]. A study of the permeation pathways of radiolabeled mannitol showed trans-scleral diffusion to be the predominant pathway for subconjunctival injection [38]; it is then necessary to understand the parameters affecting trans-scleral diffusion in order to enhance the efficiency of periocular drug delivery.

2.3.1 Trans-scleral diffusion pathway

Trans-scleral diffusion of a given molecule essentially depends on its interaction with the sclera, which in turn is a result of the specific properties of the sclera and the drug. Compared to other ocular tissue, the sclera is more permeable to a range of molecules due to its large surface area [39], porous structure [33] and reduced protease activity [40]. Permeability however is still limited by the specific architecture of the sclera – a matrix of predominantly Type-1 collagen fibers with a sparse population of fibroblasts, and proteoglycans [41]. The collagen fiber arrangement is interspersed with pores that allow

the diffusion of molecules into the sclera; trans-scleral transport is then limited by the size of these pores. The proteoglycans further confer a net negative charge to the sclera which affects the transport of charged molecules. Binding interactions may also arise from specific interactions of drugs with various components of the sclera. Table 2.3 lists various drug properties and their effect on trans-scleral transport as well as possible mechanisms of interaction.

Table 2.3: Physiochemical properties of drugs and their effect on trans-scleral transport

Property of the drug	Effect on permeability	Possible mechanism
Hydrodynamic size [42]	Permeability decreases exponentially with increase in hydrodynamic size	Limited by pore size and intracellular space of sclera [42]
Surface charge (for a given hydrodynamic size) [43]	High scleral permeability of negative molecules Intermediate permeability of neutral molecules Low scleral permeability of positive molecules	Electrostatic interaction with negatively charged proteoglycans [44] in sclera
Lipophilicity (for a given hydrodynamic size) [43]	Permeability decreases with increase in lipophilicity	Melanin binding [45] / easier passage along aqueous proteoglycans [46]

Trans-scleral transport of nanoparticles could also potentially follow the patterns described in Table 2.3. Considering the permeable nature of the sclera, the use of nanoparticles for periocular drug delivery would aim to increase bioavailability and achieve significant sustained release. To this end, either the nanoparticles are capable of penetrating through the various ocular tissue to reach the target while keeping its drug

cargo intact (increased bioavailability) or they form a drug depot from which the drug is released at therapeutic levels over a prolonged period of time (sustained release).

2.3.2 Nanoparticles for periocular drug delivery

Studies of periocular drug delivery using nanoparticles mainly focus on polymeric hard-spheres of varying sizes and surface modifications. Nano-sized particles (<50nm) have been found to diffuse across sclera [47] but their low drug loading capacity and burst release profiles as well as their rapid clearance by the periocular and choroidal vasculature [48] limit their effectiveness as periocular drug delivery systems to improve bioavailability. Larger polymeric particles (>200nm) [47, 49, 50], however, are retained in the periocular space for prolonged periods (60 days for polystyrene microspheres) to form a periocular drug depot (Fig. 2.6 A) for significant sustained release of drugs [47]. This, along with the higher loading capacity and non-burst release profile makes them a suitable candidate for periocular drug delivery. However, micron-sized particles in the periocular space may trigger an inflammatory response that could contribute to the degradation/clearance of the particles [51].

An alternative strategy is to exploit the interaction of nanoparticles with the sclera to form an intrascleral drug depot (Fig. 2.6 B). The avascular nature of the sclera and its lack of macrophages [52] results in a tissue that is rather tolerant of embedded foreign particles. The formation of an intrascleral depot however requires the movement of the particles into the sclera; this could be achieved either through direct intrascleral administration [53, 54] or through passive diffusion from the periocular space. Direct intrascleral administration however may require the use of specialized equipment and training [54, 55] compared to more common periocular injection methods, making passive diffusion a more favored method for establishing an intrascleral depot.

Figure 2.6: Schematic of A) periocular depot, B) intrascleral depot formed for nanoparticles.

Inducing an intrascleral depot via passive diffusion requires nanoparticles that are able to diffuse into the sclera and then bind to scleral components. Such behavior has been demonstrated by self-assembled vesicles such as liposomes [56] making them an attractive carrier for periocular drug delivery. The dynamic nature of the liposomal bilayer confers a certain degree of flexibility to the particles which allows it to penetrate the sclera at higher size thresholds than solid polymeric particles; within the sclera, binding interactions and pore size restrictions allow the particles to form an intraocular depot from which drugs may be released slowly [56] into the posterior segment of the eye. However, while liposomes show relatively high drug loading for hydrophobic drugs, loading capacity of hydrophilic drugs is low and often results in burst release profile. Polymersomes, as synthetic analogs of liposomes, may offer a solution to this due to their thicker bilayers and enhanced structural stability. Further, the vesicular nature of polymersomes may allow them to form intrascleral depots in a manner similar to liposomes; identifying the parameters affecting trans-scleral transport of polymersomes would then aid in the design for polymersome formulations with improved drug loading and release profiles that form intrascleral depots.

2.3.3 Translocation of polymersomes

The formation of intrascleral depots by polymersomes requires the transport of polymersomes into the sclera; to date, no work has been to study the potential of polymersomes in this area, thus making it difficult to predict possible outcomes. However, the translocation of polymersomes across synthetic membranes have been studied, providing some idea about the factors that could affect trans-scleral transport of

polymersomes. Translocation of poly (2-(methacryloyloxy) ethyl phosphorylcholine)-b-poly (2-(diisopropylamino) ethyl methacrylate) [PMPC-PDPA] and poly (ethylene oxide)-b-poly (2-(diisopropylamino) ethyl methacrylate) [PEO-PDPA] polymersomes across polycarbonate membrane with 50nm diameter pores was studied in terms of particle size and hydrophilic block chemistry [14]. Larger PMPC-PDPA polymersomes (~400nm) were shown to translocate intact across the membrane at a higher degree compared to smaller particles (~200nm and ~100nm), possibly due to their increased flexibility. The less hydrophilic PEO-PDPA polymersomes were shown to fragment on translocation to greater degree than PMPC-PDPA polymersomes with highly hydrated hydrophilic brushes. The ex-vivo translocation of these formulations were then studied across human skin, showing enhanced transport of FITC-labelled dextran when encapsulated within polymersomes [14]. Other tissue permeation studies of polymersomes involve in-vivo experiments that involve complex polymersome designs to ensure translocation into specific targets [8, 57, 58], with observed translocation behaviours often attributed to specific polymersome-tissue interactions. These studies thus cannot predict trans-scleral transport of polymersomes; however, they do provide a framework for designing a comprehensive study of the same.

2.4 Rationale for research work

The culmination of the review of literature on the treatment of PSEDs using nanoparticles leads to the overall rationale of this research work, namely:

1. The dynamic nature of polymersomes opens up the possibility of using these nanoparticles for periocular drug delivery via the formation of periocular or trans-scleral drug depots.
2. Properties of a polymersome particle such as particle size, bilayer rigidity, surface charge, hydrophilicity etc. can affect the particle's trans-scleral transport behaviour and it is necessary to understand these effects as it opens up avenues to control polymersome particle design.

This project thus aims to develop a framework for designing drug-loaded polymersome systems for sustained drug delivery to the posterior segment of the eye with emphasis

placed on understanding the underlying mechanisms driving trans-scleral transport behaviour of polymersomes.

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Chapter 3

Experimental Methodology

This chapter gives an overview of the materials used in this research project along with various experimental protocols and techniques. The method of formulation of polymersome particles and the techniques used to characterize the particles thus formed are explained in detail, along with the underlying rationale. The trans-scleral transport setup has also been described along with the analysis protocol, to facilitate understanding of the methodology used to define the trans-scleral transport behaviour of polymersomes.

3.1 Rationale for Material Selection

Poly (butadiene-*b*-ethylene oxide) was selected as the block copolymer for this project as it is biocompatible [1]. Further, the presence of poly (ethylene oxide) leads to lower protein adsorption due to steric hindrance which in turn leads to improved circulation time [2] and delayed clearance [3]. The block copolymer also generally has glass transition temperatures (T_g) below room temperature; the polymer is thus quite fluid at room temperature, allowing for formulation techniques that do not require elevated temperatures. This presents a significant advantage when working with temperature-sensitive therapeutic agents such as proteins and peptides. Charged polymersomes were formulated by blending varying amounts of a poly (butadiene-*b*-acrylic acid) block copolymer to achieve different surface charges. Care was taken to ensure that adding the charged polymer into the bilayer would not disrupt self-assembly; to this end, the hydrophobic block of the charged polymer was restricted to poly (butadiene) and the overall polymer chain length was much lesser than that of the neutral polymer.

Table 3.1 shows the block co-polymers under consideration. The specific polymers have been chosen based in fraction of hydrophilic block f_{peo} / f_{paa} that has been reported in literature to form vesicles (Bd-EO: $f_{peo} \sim 0.35 \pm 0.03$, Bd-AA: $f_{paa} \sim 0.39$).

Table 3.1: Poly (butadiene) based block co-polymers under study (Polymer Source).

Polymer	Chemical structure	M_n (kDa)
Bd(1.2)- <i>b</i> -EO(0.6)	$\begin{array}{c} \text{1, 2 addition} \\ \left[\text{CH}_2 - \underset{\begin{array}{c} \\ \text{CH} \\ \\ \text{CH}_2 \end{array}}{\text{CH}} \right]_n - b - \left[\text{CH}_2 - \text{CH}_2 - \text{O} \right]_m \end{array}$	1.962
Bd(1.7)- <i>b</i> -EO(1)		2.808
Bd(1.3)- <i>b</i> -EO(0.6)	$\begin{array}{c} \text{1, 4 addition} \\ \left[\text{CH}_2 - \text{CH} = \text{CH} - \text{CH}_2 \right]_n - b - \left[\text{CH}_2 - \text{CH}_2 - \text{O} \right]_m \end{array}$	2.071
Bd(0.63)- <i>b</i> -AA(0.4)	$\begin{array}{c} \text{1, 4 addition} \\ \left[\text{CH}_2 - \text{CH} = \text{CH} - \text{CH}_2 \right]_n - b - \left[\text{CH}_2 - \underset{\begin{array}{c} \\ \text{C} = \text{O} \\ \\ \text{OH} \end{array}}{\text{CH}} \right]_m \end{array}$	1.236

3.2 Formulation of rhodamine tagged polymersomes

Rhodamine tagged bare polymersomes were formulated using the thin film rehydration method. Thin film hydration involves the formation of a thin film of polymer which is then rehydrated with an aqueous buffer to induce the self-assembly of the block copolymers into bilayered vesicular structures. The thin, uniform film is formed from a solution of polymer in organic solvent using a rotary evaporator. A rotary evaporator uses heat, reduced pressure and mechanical rotation to gently remove the solvent [4]; specific parameters used depend on the solvent under consideration. Bare Bd-EO polymersomes were made using this method with Rhodamine tagged lipid (Fig. 3.1, Avanti Polar Lipids) as fluorophore in order to study the transport of polymersomes across the sclera. The rhodamine tagged lipid is found to be strongly associated with the polymersome particles as evidenced by lack of leaching under concentration gradient over 4 days (1ml of fluorescent polymersomes in 30ml buffer with daily buffer change). Insertion of these lipid chains into the polymersome bilayer was shown to not affect particle properties (particle size, PDI and morphology).

Figure 3. 1: 1, 2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt); the lipid chain associates strongly with the polymersome (possibly the bilayer due to hydrophobic-hydrophilic interactions) and tags the particle with red fluorescent signal.

3.2.1. Formulation protocol:

A polymersome formulation of 1mM concentration was prepared by weighing out required amount of polymer in a round bottom flask and dissolving it in 2ml of chloroform (Fig. 3.2). Rho-tagged lipid (1mg/ml solution in chloroform) was added to the polymer solution such that the final formulation contains 1% mole fraction of the

rhodamine-tagged lipid. The round bottom flask containing the solution was then placed in a rotavap (Vacuum pressure: 247mbar, bath temperature: 60°C, chiller temperature: 5°C, rotation: setting 7) to obtain a thin film. The thin film was further dried overnight in a vacuum oven to remove any residual solvent.

Once the polymer film was completely dry, it was rehydrated with PBS with rapid stirring using a magnetic stirrer for approx. 4hrs or until a homogeneous suspension was obtained. The homogenous suspension was subsequently extruded through a 0.4µm filter followed by 0.2µm and 0.05µm filters (4 times each) to obtain unilamellar polymersomes of the required size and polydispersity.

Figure 3. 2: Schematic representation of polymersomes formulation protocol.

3.3 Characterization of rhodamine tagged polymersomes

In order to understand the interaction of polymersomes with scleral tissue, it is important to determine specific properties of the particles under study. In particular, it is necessary to know the size, polydispersity and surface charge of a given polymersome formulation as well as to confirm the formation of vesicle structures (morphology). Other properties of interest could be the glass transition temperature of the particles, bilayer thickness etc. Each of these properties may or may not affect transport properties; identifying the crucial properties for transport would then allow for greater control over particle design.

3.3.1 Size, polydispersity and surface charge: Dynamic light scattering

Dynamic light scattering (DLS) techniques use fluctuations in scattered light intensity to estimate the velocity of Brownian motion of particles to obtain particle size and polydispersity [5]. Particles suspended in a solvent undergo Brownian motion - random movement of particles due to collision with solvent molecules. The velocity of this movement is size-dependent with smaller particles having higher velocity due to greater displacement by solvent molecules [5]. The size of a particle is related to its Brownian motion velocity by the generalized Stokes-Einstein relation / Smoluchowski equation. Statistical analysis of the sizes thus determined can then be used to get an idea of the polydispersity (PDI) of sizes within a given formulation. Size and PDI data of formulations under study was obtained from the Malvern Zetasizer Nano (Malvern Panalytical, Spectris plc, UK, Fig. 3.3) system using undiluted samples in disposable polystyrene cuvettes.

Figure 3. 3: A) DLS machine (Malvern Zetasizer Nano), B) hydrodynamic size measurement from DLS, the width of the peak determine PDI of the sample.

In addition to hydrodynamic size, current DLS machines can also be used to measure zeta potential. Zeta potential is the potential difference between free solvent molecules and the layer of solvent molecules associated with the surface of a suspended particle [6]; zeta potential can then be considered a function of the surface charge of the particle (Fig. 3.4). Zeta potential of formulations under study was obtained from Malvern Zetasizer Nano

(Malvern Panalytical, Spectris plc, UK) system using 10x diluted samples in folded capillary zeta cells.

Figure 3. 4: A) Schematic showing zeta potential arising from surface charge of nanoparticles, B) zeta potential measurement from DLS.

3.3.2 Morphology and molecular weight: Static light scattering

Morphology and molecular weight of nanoparticles can be determined using static light scattering (SLS). While DLS techniques use the fluctuations in scattered light intensities, SLS uses the absolute mean intensity of scattered light at different angles relative to incident light to predict the radius of gyration (R_g) [7]. The ratio of R_g over R_h (hydrodynamic radius; obtained from DLS measurements) gives us the shape factor of the particles under consideration and can be used to predict the morphology of the particles under investigation (Table 3.2). The technique can be expanded to obtain molecular weight of an individual particle by analyzing the scattering patterns of particles at different concentrations. Shape factor (R_g/R_h) of formulations under study was obtained using the Brookhaven SLS system (Fig. 3.5, BI-200SM, Brookhaven Instruments Cooperation, NY, USA).

Figure 3. 5: A) SLS machine (BI-200SM, Brookhaven Instruments), B) R_g measurement.

Table 3.2: Shape factor (R_g/R_h) associated with different morphologies of nanoparticles.

Morphology	Shape factor (R_g/R_h)
Solid sphere (including multilamellar vesicles)	0.755 [8, 9]
Hollow sphere (unilamellar vesicles)	1 [8, 9]
Worm-like chains	1.5 [8, 9]

Additional confirmation of morphology as determined by SLS was done using cryo-transmission electron microscopy (cryo-TEM) with a robust correlation found between R_g/R_h based predictions and observed cryo-TEM images (Fig. 3.6).

Figure 3. 6: Cryo-TEM images of polymersomes confirming shape factor (R_g/R_h) based morphology predictions; A) vesicles observed in sample with $R_g/R_h \sim 1$, B) worm-like chains observed in sample with $R_g/R_h \sim 1.5$.

3.3.3 Morphology and bilayer thickness: Cryogenic transmission electron microscopy

Transmission electron microscopy (TEM) uses variation in electron transmission through different materials to obtain images in the atomic scale [10]. TEM images of polymersomes can be used to confirm morphology of the self-assembled structures and give additional information such as bilayer thickness which cannot be determined by light scattering techniques such as SLS [11].

TEM imaging of polymersomes however present a unique challenge due to their nature of self-assembly; as self-assembly is driven by solvent interactions, the structure of polymersomes collapse as formulations are dried in the copper grids used for TEM imaging. A way around this is cryo-TEM imaging. Cryo-TEM was first introduced as a way to preserve biological specimens against damage from the electron beam [12] by vitrifying samples in amorphous ice [13]. Plunge-freezing polymersome samples in a coolant (e.g. liquid ethane at -173°C) fixes the self-assembled structure within the ice layer allowing for imaging of the particles in the native state [14]. The images thus obtained can provide vital information regarding the morphology and bilayer thickness of polymersomes.

Cryo-TEM images of polymersome formulations were obtained using the Carl Zeiss Libra 120 Plus system (Fig. 3.7, Carl Zeiss, Jena, Germany) at 120kV with Gatan 626 cryo-holder (Gatan, Inc., Pleasanton, CA). Samples were prepared for imaging via the plunge freezing method where $3\mu\text{l}$ of sample was placed onto a 300 mesh Lacey carbon grid (Ted Pella Inc., Redding, CA), blotted and then plunged into liquid ethane in order to vitrify polymersomes in a thin layer of ice. The samples thus prepared were stored in liquid nitrogen prior to image acquisition to maintain vitrification. Sample temperature was maintained below -170° throughout the experiment and a 2k x 2k CCD camera (Troendle (TRS), Sharpeye) was used to record the images. The images thus obtained confirmed the morphologies of the formulations under study as predicted by the shape factor (R_g/R_h) determined by SLS. All cryo-TEM sample preparation and imaging was

done at the Facility for Analysis, Characterization, Testing and Simulation, Nanyang Technological University, Singapore.

Figure 3. 7: A) TEM machine (Carl Zeiss Libra 120 Plus, FACTS lab NTU), B) cryoplunge for cryo-TEM sample preparation, C) cryo-TEM images of polymersomes.

3.3.4 Bilayer rigidity: Differential scanning calorimetry

The bilayer rigidity of polymersomes is affected by 1) fluidity of constituent block copolymers and 2) self-assembly of polymer chains within the bilayer (interdigitation, entanglement etc.). The fluidity of constituent block copolymers can be studied using differential scanning calorimetry (DSC) which measures the change in heat flow to a sample over a range of temperature, thus detecting thermal transitions in the material [15].

For a semi-crystalline polymer, these thermal transitions correspond to glass transition of its amorphous regions from a glassy to a flexible (rubbery) state and melting transition of its crystalline regions. The temperatures at which these transitions occur are defined as T_g and T_m respectively, and are a consequence of the chemical structure of the polymer.

Identifying the T_g and T_m of the block copolymers used to form polymersomes can give some insight into the flexibility of polymersome bilayers under experimental conditions. For example, a polymer with T_m above experimental temperature (37°C) would form polymersomes with a rigid bilayer compared to a polymer with T_m below experimental temperature. DSC thermograms of different polymers used to form polymersomes were obtained by rapidly quenching a known amount of polymer to -90°C and then heating to 100°C at $1^\circ\text{C}/\text{min}$ using a differential scanning calorimeter (Fig. 3.8, DSC Q-10, TA Instruments, Delaware, USA).

Figure 3. 8: A) DSC machine (DSC Q-10, TA Instruments), B) DSC thermogram of polymer.

3.3.5 Bilayer rigidity: bilayer thickness measurements

Using thermal transition of block copolymers (as identified by DSC thermograms) to infer polymersome bilayer rigidity is limited in the case of polymers with transitions below room temperature as these polymers are viscous liquids at the experimental temperatures (37°C) regardless of changes in chain length or microstructure. In such cases, most studies of bilayer properties use direct measurement of bilayer rigidity using giant polymersomes. One common method is to measure bilayer thickness of

polymersomes and relate it to bilayer bending rigidity. The relation between bilayer thickness d and bending rigidity k_c is established as $k_c \propto d^2$ using micro pipetting experiments done in giant polymersomes [16]; this relation is further expanding into the nanoscale using coarse-grain molecular dynamics [17] in the absence of a reliable, reproducible method to directly measure bilayer rigidity of nanosized polymersomes. Thus, doubling the bilayer thickness d leads to a 4-fold increase in bending rigidity k_c , leading to greater resistance to particle deformation and thus lesser transport across a porous membrane such as sclera (Fig. 3.9).

Figure 3. 9: Schematic of movement of polymersomes across a pore; A) polymersome with rigid bilayer is resistant to deformation and unable to move across the pore, B) polymersome with a more flexible bilayer deforms and moves across the pore under diffusive forces.

Bilayer thickness of different polymersome formulations were obtained from the corresponding cryo-TEM images using an image processing software (ImageJ). For each sample, images were obtained from 3 different areas of the TEM grid and 10 particles were analyzed from each area (3 measurements per particle).

3.3.6 Hydrophilicity: Contact angle measurement

Hydrophilicity of self-assembled polymersomes arise from the hydrophilic block of their constituent block copolymer which forms the outermost corona of the particle. Changes in the chemistry of these blocks can then change the relative hydrophilicity of the particles, thus affecting trans-scleral transport.

Direct measurement of nanoparticle hydrophilicity involves the use of specialized techniques such as surface adsorption assays [18], hydrophobic interaction chromatography[19] etc. Most of these techniques however have been used for solid nanoparticles with large differences in hydrophilicity - these might not be useful for deformable polymersome particles with relatively small differences in hydrophilicity.

Alternatively, it is possible to study the relative hydrophilicity of the bulk copolymer so as to infer the properties of its self-assembled polymersomes. Hydrophilicity of bulk copolymer films (formed by drop casting polymer in chloroform on to glass slides) was studied using optical contact angle (θ) measurements (Fig. 3.10, OCA 25, DataPhysics Instruments GmbH, Germany); smaller the contact angle (θ) of the copolymer film, greater its wettability and hence its hydrophilicity.

Figure 3. 10: A) Contact angle machine (OCA 25, DataPhysics), B) schematic of contact angle of water droplet on polymer film, C) water droplet on hydrophobic film ($\theta > 90^\circ$), D) water droplet on hydrophilic film ($\theta < 90^\circ$).

All characterization techniques described so far are used to 1) understand and optimize properties of polymersome formulations and 2) observe the effect of these properties on trans-scleral behaviour.

3.4 Trans-scleral transport study of rhodamine tagged polymersomes

Trans-scleral transport behavior of polymersomes across excised porcine sclera was studied using Horizontal Ussing chambers (NaviCyte, Harvard instruments). Porcine sclera was chosen as the tissue model for study as it has been shown to have cell distribution, collagen architecture and water content similar to human sclera [20, 21]. Permeation studies of model molecules (small molecules, polysaccharides, protein etc.) do show reduced permeation in porcine sclera compared to human sclera but this difference can be attributed to the greater thickness of porcine sclera compared to human [20]. This, coupled with greater availability of porcine tissue over human, made porcine sclera a practical ex-vivo tissue model. Porcine eyes were obtained from a local slaughterhouse (Primary Industries, 2 Buroh Lane, Singapore) with permission from the Agri-food and Veterinary Association (AVA), Singapore. The sclerae were excised on the same day for storage at -80°C; the tissue remained viable for up to a month mainly due to the acellular character of the sclera.

3.4.1. Trans-scleral transport protocol:

Thawed sclerae were cut into circular sections of approx. 1cm diameter and each section was placed in the sclera holder. PBS (400 μ l) was placed on the episcleral surface, both to keep the sclera hydrated and to check for leakage. The receiver chamber of the Ussing chamber was filled with 2ml of PBS containing sodium azide (0.05% w/v) and antibiotic (5% v/v HyClone Antibiotic Antimycotic, GE). The sclera holder was then gently placed at the receiver chamber opening, taking care to avoid formation of bubbles in the receiver chamber under the sclera.

Residual PBS was removed from the surface of the sclera and 400 μ l of the sample under study was placed on the episcleral surface. Subsequently, the chamber cap was tightly secured over the tissue holder (Fig. 3.11) and all openings in the setup were secured to

reduce evaporation of sample/buffer. Each horizontal setup thus prepared was then placed in an incubator at 37°C and left undisturbed for 96 hours to study passive transport through the sclera.

Figure 3. 11: A) Horizontal ussing chamber for studying passive transport of rhodamine-tagged polymersomes across porcine sclera, B) schematic of trans-scleral transport setup, C) sclera embedded in OCT prior to sectioning, D) cryomicrotome for sectioning sclera for imaging, E) fluorescent image of scleral cross-section showing polymersome trans-scleral transport.

3.4.2 Post trans-scleral transport – tissue fixing and imaging protocol:

Scleral sections were removed from the setup at the 96hr time point and fixed overnight in 4% paraformaldehyde solution. After fixing, the scleral sections were washed in PBS and embedded in optimal cutting temperature compound (FSC 22 Frozen Section Compound, Leica), before freezing at -80°C for at least 1hr.

The embedded sclera was sectioned in a cryo-microtome (RM2255, Leica) at -25°C to obtain cross-sectional slices, which were then stained with DAPI to visualize resident cells in the sclera. Imaging of the prepared cross-sections was carried out using red and

green channels of an epifluorescent microscope in order to see the overall cross-section (DAPI, blue signal) and the polymersomes (Rhodamine, red signal).

Trans-scleral transport studies done in this manner does not reproduce in-vivo clearance processes in the periocular space or the vitreous humour (lymphatic and systemic clearance [22], efflux transporter [23]) , neither does it account for other factors affecting transport such as intraocular pressure (increased pressure leads to reduced transport [24]). As a result, such ex-vivo studies represent a simplified view of the scleral transport process and hence are not a good predictor of the in-vivo fate of the particle. Even so, the setup can be used to understand basic interactions between the sclera and polymersomes or the drugs under study which in turn can be used as a preliminary screening test of formulations for future in-vivo experiments.

3.5 Statistical data analysis

All formulations were prepared in triplicates to obtained size and morphology data. Trans-scleral transport studies were done in triplicates for every sample of a given formulation such that $n = 9$. Statistical data analysis was carried out using Student's t-test, with $p < 0.05$ as the minimal level of significance.

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Chapter 4

Trans-scleral Transport of Polymersomes

Transport studies were carried out for neutral and charged polymersome formulations in order to determine the factors affecting trans-scleral transport. It was found that intrinsic characteristics of the block copolymer (chemical structure, molecular weight etc.) as well as formulation methods (extrusion to downsize polymersome size) could be optimized to achieve enhanced transport of neutral polymersomes. Building on this, negatively charged polymer was incorporated into neutral polymersomes to study the effect of surface charge and/or hydrophilicity on trans-scleral transport. Trans-scleral depots were formed by several formulations and the factors enabling this depot formation were identified.

4.1 Introduction

As described in chapter 2, nanoparticle drug delivery systems can enhance periocular drug delivery by forming intrascleral drug depots that release ocular drugs into the posterior segment of the eye in a sustained manner. The formation of such depots require the nanoparticles in question to permeate into the sclera, ideally through passive diffusion from the periocular space. The dynamic nature of self-assembled polymersomes make them potential candidates for this passive diffusion into the sclera as they have been shown to diffuse intact across porous structures [1]. Understanding polymersome properties that affect trans-scleral transport can then be used to design formulations to form intrascleral depots for ocular drug delivery to the posterior segment of the eye.

Polymersome properties under study are:

1. Hydrodynamic size of polymersome
2. Chemical structure of block co-polymer
3. Molecular weight of block co-polymer
4. Effect of incorporating negatively charged polymer

4.2 Experimental Methods

Thin-film rehydration (as described in section 3.2) was used to prepare rhodamine tagged polymersomes with varying intrinsic (molecular weight and microstructure of copolymer) and extrinsic (hydrodynamic size, surface charge, hydrophilicity of particle) properties. The formulations were characterized using light scattering techniques and cryo-TEM imaging to establish patterns in size, morphology, zeta potential and bilayer thickness (section 3.3). The trans-scleral transport behaviour of these formulations were then studied using an *ex-vivo* setup modelling periocular conditions (section 3.4). The porcine sclera used in each trans-scleral experiment was fixed using 4% paraformaldehyde (PFA), sectioned and imaged using epifluorescence microscopy in order to study the trans-scleral distribution of polymersomes at the end of each *ex-vivo* experiment. The specific distributions thus observed were found to be dependent on the unique characteristics of different polymersome formulations.

4.3 Results and Discussion

4.3.1 Effect of hydrodynamic size of polymersome

In order to study the effect of particle size on trans-scleral transport, Bd (1.2)-b-EO (0.6) polymersomes of two different hydrodynamic sizes (as measured by DLS) were prepared via thin film hydration method. The different size profiles (Fig. 4.1) were obtained by changing pore size of the filter used for extrusion; A100 samples were extruded through a 0.2 μ m filter to give sizes above 100nm while B100 samples were further extruded through a 0.05 μ m filter to give sizes below 100nm. Both formulations were found to have a shape factor R_g/R_h of ~ 1.15 , indicating a vesicular morphology and zeta potential ~ 2.22 mV.

Figure 4. 1: A) Hydrodynamic size and B) PDI of Bd (1.2)-b-EO (0.6) polymersomes; significantly different particle sizes ($p < 0.05$) were obtained by using extrusion filters of different pore sizes (A100 – 0.2 μ m filter, B100 – 0.05 μ m filter).

On running transport experiments for these formulations, it was observed that B100 samples with average hydrodynamic size of 78.6nm showed significantly greater trans-scleral transport compared to A100 samples with average hydrodynamic size of 167.5nm (Fig. 4.2) over 96hrs; it is clear then that hydrodynamic size of the polymersome has a profound effect on the extend of trans-scleral transport. This could be explained in terms of the size dependency of simple diffusion; under identical concentration gradient, experimental time scale and temperature, smaller particles permeate further [2]. All transport studies henceforth were done with sized-down polymersomes (< 100 nm) in order to see observable differences in trans-scleral transport arising from different particle/copolymer characteristics.

Figure 4. 2: Trans-scleral transport of A) A100 and B) B100 formulations of Bd (1.2)-b- EO (0.6); B100 samples with avg. size of 78.6nm showed significantly greater trans-scleral transport compared to A100 samples with avg. size of 167.5nm (n = 9).

4.3.2 Effect of chemical structure of block co-polymer

Having established the hydrodynamic size effect on trans-scleral transport, the effect of chemical structure of the block copolymers on trans-scleral transport was considered. The isomeric nature of poly (butadiene) means that a given Bd-b-EO copolymer sample can contain predominantly either 1, 4 (cis or trans) or 1, 2 side vinyl isomers depending on the polymerization method [3]. This change from a linear polymer chain (1, 4) to a branched one (1, 2) could in turn change the packing of the polymer chains in the polymersome bilayer, thus intrinsically changing its properties.

In order to study this effect of chemical structure on trans-scleral transport, polymersomes were formulated from Bd (1.2)-b-EO (0.6) [1, 2] and Bd (1.3)-b-EO (0.6) [1, 4] block copolymers. Sizes below 100nm were obtained for both formulations with $R_g/R_h \sim 1$, indicating the formation of vesicles which was confirmed by cryo-TEM imaging (Fig.4.9, A & B). However, the size profiles (Fig. 4.3) varied significantly ($p < 0.05$), with Bd (1.3)-b-EO (0.6) forming polymersomes with an average size of 93.21nm against the 78.58nm particles formed by Bd (1.2)-b-EO (0.6). Zeta potential of both formulations was found to be ~ -1.2 mV.

Figure 4. 3: A) Hydrodynamic size and B) PDI of Bd (1.2)-b-EO (0.6) and Bd (1.3)-b-EO (0.6) polymersomes, chemical structure of the block copolymer seems to affect self-assembly as evidenced by the significant difference in size and PDI ($p < 0.05$).

DSC analysis of both block copolymers was done to study bilayer fluidity at experimental conditions (37°C). The thermograms obtained showed melting transition at $T_m \sim 28^{\circ}\text{C}$ with no apparent glass transition (Fig.4.4) for both block copolymers; thus at experimental temperature of 37°C , both polymers were highly fluid and there seemed to be no difference in polymersomes bilayer rigidity between the formulations. In fact, based on this DSC analysis, it would seem that polymersomes of both block copolymers would exhibit similar trans-scleral transport behaviour. The identical thermograms of structurally different 1, 2 and 1, 4 addition polymers can be attributed to the relatively short poly (butadiene) chains of both polymers; any difference in thermal behaviour is thus too small to be detected via DSC.

Figure 4. 4: DSC thermograms of A) Bd (1.2)-b-EO (0.6) [1, 2 addition] and B) Bd (1.3)-b-EO (0.6) [1, 4 addition] block copolymers.

However bilayer thickness measurements of the cryo-TEM images of Bd (1.2)-b-EO (0.6) [1, 2 addition] polymersomes and Bd (1.3)-b-EO (0.6) [1, 4 addition] polymersomes found a statistically significant difference between each formulation. Both copolymers were found to form polymersomes with bilayer thickness $d < 8\text{nm}$; this, along with the low molecular weight of the polymers indicate the chains are fully stretched in the polymersome bilayer [4]. Bilayer thickness however differed between formulations, with polymersomes formed by Bd (1.3)-b-EO (0.6) [1, 4] found to have thicker bilayers compared to those formed by Bd (1.2)-b-EO (0.6) [1, 2] (Fig.4.5). The thicker bilayer of the 1, 4 addition polymersomes could be attributed to the specific microstructure of its polybutadiene block; when chains are elongated, the linear microstructure of 1,4 addition polybutadiene leads to longer chains compared to 1,2 addition polybutadiene with branched microstructures (Fig.4.8 E & D). The difference in microstructure can also lead to differences in bilayer packing with the linear 1, 4 addition block copolymer being much more densely packed in the bilayer and thus forming a more rigid assembly compared to the loosely packed bilayer of the branched 1,2 addition block copolymer.

Figure 4. 5: Cryo-TEM images of A) Bd (1.2)-b-EO (0.6) [1, 2 addition] polymersomes and B)) Bd (1.3)-b-EO (0.6) [1, 4 addition] polymersomes; C) bilayer thickness of Bd (1.2)-b-EO (0.6) and Bd (1.3)-b-EO (0.6) polymersomes ($p < 0.05$); microstructures of D) 1,2 addition polybutadiene (linear length of monomer = $4.569 \text{ \AA}$) and E) 1,4 addition polybutadiene (linear length of monomer = $7.403 \text{ \AA}$).

On studying trans-scleral transport behaviour of Bd (1.2)-b-EO (0.6) [1, 2 addition] and Bd (1.3)-b-EO (0.6) [1, 4 addition] polymersomes, significant difference in transport was observed, with Bd (1.2)-b-EO (0.6) polymersomes showing transport up to 30% of scleral thickness (Fig. 4.6). In the absence of difference in size or surface chemistry (charge/hydrophilicity), this difference in trans-scleral transport can only be attributed to difference in bilayer rigidity. However, DSC data showed no difference in chain fluidity between both block copolymers and so cannot explain the observed transport behaviour;

increased transport of the 1, 2 addition polymer is then a consequence of its specific bilayer assembly.

However, a reasonable explanation of the observed difference in trans-scleral transport between the branched Bd (1.2)-b-EO (0.6) [1, 2 addition] polymersomes and the (1.3)-b-EO (0.6) [1, 4 addition] polymersomes can be derived when looking at the difference in polymersome bilayer thickness between the two formulations. The translocation of polymersomes under the influence of diffusive forces requires the particles to bend in order to squeeze through the scleral pores [1]; higher the bilayer bending rigidity (k_c), lesser the ability of the polymersome to deform sufficiently so as to allow trans-scleral diffusion (Section 3.3.5, Fig.3.5). The bilayer bending rigidity (k_c) of polymersomes has been shown to be quadratically proportional to bilayer thickness d ($k_c \propto d^2$) [5], allowing even small changes in bilayer thickness to have a significant effect on bilayer bending properties. In this light, the thicker bilayer of Bd (1.3)-b-EO (0.6) [1, 4 addition] polymersomes leads to an increase in the bilayer bending rigidity (2.4 times that of Bd (1.2)-b-EO (0.6) polymersomes) that inhibits their trans-scleral diffusion. The difference in bilayer packing of these block copolymers as a result of different polymer chain structure can also be considered to affect the trans-scleral transport behaviour as branched 1, 2 addition polymer chains can lead to loosely packed, fluid bilayers compared to the linear 1, 4 addition polymers; this however has to be verified using techniques such as small-angle x-ray or neutron scattering (SAXS/SANS).

Figure 4. 6: Trans-scleral transport of A) Bd (1.2)-b- EO (0.6) and B) Bd (1.3)-b-EO (0.6) polymersomes; Bd (1.2)-b- EO (0.6) formulation shows significant transport (up to ~30% of total sclera thickness) while Bd (1.3)-b- EO (0.6) formulation shows no transport due to thicker bilayer and corresponding increase in bilayer bending rigidity (n=9).

Further studies of trans-scleral transport focused on polymersomes formulated from block copolymers with 1, 2 vinyl isomers of poly (butadiene).

4.3.3 Effect of molecular weight of block co-polymer

Having studied the effect of particle size and block copolymer architecture on transport, work was done to understand the effect of molecular weight of block copolymer on trans-scleral transport. Polymersomes ($R_g/R_h \sim 1.02$) were formulated using Bd (1.2)-b- EO (0.6) and Bd (1.7)-b- EO (1); despite the increased molecular weight of Bd (1.7)-b- EO (1) polymer chain, no significant difference was observed in the size profile (Fig. 4.7).

Figure 4. 7: A) Hydrodynamic size and B) PDI of Bd (1.2)-b-EO (0.6) and Bd (1.7)-b-EO (1) polymersomes, no significant difference in size and PDI ($p < 0.05$).

DSC analysis of both block copolymers was done to study bilayer fluidity at experimental conditions (37°C) and again, the thermograms obtained showed melting transition at $T_m < 37^\circ\text{C}$. No glass transition (Fig. 4.8) was found for the smaller polymer but the higher molecular weight polymer showed glass transition at $T_g \sim -21.48^\circ\text{C}$. This presence of a glass transition along with the 1°C increase in T_m of higher molecular weight polymer, indicates that increasing polymer chain length does affect chain mobility. However, T_m is still lower than the experimental temperature of 37°C, indicating both polymers are highly fluid at this temperature and there seems to be no difference in polymersomes bilayer rigidity between the formulations.

Figure 4. 8: DSC thermograms of A) Bd (1.2)-b-EO (0.6) and B) Bd (1.7)-b-EO (1) block copolymers.

Cryo-TEM imaging of both Bd (1.2)-b- EO (0.6) and Bd (1.7)-b- EO (1) formulations confirmed the formation of polymersomes (Fig. 4.9). However, there is an increase in bilayer thickness of Bd (1.7)-b- EO (1) polymersomes compared to Bd (1.2)-b- EO (0.6) polymersomes (Fig. 4.9C). This observation is in agreement with previous studies in literature that show increase in bilayer thickness d with molecular weight M_w in accordance with the power law $d \propto M_w^\alpha$ (α indicates the conformation of polymer chains within the bilayer) [6]. Polymersomes formed by low molecular weight polymer chains with bilayer thickness $d < 8\text{nm}$ tend to have α values approaching 1, indicating a stretched chain conformation [4].

Figure 4. 9: Cryo-TEM images of A) Bd (1.2)-b-EO (0.6) polymersomes and B) Bd (1.7)-b-EO (1) polymersomes; C) bilayer thickness of Bd (1.2)-b-EO (0.6) and Bd (1.7)-b-EO (1) polymersomes ($p < 0.05$).

Trans-scleral transport studies of the two formulations showed decreased trans-scleral transport for the Bd (1.7)-b-EO (1) polymersomes (Fig. 4.10). As described in sections 3.3.5 and 4.3.2, this difference in trans-scleral transport can be explained in terms of increase in bilayer bending rigidity k_c with increase in bilayer thickness d of Bd (1.7)-b-EO (1) polymersomes with higher molecular weight, which in turn limits the polymersome's ability to navigate the collagen network of the sclera.

Figure 4. 10: Trans-scleral transport of A) Bd (1.2)-b- EO (0.6) and B) Bd (1.7)-b-EO (1) polymersomes; Bd (1.7)-b- EO (1) formulation shows reduced transport compared to the Bd (1.2)-b- EO (1) formulation due to the increase in bilayer bending rigidity due to increase in bilayer thickness ($n = 9$).

All in all, trans-scleral transport studies of neutral Bd-b-EO polymersomes showed that intrinsic characteristics of the block copolymer (chemical structure, molecular weight etc.) as well as formulation methods (extrusion to downsize polymersome size) can be optimized to achieve significant trans-scleral transport. Bd (1.2)-b-EO (0.6) polymersomes of hydrodynamic size $\sim 78.6\text{nm}$ was found to show maximum transport through the sclera transport (up to $\sim 30\%$ of total sclera thickness) and could be a viable candidate for trans-scleral drug delivery.

4.3.4 Effect of incorporating negatively charged polymer

Expanding on the trans-scleral transport behavior of neutral Bd-b-EO polymersomes, the focus of study now shifted to the effect of surface charge of polymersome on transport. To this end, charged polymersomes were formulated by blending Bd (1.7)-b-EO (1) with a poly (butadiene-b-acrylic acid) block copolymer [Bd (0.63)-b-AA (0.4); $M_w = 1.442\text{kDa}$; $f_{\text{paa}} = 0.39$] at varying mole fractions. The replacement of the PEO block with a PAA results in a negatively charged block copolymer; blending it with the neutral block copolymer introduces negative surface charge to the self-assembled polymersomes. The larger molecular weight polymer Bd (1.7)-b-EO (1) was chosen as the neutral polymer in order to ensure minimal disruption of vesicular self-assembly with the addition of the charged polymer. Furthermore, preliminary drug loading studies with dexamethasone

showed higher drug encapsulation by Bd (1.7)-b-EO (1) polymersomes ($67.01 \pm 10.31\%$) compared to Bd (1.2)-b-EO (0.6) polymersomes ($30.02 \pm 4.72\%$). In this light, Bd (1.7)-b-EO (1) polymersomes would be a more viable candidate for trans-scleral drug, should it be possible for the particles to form a trans-scleral drug depot.

Formulations containing 0%, 5%, 10%, 15%, 50% and 100% (mol %) of Bd (0.63)-b-AA (0.4) blended with neutral Bd (1.7)-b-EO (1) were prepared and particle characteristics such as size, polydispersity, zeta-potential and morphology were studied. The incorporation of the charged Bd-AA polymer in the polymersome bilayer was evident from the consistent increase in zeta-potential ($p < 0.05$) as mol% of Bd-AA in the blend increased (Fig. 4.11.A). No statistically significant difference in size and PDI (Fig. 4.11. B & C) was observed between 0%, 5%, 10% and 15% blends; the 50% and 100% blends however showed a significantly different size profile compared to the lower AA% blends, indicating possible disruption of self-assembly. R_g/R_h value obtained from SLS indicated the formation of vesicles by the 0%, 5%, 10%, and 15% blends (R_g/R_h ; ~ 1.03); the 50% blend in contrast gave an R_g/R_h value indicative of rod-like micelles (R_g/R_h ; ~ 1.40) and 100% blend gave an R_g/R_h value indicative of spherical micelles (R_g/R_h ; ~ 0.78). The predicted morphologies were confirmed via cryo-TEM imaging (Fig. 4.12).

Figure 4. 11: A) Zeta-potential, B) hydrodynamic size and C) PDI of Bd (1.7)-b-EO (1) + Bd (0.6)-b-AA (0.43) blended polymersomes, significant difference in size, PDI and zeta potential across different blends (0%, 5%, 10%, 15%, 50% and 100% Bd (0.6)-b-AA (0.43)).

Figure 4. 12: Cryo-TEM images of A) 0%, B) 5%, C) 10%, D) 15%, E) 50% , F) 100% Bd (0.6)-b-AA (0.43) blended polymersomes; morphologies observed confirm Rg/Rh predictions.

Trans-scleral transport studies of the blended formulations (Fig. 4.13) showed no transport in all formulations- except the 10% blend- with particles sticking to the episcleral surface. In contrast, the 10% formulation showed transport up to 67.51% of total scleral thickness (n=9); this is a marked improvement compared not just to the transport of the other blended formulations but also the maximum transport observed in the neutral Bd (1.2)-b-EO (0.6) formulation (Fig. 4.14).

Figure 4. 13: Trans-scleral transport of blended formulations of A) 0% AA, B) 5% AA, C) 10% AA, D) 15% AA, E) 50% AA and F) 100% AA (n = 9); only the 10% AA formulation shows transport into the sclera (67.51 ± 7.36 % of total sclera thickness).

Figure 4. 14: Comparison of trans-scleral transport observed for neutral Bd (1.2)-b-EO (0.6) vs. 10% Bd-AA/Bd-EO blend (n=9 where n refers to number of transport studies done using sclera sections from different porcine eyes); significant enhancement of transport is observed in the 10% blend.

The observed trans-scleral behaviour of the Bd-AA/Bd-EO blends do not follow a simple pattern dependent on surface charge, indicating that the incorporation of Bd-AA into the Bd-EO bilayer leads to changes in polymersome properties aside from a simple introduction of negative charge on to the polymersome surface.

4.3.4.1 Understanding trans-scleral transport behaviour of Bd-EO/Bd-AA blends

The addition of the negatively charged Bd-AA polymer into the bilayer of neutral Bd-EO polymersomes changes the properties of the particles, thus affecting trans-scleral transport.

The following polymersomes properties can be affected by the addition of Bd-AA:

1. Bilayer bending rigidity
2. Morphology
3. Surface charge
4. Hydrophilicity

Understanding the observed trans-scleral transport behaviours of Bd-AA blended polymersomes would then require an understanding of the specific changes in these polymersome properties and the way these changes affect particle-sclera interactions.

The trans-scleral behaviour of Bd-AA/Bd-EO blends can be broken down into four different observations which are then explained in terms of polymersome properties.

Observation 1: 0% AA polymersomes does not show any trans-scleral transport

The lack of trans-scleral transport of 0%AA polymersomes is attributed to the large molecular weight of the Bd-EO polymer chain which leads to a thick bilayer and hence high bilayer bending rigidity (section 4.3.3).

Observation 2: 10% AA polymersomes shows enhanced trans-scleral transport compared to 5%AA and 0% AA polymersomes

The incorporation of 10% Bd-AA into the Bd-EO bilayer leads to enhanced trans-scleral transport (67.51% of total scleral thickness) compared to the 0% AA and 5% AA polymersomes (no transport). This behaviour can occur due to 1) decrease in bilayer bending rigidity and/or 2) increase in hydrophilicity with addition of Bd-AA polymer.

Examining bilayer bending rigidity of the 0% AA, 5% AA and 10% AA polymersomes, it is evident that the low T_m values of both Bd-EO and Bd-AA polymers lead to highly fluid bilayers across all formulations. The incorporation of Bd-AA into the Bd-EO bilayer may lead to disruption in polymer chain packing due to differences in the preferred interfacial curvature of PAA and PEO; however Bd-AA mol% up to 10% was shown to not induce structural changes in Bd-EO/Bd-AA blended polymersomes [7]. This, in addition with the comparable bilayer thickness measurements obtained from cryo-TEM images (Fig. 4.12, $D \sim 8.85 \pm 1.3$ nm), show that addition of Bd-AA does not affect bilayer bending rigidity; while this is not sufficient evidence to completely rule out the possibility of different bilayer packing in the presence of Bd-AA, it can be assumed that the effect of

such differences are not significant at Bd-AA mol% of 10% and below. In this light, the enhanced trans-scleral transport of 10% AA polymersomes could then be explained in terms of increased hydrophilicity of the particles.

The hydrophilicity of polymersomes arise from the hydrophilic corona of the particles formed by the hydrophilic blocks of the constituent block copolymers. In the case of 0% AA polymersomes, this corona contains poly (ethylene oxide) [PEO] blocks; the 5% A and 10% AA have an increasing amount of poly (acrylic acid) [PAA] blocks as well, dispersed between the PEO chains. While both PEO and PAA are hydrophilic in nature, the fundamental difference in their chemistry can lead to different polymer properties. In particular, PAA (contact angle $\theta \sim 0^\circ$) has been shown to be more hydrophilic than PEO (contact angle $\theta \sim 22^\circ$) [8] - incorporating PAA chains into the hydrophilic corona of polymersomes could be increasing the hydrophilicity of the particles, thus enhancing trans-scleral transport.

This increase in hydrophilicity is evident from contact angle measurements of Bd-EO/Bd-AA blended films (Fig.4.15). Contact angle (θ) decreases from 92° in 0% AA blend to 40° in 10% AA blend before plateauing out for the higher %AA blends. This increased hydrophilicity leads to reduced friction [9] and higher permeation [10], which allows the 10%AA polymersomes to transport into the sclera to a much greater degree than 0% AA or 5% AA polymersomes with lower hydrophilicity.

Figure 4. 15: Contact angle (θ) measurements of Bd-EO/Bd-AA blended films; θ values decrease (indicating increase in hydrophilicity) up to 10% AA blend and then plateau out.

Observation 3: 15% AA polymersomes do not show trans-scleral transport

As incorporating Bd-AA into the Bd-EO bilayer leads to increased hydrophilicity of the polymersome, it stands to reason that increasing the amount of Bd-AA in the polymersome bilayer will lead to further enhancement of trans-scleral transport. However this is not the case with 15% AA polymersomes which show not trans-scleral transport despite having hydrophilicity comparable to the 10% AA blend.

This lack of trans-scleral transport of 15% AA polymersomes can be due to 1) change in morphology to non-vesicular structures or 2) electrorepulsive interactions with the sclera. R_g/R_h values (~ 1.02) and cryo-TEM images (Fig. 4.12) however confirm the vesicular structure of 15% AA polymersomes, thus indicating that electrorepulsive interactions are the likely cause of the observed trans-scleral behaviour.

Electrorepulsive interactions of negatively charged polymersomes with the sclera arise from the negatively charged nature of the sclera. The sclera contains negatively charged

proteoglycans interspersed within a porous collagen mesh that contribute to the net negative charge of the sclera. On a microscopic level, this negative charge can be understood in terms of the Debye length of the proteoglycans. Charged surfaces interact with ions in surrounding buffer to form an ion cloud, the length of which is defined as the Debye length ($1/\kappa$); debye length is then the maximum range of influence of the surface charge of a material [11] and is directly proportional to this surface charge. The sclera mesh of intertwined collagen and proteoglycans can then be imagined as a network of charged fibres, each having its own ionic cloud (Fig. 4.16 B). The effective charge-free pore size then is lesser than the actual physical pore size measured using techniques like SEM or confocal microscopy.

Any charged particle passing through this negatively charged mesh escapes electrostatic effect of the charged fibres if its own ionic cloud does not overlap with that of the fibres (Fig. 4.16 C). However, if the ionic clouds overlap (Fig. 4.16 D), the particles undergo electrostatic repulsion or attraction depending on the specific charge of the particle; a negatively charged particle will be repelled away while a positively charged particle will be attracted and stick to the fibres.

Figure 4. 16: Schematic of effect of negatively charged proteoglycans (black) in sclera and its interaction with charged particles (red); A – physical size of the pore (shaded) in the absence of charge on the fibres; B – ionic clouds of charged fibres reducing effective charge-free area (shaded) of the pore; C - interaction of particle with small surface charge (i.e. small ionic cloud) with charged mesh, the particle is able to slip into the charge-free area of the pore, thus avoiding electrostatic interactions; D - interaction of particle with large surface charge (i.e. large ionic cloud) with charged mesh, overlapping of ionic clouds lead to electrostatic interactions.

The incorporation of increasing amounts of Bd-AA into the Bd-EO bilayer leads to an increase in the net negative surface charge of polymersome particles (Fig.4.11 A); thus the increased negative charge of the 15%AA polymersomes ($\sim -25\text{mV}$) leads to electrorepulsion of these particles which prevents their transport into the sclera despite the increase in hydrophilicity.

Observation 4: 50% AA and 100% AA blends do not show trans-scleral transport

The increased amounts of Bd-AA in the 50% AA and 100% AA blends leads to disruption of self-assembly, resulting in the formation of rod-like (50% AA, Fig. 4.12 E) and spherical (100% AA, Fig. 4.12 F). These morphologies lack the deformability of vesicular assemblies like polymersomes; this, along with the increased negative surface charge which leads of electrorepulsion, can explain the lack of trans-scleral transport observed for these formulations.

4.4 Conclusions

The trans-scleral transport studies of neutral Bd-EO and negatively charged Bd-EO/Bd-AA polymersomes show that trans-scleral transport of polymersome particles into the sclera depends on particle size, bilayer bending rigidity and surface chemistry. Controlling these parameters allows for the design of polymersome formulations that permeate into the sclera to form intrascleral depot; to do so, it is necessary to understand which parameters have a greater effect than the others. In general, the basic design hierarchy is as follows:

1. Surface charge $< -25\text{mV}$; zeta potential $\zeta > -25\text{mV}$ leads to electrorepulsive interactions that prevent trans-scleral transport regardless of decrease in particle size and bilayer rigidity or increase in hydrophilicity
2. Particle size $< 100\text{nm}$; particle sizes greater than 100nm do not permeate into the sclera regardless of decrease in rigidity or increase in hydrophilicity

For Bd-EO based polymersomes, other considerations include:

1. Bilayer rigidity; lower bilayer rigidity can be achieved by choosing lower polymer chain lengths and/or branched microstructure that allow for looser bilayer packing and hence greater deformability
2. Surface hydrophilicity of the polymersome particle

Increase in surface hydrophilicity of polymersome particles greatly enhance trans-scleral

transport and can even override the limiting effect of bilayer rigidity. Further studies are needed to understand the exact reasons behind this and to also test if hydrophilicity can enhance transport for larger Bd-EO polymersomes, thus overcoming the size limitations for the formation of intrascleral transport.

All in all, polymersomes show promise as a potential drug delivery system for periocular drug administration via the trans-scleral route. A neutral Bd-EO formulation and a negatively charged Bd-EO/Bd-AA blend has been shown to form intrascleral depots; however, the other formulations do stick to the episcleral surface and can be used as periocular depots. Further, several parameters affecting trans-scleral transport of polymersomes have been identified here, presenting a potential guideline for designing polymersome formulations for trans-scleral drug delivery.

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Chapter 5

Discussion and Future Work

This chapter provides an overview of the overall research work and revisits the outcomes laid out in chapter 4, with reference to initial hypothesis. The impact of the work is examined and a concise summary of the conclusions is provided. As a whole, the results of this research work show the viability of polymersomes as nanoparticles for periocular drug delivery and prove the initial hypothesis that certain structural features of polymersomes facilitate their permeation into the sclera to form intrascleral drug depots for periocular drug delivery. Limitations of experimental technique and design are acknowledged, along with possible strategies to overcome the same. Finally, recommendations for future work are suggested, in order to further expand the scope of this area of research.

5.1 Overview of research outcomes

Polymersomes are synthetic analogues of liposomes formed by the self-assembly of amphiphilic block copolymers as a result of hydrophobic-hydrophilic interactions with the surrounding solvent. These particles retain the drug loading versatility of liposomes while exhibited enhanced membrane toughness and reduced permeability [1]. As a relatively ‘young’ nanoparticle system, most work in the field of polymersomes focus on designing unique polymersome assemblies via synthesis of new polymers [2, 3], new formulation techniques [4], particle functionalization [5] etc. There is then a need to develop polymersome technology towards practical applications.

In this light, this research work aimed to study the suitability of polymersomes for ocular drug delivery via periocular route of drug administration. Periocular drug delivery presents an alternative treatment strategy for posterior segment eye disease (PSEDs) by administering drugs into the periocular space surrounding the eye orbit [6]. Drugs then diffuse from this space across the sclera and choroid into the posterior segment of the eye, thus allowing for more effective treatment of PSEDs like glaucoma, age-related macular degeneration and diabetic macular edema [7]. Drug loaded nanoparticles further enhance this treatment strategy by forming periocular [8] or intrascleral drug depots capable of releasing a sustained dose of drug over extended time periods. Such drug depots were shown to be formed by self-assembled liposomes loaded with anti-VEGF molecules [9]; as polymersomes share the dynamic self-assembly properties of liposomes, it was then possible that polymersomes too could form intrascleral drug depots allowing for further enhancement of periocular drug delivery.

In order to study the potential of polymersomes for periocular drug delivery via the formation of intrascleral drug depots, poly (butadiene – b- ethylene oxide) polymersomes of varying particle properties were formulated. Intrinsic properties such as block copolymer molecular weight and microstructure as well extrinsic particle properties such as hydrodynamic size, bilayer bending rigidity, surface charge and hydrophilicity were studied. All formulations were meticulously characterized in order to understand the effect of polymersome properties on trans-scleral transport behaviour as observed by *ex-*

vivo passive transport experiments across porcine sclera.

Polymersomes were found to form periocular depots in the case of most formulations with a two formulations [neutral Bd (1.2) –b- EO (0.6) and 10% Bd (0.6)- b- AA (0.43) blended with Bd (1.7)-b-EO(1)] forming intrascleral depots. These results show the viability of polymersomes as nanoparticles for periocular drug delivery. Further, the formation of intrascleral depots by two polymersome formulations prove initial hypothesis that *there are certain structural features of polymersomes that facilitate their permeation into the sclera to form an intrascleral drug depot for periocular drug delivery*. This observation however is not ubiquitous across all studied formulations, indicating the need for careful design of polymersomes systems to achieve required results; in this case, the formation of an intrascleral depot. Table 5.1 lists the polymersome properties that affect trans-scleral transport of polymersomes and ways to manipulate these properties to enhance transport.

Table 5.1: Polymersome design strategies to enhance trans-scleral transport.

Polymersome property	Effect on transport	Design strategy
Hydrodynamic size	Smaller the particle size, greater the degree of transport	Choose fluid polymers (low Tg and Tm) that can be downsized via formulation techniques to sizes <100nm
Bilayer bending rigidity	Lower the bilayer bending rigidity, greater its deformability and greater the degree of transport	Choose fluid polymer chains with low molecular weight and increased free space (branching) to form thin, fluid bilayers

Surface charge	Negative surface charge induces electrorepulsive interactions with the sclera, impeding trans-scleral transport	Adjust the chemistry of hydrophilic block of polymer chain to avoid net negative surface charge; if the total elimination of negative charge is impossible, limit surface charge to $< -20\text{mV}$
Hydrophilicity	Greater the hydrophilicity, greater the degree of transport	Adjust the chemistry of hydrophilic block of polymer chain to allow for greater hydration of hydrophilic corona; alternatively, incorporate more hydrophilic moieties onto polymersome surface to increase hydrophilicity

All in all, this body of work examines the trans-scleral transport behaviour of polymersomes and provides a framework for designing drug-loaded polymersome systems for sustained drug delivery to the posterior segment of the eye.

5.2 Experimental limitations

While outcomes of the study of trans-scleral transport of polymersomes are significant, it is necessary to understand the limitations in current experimental techniques and to place the results within the framework of these limitations.

Polymersomes are nanosized particles which leads to limitations in the direct characterization of particle properties. For example, bilayer bending rigidity of polymersomes are often studied using roundabout methods such as bilayer thickness and thermal transitions due to the difficulty of direct measurements at the nanoscale. Problems also arise from the self-assembled nature of the particles; for example, direct

measurement of polymersome hydrophilicity is not possible with techniques such as hydrophobic interaction columns as particle assembly is disrupted. All this leads to characterization often done on a relative rather than absolute scale and on bulk material with the observed patterns then extrapolated to the nanoscale; this approach may not be inherently wrong, however it is necessary to further develop fundamental nanoscale characterization techniques such as atomic force microscopy (AFM) and small angle x-ray scattering (SAXS) for the study of self-assembled polymersome structures.

Another significant limitation arises from the design of the *ex-vivo* trans-scleral transport experiment which uses porcine sclera in horizontal ussing chambers to model the local environment of the periocular space. While this setup does an admirable job of using available resources, it is fundamentally different from the *in-vivo* conditions of the human periocular space; the setup does not reliably model clearance mechanisms, active transport pathways or even the basic geometry of the periocular space. Therefore, trans-scleral transport behaviour observed using this *ex-vivo* setup should not be considered the final say in possible *in-vivo* behaviour; the technique however provides a way to screen formulations prior to more expensive and elaborate *in-vivo* experiments.

5.3 Recommendations for future work

Trans-scleral transport studies of poly (butadiene - b- ethylene oxide) based polymersomes show the formation of periocular and intrascleral depots by various polymersome formulations. This work could potentially be built up on and expanded further in the following ways:

1. Current study focuses on poly (butadiene – b – ethylene oxide) based polymersomes; future research can work with other block copolymers such as poly (ϵ -caprolactone- b -ethylene oxide), poly (ethylene glycol –b- diethyl aminoethyl methacrylate) etc. which introduces properties such as biodegradability, pH responsiveness etc. to polymersomes.
2. More work can be done to study the effect of particle hydrophilicity in trans-

scleral transport. For example, an increase in particle hydrophilicity has been shown to overcome the limiting effect of high bilayer bending rigidity (Section 4.3.4.1.); it would be interesting to see if a similar effect can be observed in terms of particle size, with larger particles showing trans-scleral transport when particle hydrophilicity increases. Alternatively, the hydrophilic effect of polymers other than poly (ethylene oxide) and poly (acrylic acid) such as poly (2-(methacryloyloxy) ethyl phosphorylcholine) or positively charged polymers can be studied.

3. There is a need to study drug loading and release profiles of the poly (butadiene – b – ethylene oxide) polymersomes formulations developed in this research work so as to develop a viable product. Preliminary drug loading and release studies using Aflibercept (Eylea®) has shown that neutral Bd (1.7)-b-EO (1) polymersomes encapsulate 66.8% of added drug with sustained release up to 2 weeks (Appendix).

The ability of poly (butadiene – b – ethylene oxide) polymersomes to form periocular and intrascleral depots indicates the possibility of using the nanoparticle system for periocular drug delivery. To date, this is the first time these particles have been studied for this particular application and the observed results open up a potential area of application for these polymersome formulations.

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APPENDIX

A1. Preliminary drug loading studies for neutral PBd-PEO polymersomes

Preliminary drug loading studies for PBd-PEO polymersomes were done using Eylea (Aflibercept). Bd (1.7)-b-EO (1) polymersomes were prepared via thin film hydration and Eylea was loaded at an initial concentration of 0.5mg/ml. Final formulation was found to retain 66.75% of the initial drug concentration (Fig. A1.1.D). Loading of Eylea led to an increase in particle size to 160nm (compared to 120nm bare polymersomes; Fig. A1.1.A); particles retained vesicular morphology as confirmed by SLS and cryo-TEM imaging (Fig. A1.1.C.).

Figure A1.1: A) Hydrodynamic size of bare and eylea loaded Bd (1.7)-b-EO (1) polymersomes, B) PDI of bare and eylea loaded Bd (1.7)-b-EO (1) polymersomes, C) Rg/Rh values of eylea loaded Bd (1.7)-b-EO (1) polymersomes, D) eylea encapsulation by Bd (1.7)-b-EO (1) polymersomes

Release studies for eylea loaded polymersomes (1ml in 1000kDa cellulose ester dialysis bag) were carried out over 14 days in 30 ml buffer (PBS with 0.05% Tween). 1ml of release buffer was collect every day for analysis and the remaining buffer was replaced with fresh buffer to ensure sink conditions. Collected samples were analyzed using ELISA. Encapsulating eylea in polymersomes led to slow and sustained release of the drug over a 14 day period, compared to free eylea which was completely released from

dialysis bag in 3 days (Fig. A1.2.).

Figure A1.2: Cumulative release profile of free eylea and eylea encapsulated in Bd (1.7)-b-EO (1) polymersomes (functional protein measured using ELISA); encapsulated eylea shows sustained release for up to 14 days ($p < 0.05$, statistically significant difference compared to free eylea).

A2. Viability of transport setup

Prior to trans-scleral transport studies, the transport setup and experimental protocol was validated to ensure its robustness and to get a better understanding of variability. Porcine sclera thickness (Fig. A2.1) was measured using a Vernier caliper and found to vary from 0.421mm to 1.456mm with an average thickness of 0.928 ± 0.285 mm ($n=30$). As this is a biological variability, care was taken to measure thickness of sclera prior to each transport experiment in order to account for its effect on experimental results.

Figure A2.1: Porcine sclera thickness over 30 sections

The viability of the sclera was also studied over the duration of the experiment (96hrs) using scanning electron microscopy (SEM). The time scale of the experiment as well as the temperature at which it is conducted (37°C) leads to degradation of the sclera; it is then necessary to use a buffer that can preserve the architecture of the sclera over the course of the experiment. Two buffers were tested: SA buffer - 0.05% NaN₃ in PBS (1x, pH 7.4) and AB buffer - 0.05% NaN₃ in PBS (1x, pH 7.4) with 5% by volume of antibiotic. The antibiotic used was HyClone Antibiotic/Antimycotic Solution (10,000 units of penicillin, 10,000µg of streptomycin, and 25µg of Amphotericin B per mL, GE Healthcare).

SA buffer containing sclera showed turbidity within 24hrs (Fig. A2.2 A), indicating possible degradation and bacterial growth; turgidity increased further by 96hrs. The AB buffer, however, remained clear up to 96hrs (Fig. A2.2 B).

Figure A2.2: Sclera in different buffers at 24 hour and 96 hour time points; A) SA buffer B) AB buffer ; SA buffer is cloudy at 24hr with the turbidity increasing by 96hr, thus indicating degradation of sclera while the AB buffer up to the 96hr time point.

Scleral sections from both SA and AB buffers at 24hr and 96hr time points were imaged using SEM and compared against freshly excised sclera. The sections in SA buffer showed degradation of collagen architecture and the presence of bacterial colonies at 24hr time point; the degradation was further exacerbated by the 96hr time point (Fig. A2.3). In contrast, the AB sections maintained their structural integrity over 96hrs (Fig. A2.4) with no visible bacterial growth. Subsequently, the AB buffer was used for all trans-scleral transport studies.

Figure A2.3: SEM images of sclera surface: A) & B) freshly thawed sclera (episcleral & choroidal surface) showing intact collagen architecture; C) & D) sclera in SA buffer at 24 hours (episcleral & choroidal surface); E) & F) sclera in SA buffer at 96 hours (episcleral & choroidal surface); sclera shows degradation of collagen architecture and the presence of bacterial colonies within 24 hours, with conditions worsening by 96hr time point.

Figure A2.4: SEM images of sclera surface: A) & B) fresh sclera (episcleral & choroidal surface) showing intact collagen architecture; C) & D) sclera in AB buffer at 96 hours (episcleral & choroidal surface); sclera retains collagen architecture over 96hrs with no visible bacterial growth