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Design and characterisation of an intraocular nanobubble drug delivery system

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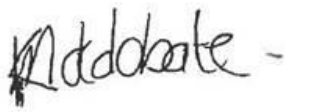
Declaration

I declare that this dissertation is my own, unaided work, except where otherwise acknowledged. It is being submitted for the degree of Master of Science in Medicine (Pharmaceutical Affairs) in the University of the Witwatersrand. It has not been submitted before for any degree or examination at any other university.

Signed this 14th day of August 2020



Kealeboga Mokolobate

Research Outputs

1. Oral Presentations

Formulation, characterisation and design of an intraocular anti-inflammatory nanoparticulate drug delivery system, oral presentation. Biennial Wits University

Research Day: 2014

Production of drug-loaded nanoparticles: Oral Presentation done at Cross Faculty

Research Day: 2013

Animal Ethics Declaration

I, Kealeboga Mokolobate, hereby confirm the study entitled “Microimaging of intraocular nanobubble system” approved by the Animal Ethics Screening Committee (AESC) of the University of the Witwatersrand with Ethics Clearance Number)...AESC:2013/44/04. The approval for the use of animal tissue samples letter from the University of the Witwatersrand is attached in Appendix D.

Abstract

The posterior segment of the eye poses many challenges in terms of drug delivery. Procedures such as intraocular injections are invasive, are associated with low compliance, and may yield health hazards such as cataracts. This study aimed to design a layer-by-layer-originated lipopolymeric intraocular nanoparticulate system, administered in the cul-de-sac of the eye, for the treatment of uveitis. The main aim is for the formulation to have theranostic capability: the ability to treat the disease, and also visually track the movement of the nanoparticulate systems through ultrasound the use of Vevo®2100 high resolution ultrasound imaging system. These nanoparticulate systems were produced by dissolving the lipids (cholesterol and soybean oil), buffer pH 7.4 and drug in chloroform. This emulsion was sonicated and the organic solvent was removed. The emulsion was then freeze-thawed. Following that, a layer of chitosan was added to the nanoparticles, allowed to deposit, and finally, a layer of polyethene glycol was added and allowed to deposit. Zetasizing was used conducted to ensure stable particles within the nanometre range (below 200nm). Drug release studies were conducted on the formulations and images through atomic force microscopy highlighted nanoparticles with a thick shell due to the reinforced layering, effectively entrapping the drug. This study illustrated how the variables (time of sonication, number of freeze-thaw cycles and amount of polymer [soybean oil) affected the formulation and enabled determination of an optimised formulation. The chemical stability of the formulations was examined by Fourier Transform Infrared(FTIR) and X-Ray Crystallography. Differential Scanning Calorimetry and Thermogravimetric Analysis assessed the formulation's reaction to heat, and thus stability. Rheological studies assisted in elucidating rheological behaviour and likely behaviour in the eye. This formulation (once incorporated into a suspension) will circumvent the need for invasive procedures regarding infections in the posterior segment of the eye in treating uveitis.

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I would like to extend an extra special thank you to the Wits Advanced Drug Delivery Platform. Professor Pillay, Professor Lisa Du Toit, Professor Yahya Choonara, Professor Pradeep Kumar, Dr. Pierre Kondiah – thank you for introducing me to this new world in science. This is such a rare skill and I am thankful for this exposure. An extra special thank you for your guidance and patience.

Dr. Femi Akilo and Dr. Mduduzi Sithole, whether it has been showing me how to use a machine or just encouraging me to carry on and persevere, I really appreciate your assistance. You have been so instrumental in ensuring that I hand this work in, and I really appreciate the encouragement. I hope we remain lifelong friends.

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To my partner, Neo Molefe, thank you for inspiring me, by persevering in your own studies and encouraging me in mine. Your solid work ethic and ambition have inspired me and inspired reflection in my own ambitions. Thanks for always checking that I am on track with my studies.

I cannot ever forget my family and the instrumental role you have played in ensuring that I complete this. Mum, thanks for the encouragement, and I am so pleased that you are pursuing your own ambitions and completing your Masters Degree. To my sister, Tumi, thanks for always believing in. To my niece, Reabeatswe, I hope this is an inspiration for you.

Dedication

I would like to dedicate this dissertation to my mother and my family broadly. Mother, thank you for all the love, support and all the sacrifices.

Thank you for giving me opportunities that you only received much later in life because of all your sacrifice. I wish you all the best in your studies.

I love you and my love for learning and education can only come from you. I hope I have made you proud.

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CHAPTER 1
INTRODUCTION TO OCULAR DRUG DELIVERY

1.1. BACKGROUND

The eye remains one of the most challenging organs in terms of drug delivery (Weng et al., 2017). It is divided into the anterior and posterior segments. Each segment has its own elaborate nutrient transport system designed to keep drugs and xenobiotics out of the eyes. However, as with any part of the body, it is susceptible to inflammation. If an inflammation to the eye or disease is not treated, it could result in blindness. Consequently, the necessity to deliver drugs to desired sites of the eye is pertinent. Furthermore, there is a necessity to track the drugs destined for the posterior segment of the eye. This task has also proven challenging as there is no known mechanism of doing this. As a result, there is a need to produce a drug delivery system that is able to bypass all the barriers found in the eye and has the ability to be tracked. Glaucoma, acute macular degeneration and inflammation such as uveitis remain the most damaging and pertinent disorders that damage either the posterior segment or the anterior segment of the eye (du Toit et al., 2011). However, uveitis is the main point of focus of this dissertation. Uveitis refers to inflammation in the eye, and the main sites are generally the ciliary body, choroid, vitreous body and retina (Yan et al., 2017).

Drug delivery to the anterior segment of the eye is hindered by the tear film and enzymes in the aqueous humour. On the other hand, drug delivery to the posterior segment of the eye is hindered by the vitreous humour, its associated enzymes and the nutrient delivery systems that keep xenobiotics out. The blood retinal barrier (BRB), the retinal pigment epithelium and a number of multi-drug resistant proteins, along with active transport mechanisms affect drug transport to the posterior segment of the eye (Kansara et al., 2007, Vellonen et al., 2018). The multi-drug resistant proteins present a challenge where they possess efflux pumps that keep foreign compounds out.

Further, multidrug resistant proteins are expressed in cases of retinoblastoma, making disease management using therapeutic agents (Shukla et al., 2017) as they ultimately

neutralise and pump out therapeutic agents. Furthermore, the tight junctions found in the corneal epithelial layer are effective in keeping hydrophilic substances out.

The easiest and least painful method of drug delivery to the eye is topical administration using eye drops or a gel formulation. However, topical administration is associated with a few challenges, the most basic being the formation of tears, which washes out drugs that are applied topically (Prosperi-Porta et al., 2016). Other systems used to ensure effective drug delivery to the eye and avoidance of tear-film formation are biodegradable implants. However, implants are invasive, uncomfortable and not user friendly. Nanotechnology may play a significant role in drug delivery to the eye (Trabado et al., 2018) and nanobubbles could be considered as an alternative viable method to enhance drug transport in the eyes. Nanobubbles can be defined as bubbles composed of lipids, such as soybean lipid, coated with a polymer and possessing a gas-filled core. These bubbles are especially suited for drug delivery as they are composed of agents that mimic normal cell membranes. These bubbles are between 10-1000nm in diameter and are filled with gas and may be loaded with a drug of choice. Nanobubbles may present a major advantage because of their ability to be tracked using ultrasound techniques. These bubbles also have the ability to bypass barriers to the eye by forming transient pores in cells as they enter or pass through them (Hirabayashi et al., 2017).

Ultrasound microimaging is quite effective in monitoring the movement and targeting efficacy of these bubbles. Ultrasound imaging has advantages which include allowing observation of nanobubbles in real time through the use of contrasting agents and probes (Zhang et al., 2018). Nano-agents are compounds that enhance the ability to view nanobubbles. Thus, the use of these nano-agents (such as Tween®80) is important. Tween®80 enhances the ability to visualise the nanobubble because of its ability to enhance echogenicity (the ability to echo or reflect a sound) and thus enhance the ultrasound influence on the nanobubble.

Imaging and tracking of the nanobubbles is greatly affected by the contrasting agents that are used (i.e. the gas incorporated into the nanobubble) because it may improve on their visualisation (Wang et al., 2010). The integrity of the gas core will also affect collision with other organelles of the eyes (Adhikar et al., 2016; Ferrera et al., 2008). Furthermore, the type of gas used will affect nanobubble half-life and size due to the pressure and

surface tension (Brenner and Lohse, 2008). The choice of gas can also affect the stability of longevity of the nanobubbles (reviewed in Khan et al., 2018).

Oxygen or gas delivery can be enhanced using factors such as frequency and sonication (Khan et al., 2018). Echogenicity, the ability to echo or reflect sound waves is an important aspect in the imaging and is strongly related to oscillation of nanobubbles. Echogenicity has to be enhanced by the lipid formulation and echogenic agents used as part of the formulation. The bubbles need to be small, yet sizeable enough to form the transient pores in the barriers that need to be surpassed, a process known as sonoporation (Adhikari et al., 2016). Oscillation caused by a sound wave greatly affects drug delivery (Ferrara et al., 2008) and will need to be enhanced, as it affects membrane permeability. Consequently, the polymer chosen to coat the nanobubble must facilitate effective oscillation.

1.2. RATIONALE AND MOTIVATION FOR THE STUDY

Ensuring that a nanoparticulate system is able to pass through barriers in the eye, is able to be viewable in real time using ultrasound techniques was an important motivating factor for this study. Ensuring that the introduction of the nanoparticulate system does not cause adverse immunogenic effects required examination. It was also important to study the most appropriate polymers to use, as they will be able to carry the drugs.

An important aspect of nanobubble production is the fact that they need to be produced such that they do not activate the immune system. The most favourable nanobubbles are able to mimic bilayers of most cells. Thus, the nanobubble membranes also need to have a structure that mimics the hydrophilic heads and hydrophobic tails of the phospholipid bilayer. This can be achieved using long acyl chains amongst other compounds. The bilayers contain compounds which can be ubiquitously found in the body, such as cholesterol. Nanobubbles being produced using lipid or fats as part of the layering, can be called lipobubbles (Wang et al., 2010.)

The mechanism of operation of nanobubbles needs to be tested under the harsh, almost impermeable climate of the eye. A proposition that seems feasible is topical administration of the nanobubble with the use of a pressure vessel where the nanobubbles are pushed out through a small opening and sprayed into the eye (i.e.

aerosol spray). This will ensure that the pressure forces the nanobubbles out at a high speed. This will also increase the kinetic and potential energy of the bubbles. Alternatively, the nanobubbles could be incorporated into a gel formulation that enables adherence to the corneal surface in the presence of tears, thus preventing the drug from being washed out from the ocular surface, as illustrated in Figure 1.1. A change in viscosity on exposure to a change in temperature or pH may be considered to activate the release of the nanobubbles.

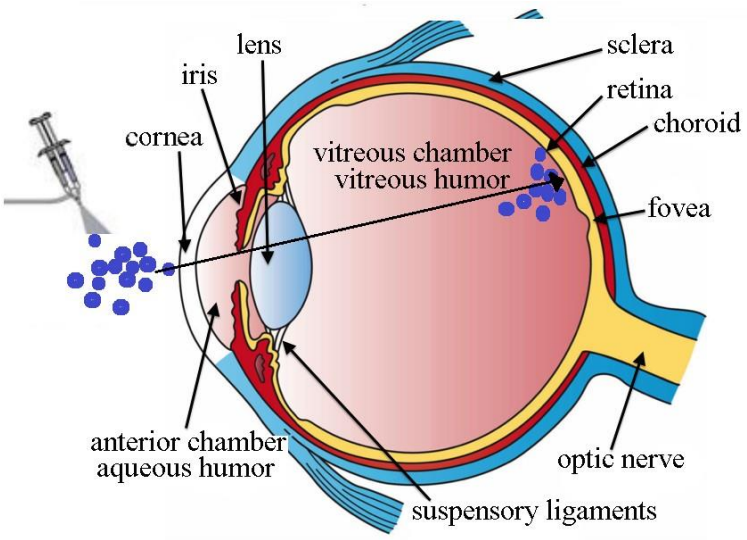

Figure 1.1: Schematic diagram illustrating the use of the intra-ocular nanobubble systems (IONBS). This diagram also illustrates all the layers in the eye which play a role in hindering effective drug delivery in the eye. The likely path would be the movement of the nanobubbles through the cornea into the anterior chamber, into the posterior chamber and into the vitreous body. The other path is the movement around the eyeball and into the sclera. The varying thickness of the sclera may allow for drug to pass through into the choroid and into the posterior eye (Kumari, 2020).

1.3. AIM AND OBJECTIVES

The aim of this study was to design an intraocular nanobubble system (IONBS), a novel topically administered ocular targeted drug delivery system for the posterior segment of the eye. The multilayered intraocular nanobubble system was composed of polymers that were not immunogenic. The process entailed producing a layer-by-layer nanoliposomes. These nanoliposomes had gas, sulphur hexafluoride introduced through displacement. The study further aimed to undertake real-time imaging of the system *in vivo*. The

proposed novel drug delivery system employed gas-filled nanobubbles. In order to achieve this aim, the following objectives were proposed:

1 To synthesise drug-loaded stable nanoparticles with the potential to deliver anti-inflammatory drugs to the posterior segment of the eye and with potential targeting and nano adhesive properties. Once layer-by-layer nanoliposomes were produced, a gas, sulphur hexafluoride, would be produced to produce nanobubbles. The sulphur hexafluoride would allow real-time viewing using ultrasonic techniques. The intention was to achieve this through selection of suitable echogenic agents, lipid (e.g. soybean lipid) and polymers such as poly-co-glycolic acid (PLGA), which are susceptible to changes in viscoelasticity, pH or temperature for nanobubble formulation. These nanobubbles would have theranostic capabilities, a diagnostic element and the ability to treat uveitis.

2 Determination of size and surface stability of the nanoparticles. Furthermore, the intention was to use Scanning Electron Microscopy (SEM) to characterise the surface morphology of the nanoparticles. The size is important as a small size would ensure that the nanobubbles are able to pass through the various ocular barriers.

3 Viewing of the nanobubble lifetime using ultrasound techniques. Simulated vitreous humour was to be used to view the nanobubble ocular drug delivery system in vitro. The Vevo®2100 high resolution imaging system was to be used for ultrasound imaging and assisted in determining the stability of the nanobubbles. Ensuring the nanobubbles could be viewed is important it would allow real time tracking of the nanobubbles.

4 Incorporation of the nanobubbles into a suitable drug delivery system.

5 Evaluation of the drug delivery system (in aerosol or gel form) *in vivo* employing the New Zealand white rabbit model in which ocular inflammation has been induced. The Vevo®2100 system would be used to track the nanobubbles and to determine biodistribution. This aspect is important as it would assess whether tracking of the nanobubbles is possible in real time, using ultrasound.

6 Determining the reaction of the eye to intraocular nanobubble system (IONBS) by checking for inflammatory markers inflammation, was to be ascertained. This

aspect is important as it would assess whether there is cellular damage due to the introduction on the nanobubbles

1.4. NOVELTY OF THIS STUDY

1. It is important to understand the positioning of this study in ocular drug delivery work. In further chapters, the literature review and small comparative overviews in other chapters reveals that currently most ocular nanoparticulate systems are mono-layered, whilst this this system was multi-layered. This emphasised that the study undertaken bore importance for ensuring optimal drug entrapment.
2. Most studies intended for ocular drug delivery using nanotechnology, primarily makes of nanoparticles, and micelles, but there is not a wide body of research on incorporating a gas and thus producing nanobubbles.
3. Theranostic ocular drug delivery has also been considered in other studies, however, there is not a wide body of research on this in posterior ocular drug delivery and this speaks to the novelty of the study that was undertaken.
4. The use of surfactants is an in important aspect in producing stable nanoparticles, and the literature review and chapter of pre-formulations and experimental design will have a comparative review will reveal the importance of surfactants. However, in this study, the surfactant used, was used not only for stabilising the formulation. However, it was used also as a contrasting agent in ensuring optimal viewing of the nanobubbles. This adds to the novelty of the study.

1.5. OVERVIEW OF THE DISSERTATION

Chapter 1: This dissertation outlines the current standards around treatment of posterior ocular diseases, and introduces nanoparticulate technology advances in ocular research, as the possible future gold standard in this area. It also points to nanobubbles as the best possible drug delivery mechanism for treating posterior uveitis. This chapter outlines the aims and objectives and outlines how this work differentiates itself from similar work in the field of ocular drug delivery.

Chapter 2: This chapter forms part of the literature review in. This chapter gives an anatomical analysis of the eye and outlines why drug delivery, particularly to the

posterior eye is such a challenging feat. The chapter further gives an overview of general trends in drug delivery with regards to the posterior segment of the eye, gives advantages to these approaches, but also presents shortcomings. The chapter then introduces nanotechnology in the ocular drug delivery field and outlines progress and developmental areas. The chapter further motivates why nanobubbles might be a viable mode of drug delivery to the posterior segment of the eye.

Chapter 3: This section of the dissertation outlines the preformulation studies, sets the factors affecting nanoparticulate size and drug entrapment efficiency and outlines the results of the Box-Behnken design study that elucidated the optimised formulation. The chapter outlines the variables used to determine an optimised formulation of the nanobubbles, and Zeta-sizing studies, along with determination of zeta potential are used to assess size and stability. This chapter further presents, in better detail, a mathematical analysis of the outputs of the design. The chapter ends of by comparing what other studies have revealed in Box-Behnken designs of nanoparticulate systems for ocular drug delivery.

Chapter 4: demonstrates all the qualitative aspects of the drug delivery system using methods such as Fourier Transform Infra-Red (FTIR), Differential Scanning Calorimetry (DSC) and X-Ray Diffraction. All these studies would have been performed on the optimised formulation.

Chapter 5: demonstrates how the optimised formulation reacted *in vivo*, in a study involving rabbits and using ultrasound real-time viewing techniques. The study also takes a makes an overview of how studies have measures safety of the formulations in the rabbit eyes and the use of suspensions to aid topical administration.

Chapter 6: makes recommendations and provides final thoughts, as well as a summary of the outputs and recommendations for future research.

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CHAPTER 2
LITERATURE REVIEW: ANTERIOR TO POSTERIOR SEGMENT TOPICAL OCULAR DRUG DELIVERY

2.1. INTRODUCTION

Theranostics is the ability to dually diagnose and therapeutically treat (Jedrazk et al., 2019). Theranostics is especially important in treating posterior ocular disease because invasive techniques cause damage in the long term and require treatment to repair it. Topical treatment of which the therapeutic efficacy can be tracked and quantified is the best resort for effective treatment of infections such as posterior uveitis.

Effective ocular drug delivery to the posterior segment of the eye is challenging due to the multiple barriers and layers designed to keep drugs and xenobiotics from entering this important organ. The use of polymers has gained attention due to their vast properties and applications. Polymers can be used to improve bioavailability, can be used to ensure stable drug release, can ensure enclosed safe area for drug, and can assist with targeted drug release, to name a few uses. Drug delivery to the posterior segment of the eye is conventionally treatable using methods such as intraocular injection and implants such as Ozurdex (Whitcup & Robinson, 2015). However, intraocular injections are invasive and may lead to complications such as cataracts (Thakur et al., 2016). Ocular implants involve invasive and complicated surgery and thus the ophthalmic pharmaceutical conundrum is: is it possible to produce a non-invasive and topical route to treat inflammation and ocular disorders associated with the posterior segment of the eye?

Nanotechnology has assisted in the advancement of theranostics, the dual capability of diagnosis and therapeutics, more notably in cancer. This chapter provides a bird's eye view on ocular anatomy, the state of ocular drug delivery. Lastly, it will zoom in on nanotechnology and microtechnology with regard to ocular drug delivery in the posterior segment of the eye.

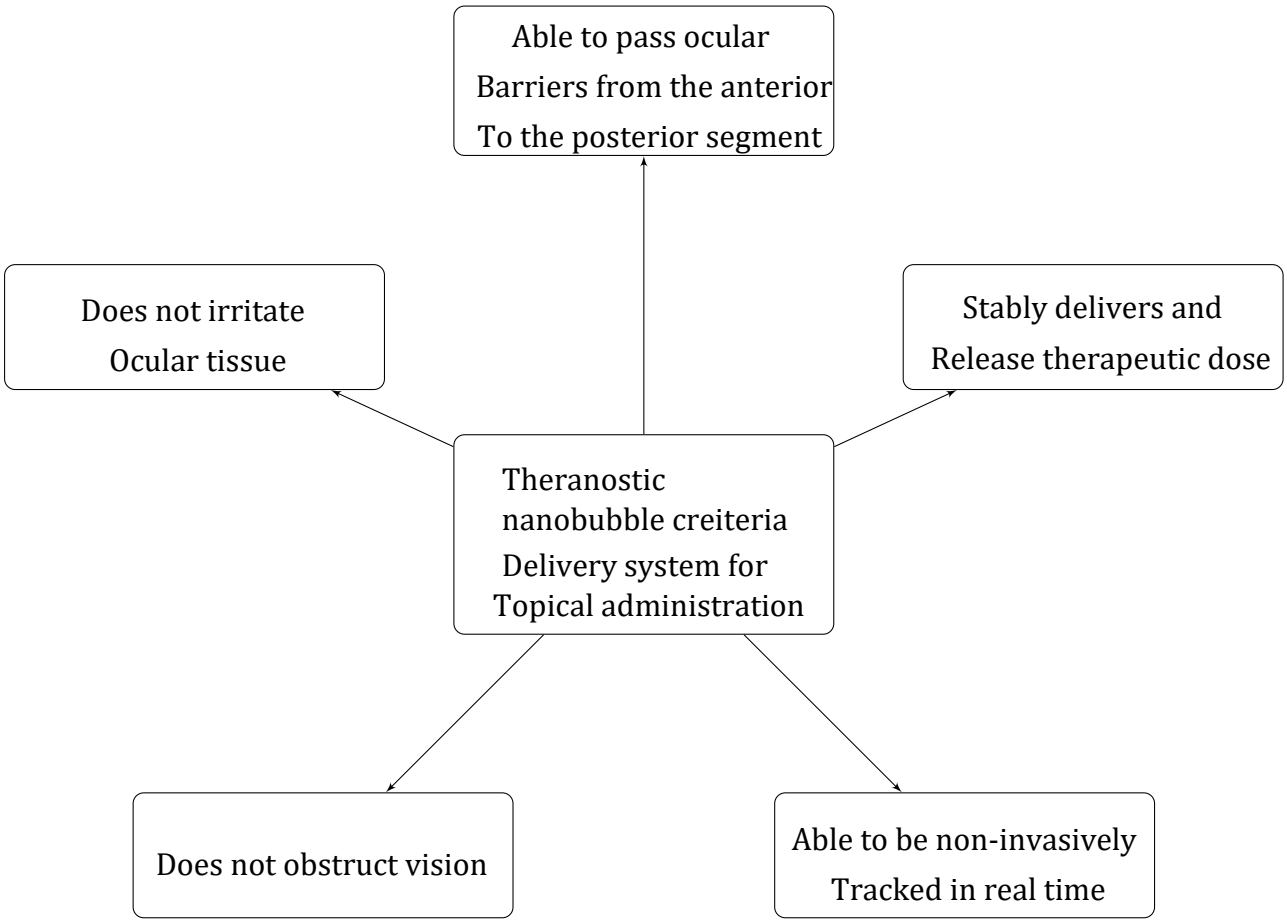

Figure 2.1: Diagram illustrating criteria for effective theranostic nanobubbles

2.1.1. Anatomy of the eye as a multilayered organ, designed to impede drug delivery

The eyes possess an elaborate nutrient transport system that impedes the movement of xenobiotics. Tissues composing of the eyes are multilayered and each layer has varying permeability to water, drugs and lipids. The tearing effect, a multilayered nature, and specialised barriers contribute to difficulties in allowing effective drug delivery to the posterior segment of the eye. The tearing effect, along with anatomical constraints allows only a maximum of 30 µl in the eye (Chan et al., 2016). This presents important limitations and considerations with regards to measuring the therapeutic dose and the maximum volume needed. As earlier discussed, there are many barriers which exist to impede drug delivery to the eye, both in the anterior segment and in the posterior segment. Most discouraging is that there is a low bioavailability of drugs to the anterior segment of the eye, with less than 5% reaching ocular tissues (Geroski &Edelhauser, 2000). There are a

number of reasons for this: the reflexes of the eye, nasolacrimal drainage, and the epitheliated cornea.

From an anterior view, the layers include the cornea, anterior chamber, the iris, core and lens. From the posterior view the following layers are present: conjunctiva, followed by the sclera, followed by the choroid and lastly the retina. All these layers all possess specialised tissues intended to perform varying functions and thus differ in their ability to absorb water and their hydrophobicity.

The sclera is made up of collagen fibrils ranging in size from 25-230 nm. The sclera is a point of interest in drug delivery to the posterior segment of the eye, using topical administration as it would be one of the first points of contact for any drug. Huang et al. (2018) posited that the sclera may be able to allow macromolecules of 20kD to 150 kD to pass through. This was tested on rabbit sclera as the rabbit eye model resembles that of humans. Topically applied nepafenac was able to inhibit choroidal and retinal neovascularization and thus offers a hope for topical application for treating diseases in posterior ocular tissues (Koevary, 2003). Drug delivery through the sclera is made possible by the fact that the sclera has varying width (as shown in Figure 2.2) and thus drug delivery can be possible in regions less thick.

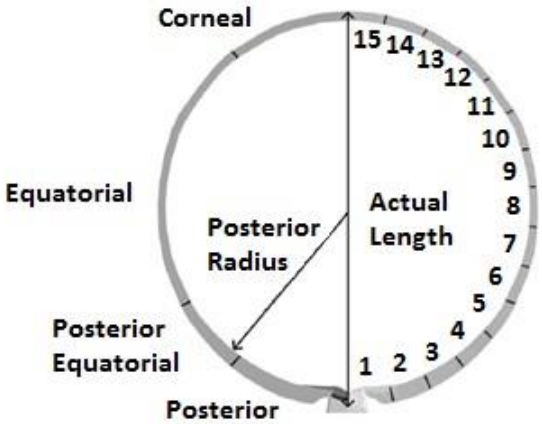

Figure 2.2: Schematic illustrating dimensions of eyes and varying widths of the sclera around the eyeball (Huang et al., 2018). This diagram shows all the barriers nanoparticulate systems need to pass in order to reach the site of therapy in order to be effective.

2.1.2. The vitreous chamber as a structure able to allow diffusion of therapeutic agents

The vitreous chamber contains a gel-like substance called the vitreous body/humour which consists of collagen fibrils, proteoglycans and glycosaminoglycans such as hyaluronic acid. The vitreous body is nutrient rich and provides these nutrients to the lens. The vitreous body is mostly gel and contains at least 98% water (reviewed in Shafaie et al., 2018); however, this gel starts to become more liquid during aging. An important consideration in achieving optimal drug delivery to the posterior segment of the eye is the structure of the vitreous body. This is because drug penetration to the posterior eye will result in drug depositing in the vitreous chamber. Drug delivery to the retina has also been achieved by injecting the drug into the vitreous region. The vitreous body is susceptible to inflammation and thus drug delivery to this region is challenging and invasive (Day et al., 2016).

The vitreous humour can be removed from the eye without causing marked damage and it does not change its form once removed from the eye. The vitreous region can be partitioned into a number of regions: the central vitreous, the basal vitreous, the vitreous cortex, the vitreoretinal interface and the zonule. The central vitreous has a low concentration of fibrils, the basal vitreous has a high concentration of fibrils, which mostly adhere to the retinal and non-pigmented ciliary epithelium of the pars plana. The vitreous cortex is a thin layer of gel distinguishable from the centre gel because it has a high concentration of collagen fibril, in comparison to the central vitreous. The vitreous cortex can be divided into the anterior portion and the posterior portion. The anterior portion separates the front of the vitreous from the lens. The posterior portion is composed of collagen fibrils parallel with the retinal surface, hence it is attached to the lamina and retina.

The fibrils are composed of type 2 collagen (Silva et al., 2017), which is also found in cartilage. The organisation of collagen in the vitreous humour and cartilage is identical (Bos et al., 2001). Another interesting aspect is the amount of hydroxylysine glycosylation in the collagen. Lysine (an amino acid) residues content is also associated with growth and aging as the lysine residues are fewer in growth plate cartilage (Bos et al., 2001). Hydroxylysine glycosylation is associated with aging, and furthermore the vitreous

humour liquefies with aging, affecting vision. Molecular structures within the eye could play a role in the aging process.

Although the vitreous humour is mostly acellular, the vitreous cortex and basal cortex contain hyalocytes. Hyalocytes possess phagocytic properties. Gärtner (1971) observed macrophage-like cells in the basal vitreous. The lens grows throughout life and it contains a nucleus which is at the centre of the lens (Ganagalum & Bhat, 2009).

The vitreous body is an important part of posterior ocular drug delivery as the collagen fibrils and glycosaminoglycans are connected to the retina. The retinal pigment epithelium (RPE) is a barrier to drug delivery. If the therapeutic agent is able to diffuse to the vitreous body and pass through the RPE, and to the choroid, it will be able to treat uveitis. Movement of molecules within a gel/liquid medium like vitreous body is through convection and diffusion. Kaiser and Maurice (1964) found that fluorescein was able to diffuse through the lens. Different parts of the lens allowed diffusion at varying rages. The lens fibers allowed diffusion easily, the cell membrane allowed slower diffusion, the lens capsule allowed easy diffusion, and the epithelium did not allow easy passage (Kaiser & Maurice,1964). In terms of drug delivery to the posterior segment of the eye, the lens's epithelial layer will form a considerable barrier to drug delivery to the vitreous humour. The lens experiences changes through aging and these changes are seen in the domains in the eye. In adult lenses, two-thirds of phospholipids are sphingolipids and dihydrosphingolipids. The composition changes with age (Mainali et al., 2017).

The movement of fluorescein once injected in the vitreous body can shed light on the likely movement of drugs in the vitreous humour. When fluorescein is injected into the vitreous body, it passes out across the entire retinal surface. Competitive inhibitors and active transport mechanisms ensure the transport of organic anions of the vitreous body by retinal capillaries and the retina's pigment epithelium. This active transport mechanism was discovered in rabbit eyes (Cunha-Vaz & Maurice, 1967). The structure of the human ocular fundus and the visualisation of the sub-retinal structure will elicit the factors that will affect drug permeation through the eye.

2.1.3. The choroid as an ocular layer susceptible to uveitis

The choroid is the layer found between the retina and the sclera. The choroid is often inflamed in cases of posterior uveitis. The choroid is an important consideration in drug delivery to the posterior eye. It has varying elasticity depending on its position. The Young's Modulus is higher (thus less elastic) in the more posterior regions as compared to the anterior region (Friberg & Lace, 1988). In the sclera, choroid and retina, collagen and elastin are present. Although it contains mostly water, the vitreous humour contains other proteins such as proteoglycans and proteinases (Skeie et al., 2015).

Ocular toxoplasmosis, caused by the parasite Toxoplasma gondii, an obligate intracellular protozoan parasite, causes posterior uveitis (Ozgonul & Besirli, 2017). Optical coherence tomography reveals that the lesion sites are hyper reflective. Retinal thickness decreases and there is evidence of macular oedema (Orefice et al., 2007). Another interesting finding is the change in the hyaloids canal during toxoplasmosis. The posterior hyaloids canal thickens and detaches. The blood retinal barrier is composed of tight junctions and retinal pigment epithelium is the outermost region. The tight junctions stop entry of other cells into the retina. This prevention of entry is disrupted in the cases of ocular toxoplasmosis (Song et al., 2017).

2.1.4. Cloquet's canal as a potential carrier of nanoparticulate drug delivery systems

The Cloquet's canal, also known as the hyaloids canal, is a tubular mass that extends from the lens to the posterior pole and optic nerve of the eye. The vitreous body routes the Cloquet's canal (Alovisi et al., 2017). The mass is not easily visible by slit-lamp examination. According to Enaida et al. (2004), the hyaloid canal does not have any vitreous gel within it and is white in appearance. In terms of exact location, it is 1-2mm wide, it extends from the optic nerve head to a point slightly nasal and inferior to the posterior pole of the lens (Cunnane et al., 2016; Sakamoto et al., 2002). In 2006, the Cloquet's canal was viewed by ultra-high resolution optical coherence tomography by Kagemann et al. (2006), and this development has been part of explaining vision problems (Altintas & Ilhan, 2018).

2.1.5. Progression of uveitis in the posterior eye

Inflammation in the posterior eye causes uveitis. The inflammatory response should generally yield positive results as it signals the body to fight pathogens. The acute phase of inflammation is characterised by the rapid influx of blood granulocytes typically neutrophils, followed swiftly by monocytes that mature into inflammatory macrophages.

These macrophages subsequently proliferate and thereby affect the functions of resident tissue macrophages.

Intraocular pressure needs to be a consideration in treating inflammation in the eye. A high intraocular will adversely affect the eye, thus anything introduced into the eye must not adversely affect intraocular pressure. In drug delivery to the posterior eye considerations, intraocular pressure needs to be taken into account.

2.2. DRUG DELIVERY APPROACHES TO THE POSTERIOR SEGMENT OF THE EYE

2.2.1. Barriers to drug delivery to the posterior segment of the eye

The blood aqueous barrier (BAB) and blood retinal barrier (BRB), aptly named the blood ocular barriers (BOB), control the movement of molecules through the eye. The BAB controls the exchange of blood and intraocular fluid involving the ciliary body, and trabecular meshwork. The BRB regulates the movement of metabolic substances from the eye and into the bloodstream. The surface of the blood-retinal barrier is the retinal pigment epithelium (Ferrington et al., 2017). The BOB has an elaborate network of organelles and protein pumps to impede entry of xenobiotics and drugs into the eye.

It is this main network that ensures the current ineffectiveness of topical suspensions or ointments for therapy to the posterior segment of the eye. Ophthalmic solutions, suspensions and ointments remain the gold standard for anterior ocular drug delivery. Furthermore, innovators such as Moosa et al. (2014), have formulated solid eye drops for glaucoma. This can be used to assist patients suffering from arthritis and other disorders causing impaired mobility and unable to squeeze medicinal bottles by themselves. This innovation and similar formulations have been gaining traction as the gold standard for anterior ocular treatment.

Technology for effective drug delivery to the posterior segment of the eye has involved mainly implants and intravitreal injections (see Table 2.1).

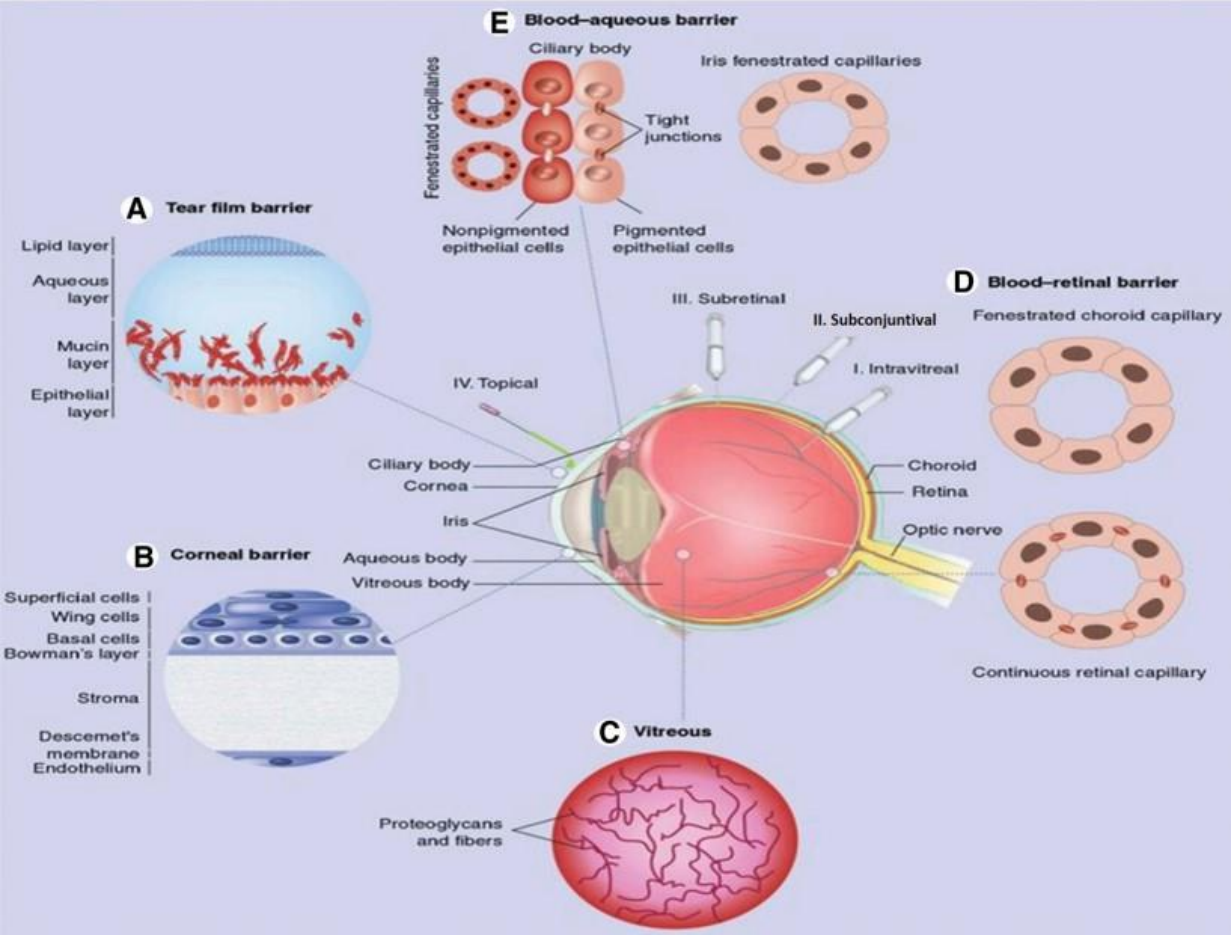

Figure 2.3: Figure illustrating the ocular barriers and (a-e), and the associated routes of drug administration (i-iv). The barriers are (a) tear film barrier; (b) corneal barrier; (c) vitreous barrier; (d) blood–retinal barrier and (E) blood–aqueous barrier. Routes of drug administration are (i) intravitreal injection, (ii) subconjuctival injection, (iii) subretinal injection and (iv) topical administration (reviewed in Gote et al., 2019). This diagram integrates the understanding of the barriers and how they are currently being overcome

Although innovative, these techniques present a number of drawbacks. Devising a technique that allows for treatment in the posterior eye using an anterior route has been challenging. This is the most desirable route as it excludes the need for invasive techniques. It allows for the patient to use the medication at home, thus lessening the burden on the healthcare system and allowing comfortable use. There are a number of requirements such a drug delivery should possess: pose no interruption to the cornea, pass through the ocular layers without causing any cytotoxicity and not eliciting the

immune system, the drug delivery system should be an effective encapsulator, only releasing the drug at the appropriate moment.

Table 2.1: Table illustrating current practices and modes of administration in treating ocular diseases

Administration	Drug Delivery System	Drug	Treatment	Reference
Topical	Nanoparticles			
	Nanospheres	Flurbiprofen	Anterior Inflammation	
	Nanomicelles	Diclofenac		Shi et al., 2015
Implanted	Vitrasert	Ganciclovir	CMV-retinitis	Dhillon et al., 1998
	Retisert	Flucinoloneacetonide		Jaffe et al., 2006
	Donut-shaped Minitablet	Ganciclovir	CMV-retinitis	Choonara et al., 2009
	Ozurdex	Dexamethasone	Uveitis, macular edema, retinal vein occlusion	
Oral		Kinase inhibitor	Retinal Neovascularization	Seo et al., 1999
Trans-Tenon				
Contact Lens	Hydrogel contact lenses	Lidocaine, Timolol Maleate	Glaucoma	Gulsen & Chuhan., 2004, Hiratan& Alvarez-Lorenzo., 2004
Topical	Mini-tabletting	Timolol Maleate	Glaucoma	Chetoni et al., 2009

2.2.2. Intravitreal injections as initial therapy to treat uveitis and circumvent ocular barriers

The current gold standard in therapeutics in treating diseases and disorders of the posterior segment of the eye is the use of intravitreal injections. Intravitreal injections

are considered effective because they are able to reach the intended site and are able to administer the recommended therapeutic dose. The main drawback to the use of intravitreal injections has been invasiveness. This procedure is associated with ocular hypertension, and after a number of injections poses a risk to ocular health (Bracha et al., 2018). A long needle is inserted through the eye from anterior to posterior. Intravitreal injections have to be performed under anaesthesia and may require multiple doses. These injections are however also associated with retinal detachment and cataracts and these disorders are associated with blindness. The multiple dose approach using intravitreal injections is also associated with low compliance.

As mentioned, the procedure of intravitreal injections is invasive and causes much discomfort. This procedure is also a causative agent of cataracts in the eye, retinal tears and haemorrhaging. Intravitreal injections are also associated with low compliance due to the invasiveness and potential for drug toxicity. Shortly after the drug has been injected into the posterior segment of the eye, the levels of drug may be high and may be toxic to neighbouring cells. However, just prior to injection of a renewed dose, the drug levels may be at an ineffective dose in the eye (Weiner et al., 2008). This change in drug dose may affect the levels of therapeutic dose available and may cause cytotoxicity.

The intravitreal injection of nanoparticles has also been considered in drug delivery to the posterior eye. Bourges et al. (2003) demonstrated a feasible drug delivery approach to the posterior eye using an intravitreal injection to deliver nanoparticles to the posterior segment of the eye. Nanoparticles as drug delivery carriers may have protective properties on the drug and assist in prolonging half-life due to relevant intermolecular and weak covalent bonds. These particles were localised on the retinal pigment epithelium. This may serve as a viable option for drug delivery. However, this does not eliminate the possible complications of retinal detachment. Intravitreal injections are used to treat posterior segment disorders (Weiner et al., 2008) such as uveitis. A further advantage to the use of polymeric drug delivery carriers is the ability to control the rate of drug release in the infected area. Controlled drug release is effective as it would allow for less doses of the drug and ensure constant release of the drug to maintain the minimum therapeutic dose without causing toxicity. However, in the case of the posterior segment of the eye, it remains to be seen whether nanoparticles can reach the posterior segment of the eye when administered topically.

Nanoparticulate size, charge and site of entry can also affect movement of particles in the quest to find a topical formulation for treatment of ocular disorders in the posterior segment of the eye. There has been some success and the success by Amrite et al. (2005) is worthy of documentation. It was shown by Amrite et al. (2005) that nanoparticles administered subconjunctivally are able to remain at the site of delivery for two months. Amrite et al. (2005), have successfully demonstrated that the size of the particles being administered affects their ability to be retained in the posterior eye due to hydrophilic and hydrophobic forces. These particles, composed of polystyrene and produced in varying sizes, revealed that particles in the micro range and 200nm had shown increased retention in periocular tissues and could be detected 60 days post administration in rats. Amrite et al. (2008) have shown that particles as small as 20nm were unable to permeate the sclera, choroid and retinal pigment epithelium. It has also been established that moisture and drying out or dehydration processes may also affect sizes of nanoparticles (Sanjay et al., 2008). Thus, nanoparticles in solution may swell in response to changes in the environment. A further study has also held that the charge of the nanoparticles plays a significant role in retinal penetration. Bourges et al. were however unable to demonstrate how this innovation would be able to lessen complications associated with the use of intraocular injections

An improvement on multiple-dose intravitreal injections has been the insertion of implants intravitreally. These implants would release an effective dose over a prolonged period of time. Ozurdex™ is an implant used to treat uveitis of the posterior segment of the eye. It is effective over 6 months and releases dexamethasone. An important aspect of sustained release drug delivery is the monitoring of the drug delivery system and its efficacy in releasing an effective dose. Sustained release requires the use of polymers or intelligent devices that impede high concentration of burst release of the drug in response to a drug concentration gradient. High concentration of a burst release poses challenges that affect the longevity and effectiveness of the drug delivery system: straight after the drug has been injected, the levels of drug may be high and may be toxic to neighbouring cells. However, just prior to injection of a renewed dose, the drug levels may be at an ineffective dose in the eye. This demonstrates the importance of polymer choice in sustained drug release.

The use of poly-co-lactide has been documented to be effective in sustained drug release as the concentration of the polymers affects drug release. Poly-co-lactide nanoparticles have also been associated with improved bioavailability in the eye and no cytotoxicity and tissue toxicity were detected (Zhang et al., 2018).

2.2.3. Intravitreal implants as a long-term therapeutic agent to treat uveitis

Topical drug delivery to the posterior eye is hampered by the washing out of the drug by the tearing effect. Elaborate protein-pump systems make use of charge differences and ATP-regulated protein subunits to dispose of drugs in the eye (Kansara et al., 2007). In the quest to improve on topically-administered drug washout and to achieve the aspiration to evade ocular damage to prolonged used of intravitreal injection, implants have a possibility of improving on current practice. Implants, with the use of the appropriate polymers and intelligent devices, may release the drug in a sustainable and controlled manner, and perhaps with more effective therapeutic outcomes, if inserted very close to the affected site. Implants possessing biodegradable and bioerodible properties are preferable as they would not need to be removed and their products can be metabolised by ocular enzymes. Non-bioerodible or non-biodegradable need to be removed but outweigh the risks of frequent use of intravitreal injections due to their ability to act over prolonged periods of time.

2.2.3.1. Vitrasert™ as an FDA approved implant in combatting posterior ocular diseases

Vitrasert™ is an implant intended for the treatment of AIDS-related cytomegalovirus (Stryjewski et al., 2019). With countries mainly in sub-Saharan Africa challenged with controlling the spread of HIV, its complications are compounded by the effects of a weak immune system. Kaposi's sarcoma, Tuberculosis and Meningitis are related to HIV AIDS related cytomegalovirus (Yarchoan et al., 2018). CMV affects HIV- infected patients with low CD4 counts and thus weak immune systems. The use of antiretroviral therapy has been effective in lowering the viral load and slowing the effects of the virus. However, research suggests that in patients with especially low CD4 counts, highly active antiretroviral treatment (HAART) is not sufficient in treating the viral load and treating the CMV (Jacobson et al., 1997) directly may prove to be more effective. Thus, further intervention is needed in treating HIV-related CMV. Implants would be apt devices for

drug delivery as they ensure constant release of the drug within the therapeutic dose. They also ensure complete compliance, leading to speedier recovery. Vitrasert remains the gold standard for treating ocular CMV. This implant lasts 8-12 months. Its creators have used a gradient index endoscope to track the actions of the implant in the vitreous cavity. Vitrasert™ is composed of polyvinyl alcohol and ethylene vinyl acetate and contains 4.5mg of ganciclovir. This implant is inserted by making a 5mm long insertion in the sclera. This implant is able to clinically stabilise cytomegalovirus retinitis. It has been recorded that once removed, the pellet used as part of the implant was covered with fibrous material but showed no obvious signs of toxicity caused by the implant. Complications due to the surgery are endopthalmitis, vitreous haemorrhage, hypotony and macular edema (reviewed in Rodrigues da Silva et al., 2010).

An implant able to stabilise cytomegalovirus retinitis has much clinical significance as many patients suffered from the disease as HIV proceeded to erode at their immune system's efficiency. The effective treatment of this disease meant effective treatment, thus preventing loss of sight, without the need for multiple invasive procedures and without over-burdening of the healthcare system.

2.2.3.2. Retisert™ – an implant to treat long-term inflammation

Retisert™ is an implant inserted through the pars plana and requires replacement once depleted. It is loaded with the drug fluocinoloneacetonide and the polymer used allows for controlled and sustained release. Flucinoloneacetonide was the drug of choice due to its low solubility and short duration in the bloodstream. This correlated to lowered side effects. Georgalas et al.'s (2014) a study examining its efficacy over 34 weeks revealed that 87% of the patients' vision improved and the use of systemic medications, periocular injections and topical corticosteroids decreased by over 40 % in some cases. Less than 10% of the patients required cataract surgery (Jaffe et al., 2006) post insertion. A further study on rabbits, running for 52 weeks by Driot et al. (2004) revealed that the drug concentration in the vitreous humour remained relatively constant all year. The drug remained at its highest levels in the vitreous and retina. Drug levels were relatively low in the aqueous humour. Very low concentrations of the drug were found in the urine and blood, revealing that there was minimal exposure to neighbouring tissue and thus indicating low probability of cytotoxicity to neighbouring tissues (Driot et al., 2004).

Retisert™ is not without its controversies: This implant was said to have caused cytomegalovirus retinitis in a patient (Ufret-Vincenty et al., 2007). In this case, a 65-year old patient with bilateral uveitis developed cytomegalovirus retinitis. The hypothesis on the cause is that the introduction of the implant had immunosuppressive properties, thus allowing for the progression of cytomegalovirus retinitis. A further case of sclera melting was reported following the use of Retisert™ 18 months after use of the implant (Georgalas et al., 2014). These cases have brought other side effects to the fore. This includes the fact that all patients require cataracts within 3 years post-introduction of the implant. Furthermore, some patients (about 40%) require a trabeculectomy due to the raised intraocular pressure after insertion. A study comparing Retisert™ with Ozurdex™ revealed that Retisert™ had a higher recurrence rate, was associated with cataract progression, glaucoma and the need for laser surgery.

Minor complications resulting from its implantation have been dissociation from its anchoring strut 5 years post-insertion

2.2.3.3. Biodegradable implant to treat ocular diseases

Retisert™ and Vitrasert™ are non-biodegradable and thus need to either be removed or replaced after use. Continuous surgery has been associated with macula oedema, cataracts and retinal detachment. Thus the progression to biodegradable was inevitable.

Ozurdex™ is a biodegradable implant which releases dexamethasone (Kumar at al.,2019). It is inserted with an injection intravitreally. It has proven successful in treating retinal vein occlusion and non-infectious posterior uveitis. The use of biodegradable polymers is a pertinent advancement as it eliminates the need for surgery to remove the implant. A study found that the implant needed to be replaced by an average of 5 months later, and it was associated with no adverse effects except with a low incidence of cataract surgery required (Querques et al., 2013).

Retisert™ and Ozurdex™ have been compared in a study, showing fairly comparable results. A study examining the recurrence rates in these two implants found a lower recurrence with the use of Ozurdex™ (Arcinue et al., 2013). However, there have been recorded cases of the implant reaching the anterior eye, thus causing damage to the cornea and lens.

2.2.3.4. Donut-shaped Minitablet as a novel biodegradable implant able to treat diseases in posterior eye

Choonara et al. (2006) have documented and created another implant to treat cytomegalovirus retinitis: A Doughnut-Shaped Minitablet (DSMT), a biodegradable drug delivery device not larger than an American Dime (as in Figure 2.3). This DSMT is a possible improvement on current practices in posterior ocular treatment as it is biodegradable and thus eliminates the need for it to be removed surgically. The hole for the doughnut shape allows for suturing to the ocular surface (see Figure 2.4). This implant lasts for 24 weeks with a biphasic drug release profile. This profile consists of an initial burst phase and a diffusional phase. Choonara et al. (2006) were able to demonstrate that the DSMT released sustainably. DSMT was composed of Resomer: consisting of polyglycolic acid and polylactic acid. Due to the differing grades of resomer available, and due to different ratios of polyglycolic acid: polylactic acid, it was demonstrated that a more viscous grade allowed for a more desirable drug release profile. These polymers in combination are responsible for the controlled drug release. Resomer 502, 503 and 504 were used in concert with Resomer L 214.

This innovation in implant technology has the ability to successfully improve life expectancy and improved vision in patients suffering from AIDS-related cytomegalovirus retinitis. Implants circumvent ocular barriers by releasing at the site and ensuring that the therapeutic release is available to the cells that require it. The added advantage is the minute incision done to add the implants. This decreases the risk of damage to the retinal tissue. However, this does not eliminate the risk of the formation of cataracts, endopthalmitis and vitreous haemorrhage.

Although this invention presents significant progress in ocular implant technology, there have been further studies regarding the energetics and heat dissipation of the tablet matrix due to possible mechanical pressure and changes in reaction to the glass transition pressure. Elevation of temperature in the eye could affect the efficacy of macromolecules such as proteins and carbohydrate complexes.

Worthy of comparison, is the DSMT and vitrasert as they both treat HIV-related CMV and they both make use of the same drug.

Figure 2.4: A digital image depicting the DSMT device in relation to a typical USA one dime coin

2.3. CONTACT LENS DRUG DELIVERY SYSTEM

This innovative solution was produced in order to increase the contact time of the drug to the eye in order to ensure drug delivery. There are a number of ways these drugs can be added to hydrogel systems to ensure maximal drug delivery. These include polymeric hydrogels as conventional contact lens, polymeric hydrogel hosed inside of conventional contact lenses, surface-modified polymeric hydrogels to immobilise drugs on the surface of the contact lenses. Hydrogel systems can be very effective in drug delivery and the different gelling mechanisms can facilitate drug delivery (Wu et al., 2019).

2.4. MECHANICAL TECHNIQUES

2.4.1. Iontophoresis: Use of electricity for ocular drug delivery

Iontophoresis is a process where an electric current facilitates drug delivery. Iontophoresis has also been used in delivery of drugs through the skin (Huang et al., 2018). Iontophoretic drug delivery was achieved with porcine eyes as an animal model. Transcorneal and Transscleral iontophoresis transported amino acid esther prodrugs of acyclovir. The amino acids were Arginine, Glycine and Tryptophan. For transcorneal delivery, the duration was for 5 min, with 5mM, 0.5mA/cm^2. For transscleral delivery, 9mM, 1.25mA/cm^2. As comparatives, passive delivery of the drugs was done for 55min and compared to the results when ionotophoretic drug delivery was conducted. This method one done transclerally, resulted in drug delivery to the retina, choroid and vitreous humour. These methods have also been done in the past using dexamethasone (Chen & Kalia, 2018).

Dexamethasone-loaded hydrogel was used as a drug delivery device and it was done by iontophoresis. This study was done on rabbits and demonstrated the viability of using

iontophoresis. The current was set at 1mA and use of the current did not exceed 4min. This administration was done near the pars plana on the conjunctival membrane or directly on the sclera. Each administration at each area was done for 2min. The results revealed high dexamethasone concentration in both the cornea and in the sclera and retina. This analysis was done employing High Performance Liquid Chromatography (HPLC) (Lam et al., 1994).

Iontophoretic patches have been used transdermally for, amongst other things, pain management. Transdermal iontophoresis has been able to preferentially transport ions such as chloride and sodium ions and compounds such as mannitol (Burnette & Ongpipattanakul, 1987). Drugs such as indomethacin have been used to prove that iontophoretic drug delivery is possible and some cases also demonstrating in rats that an increase in current corresponds to an increase in drug delivery (Kanebako et al., 2002).

Another possibility with the use of iontophoresis is in fabricating a device that is able to deliver drug over the eyes at home. The iontophoretic technique needs special consideration because applying to a current oneself at home may need a home-friendly device.

Another recent study by Thakur et al. (2017) has studied the effect of nanobubble action on retinal cells lines. These nanobubbles were loaded with macromolecules which were IgG based. Nanobubbles composed of DPPC (1,2-dipalmitoyl-sn-glycero3-phosphocholine) and DSPE-PEG were produced and the gas perfluoropropane. These nanobubbles with macromolecules were tested on 3 cell lines and there was significant transport of the macromolecules on all cell lines. These cell lines ARPE-19 (human origin retinal pigment epithelium, MIO-M1 (Muller Glia of human origin) and 661W

(mouse origin photo receptor) were able to achieve uptake although at varying capacities. The retinal pigment epithelium cells and glial cells demonstrated the most uptake. These nanobubbles had a stable shelf life under optimal storage conditions.

Microbubbles have also been used in gene therapy (Li et al., 2009). Enhanced Green fluorescent Protein (EGFP) was able to be transported into human retinal cells *in vitro* and in the rat retina *in vivo*. The procedure involves exposing the cells to ultrasound prior to introducing the microbubbles, and once the microbubbles have been administered,

ultrasound is exposed in order to destruct the microbubbles and allow for release of the genes. The use of microbubbles has also shown minimal cell toxicity. Within the rat *in vivo*, transgene expression was safely achieved with the gene expression taking place in the retinal layer. In order to ensure gene expression, adeno-associated was used as a vector.

2.4.2. Sonophoresis: The use of sound for drug delivery

Sonophoresis is the use of sound to diagnose or therapeutically treat diseases. Sound is used in everyday medical situations in instances such a sonography. Sound can also be used to deliver drug to the eye (Beena, 2019). In the use of nanobubbles, the use of sound can be used to pop the nanobubbles and thus release the drug.

Sound can also be used, along with echogenic polymers to allow sonar to be used for real-time tracking of the nanobubbles. Ultrasound has been used in tumour suppression by oscillating, collapsing nanobubbles and then generating heat and shock waves to suppress tumour growth. These nanobubbles and the method was tested in rats and found to be a success (Suzuki et al., 2016).

Nanocups are also used in the fight against cancer with the use of ultrasound. Nanocups are single cavity nanobubbles that do not dissolve in the bloodstream. When in contact with ultrasound these nanocups are able to sustain cavitation activity four times longer than microbubbles in an *in vivo* tumour model (Kwan et al., 2015). Cavitation, ultrasound-induced activity. These nanocups are especially effective because they do not self-destruct in an ultrasound field and thus are able to deliver drugs for a prolonged amount of time. This nanocup is able to sustain its cavitation activity for several minutes.

The prolonged cavitation activation is able to ensure longer drug delivery and thus eliminating the need for multiple doses of the nanobubbles. These nanobubbles are composed of polystyrene, MMA and divinylbenzene. The nanobubbles are produced by trapping air as the nanoparticle template deforms into a cup during a drying out process (Kwan et al., 2015).

Ultrasound can also be used to direct the velocity of the nanobubbles and their trajectory (Yang et al., 2015). Doppler ultrasound beam allows this to happen. This has been done on polymeric nanobubbles with sizes ranges 200-800nm.

2.5. MICROTECHNOLOGY AND NANOTECHNOLOGY AS POSSIBLE DRUG DELIVERY SOLUTIONS TO THE POSTERIOR SEGMENT: A BROAD OVERVIEW OF MICROTECHNOLOGY AND NANOTECHNOLOGY

Microtechnology has shown promise in many industries as its popularity has increased with the advent of other technologies such as LASER technology. Microtechnology has allowed for the production of minute medical devices (Mohammadi, 2011). Techniques for creating micro devices include microcutting which allows for the production of objects more than $50\mu m$ laser machining which produces products above $3\mu m$.

Microinjection moulding which is able to produce products higher than $20\mu m$ MicroSL is able to make devices in the $1\mu m$ range and two photon polymerisation able to produce within the nanometre range with the use of these technologies (Mohammadi, 2011).

2.6. NANOPARTICULATE FACTORS FOR EFFECTIVE DRUG DELIVERY TO THE POSTERIOR SEGMENT OF THE EYE

Nanoparticles have been used in medical applications numerous times. Nanoparticles, produced by sonication of a mixture of buffer and relevant polymers produce nanoparticles with an aqueous core, named nanoliposomes. However, further steps in enhancing polymeric nanoliposomes have involved incorporating a gas into the core, producing nanobubbles. This characteristic which exhibits tracking of these particles is an important feature of theranostics. This has been especially effective with the use of ultrasound. Ultrasound is widely used because it is non-invasive and uses reflection and deflection of sound waves to produce an image. Nanobubbles with an incorporated contrast and echogenic agent are able to assist in viewing the nanobubbles. Gas-filled nanobubbles possess the dual ability to treat and allow for monitoring of the target region. The generation of nanobubbles through gas generating polymeric nanoparticles (Thakur et al., 2017) is an example of how highly applicable nanobubbles can be in theranostic treatment of diseases in the posterior eye.

The most technologically advanced manner to produce nanoparticles has been through a nanospray dryer (Maged et al., 2016). Drug-loaded nanosystems for ocular therapeutics have been produced using nanospray techniques and polymers such as methyl-β-cyclodextrin and hydroxypropyl-β-cyclodextrin, with these polymers being able to ensure effective drug delivery of econazole. The nanospray dryer has advanced

nanoparticle production and yield. Maged et al. (2016) explained this technique and why it is so advanced: The nano spray drying process is a new advanced technology to produce nanoparticles. The nano spray dryer attains a revolution in spray drying technology by producing formulations with high yield and particle size in nanosize with very narrow distributions. The nano spray drying technology depends on spraying the liquid formulations through a piezoelectric driven actuator that vibrates a stainless steel mesh (at ultrasonic frequency of 60 kHz). This creates tiny aerosol droplets in a smaller size range and with a very narrow distribution compared to that obtained from classical spray dryers. A high voltage is applied between two electrodes during the spray process to produce dried fine solid particles. Those particles are deposited on the inner wall of the electrostatic stainless steel collecting cylinder. At the end of the spray drying process, the dried fine powders are conveniently collected using a scraper.

Ultrasound microimaging is quite effective in monitoring the movement and targeting efficacy of these bubbles. Ultrasound imaging has advantages which include allowing observation of nanobubbles in real time through the use of nano-agents and probes (Wang et al., 2010). Nano-agents are compounds that enhance the ability to view nanobubbles. Thus, the use of these nano-agents (such as Tween®80) is essential in real-time tracking. Tween®80 enhances the ability to visualise the nanobubble because of its capability to enhance echogenicity.

Imaging and tracking of the nanobubbles is greatly affected by the contrasting agents that are used (i.e. the gas incorporated into the nanobubble). This is because such agents improve on their visualisation (Wang et al., 2010). The integrity of the gas core will also affect collision with other organelles and the use of contrasting agents will guide real-time viewing using ultrasound and ultrasonic waves (Cavalli et al., 2016). Furthermore, the type of gas used will affect nanobubble half-life and size due to the pressure and surface tension exerted on the nanobubble (Brenner & Lohse, 2008). Oxygen or gas delivery can be enhanced using factors such as frequency and sonication (Cavalli et al., 2016).

2.6.1. At the core of a nanosystem: Nanoliposomes and nanobubbles – comparisons and consequences for ocular nanoparticulate drug delivery

Nanoliposomes and nanobubbles have both been shown to be effective and to have a place in drug delivery technology. However, their fundamental differences may affect factors such as the drug of choice to be used, or the target organism selected. The biggest difference in nanoliposome and nanobubbles is that nanoliposomes have a liquid/aqueous core, while nanobubbles employ a gaseous core. There are gases used facilitate viewing of the nanobubbles using ultrasound waves. These gases include sulphur hexafluoride, perfluorocarbons and oxygen.

An important difference between nanobubbles and nanoliposomes is the way they react to ultrasound energy. Ultrasound waves directed at nanobubbles cause oscillation and echogenic properties of the core and eventual drug release and extravasation (Perera et al, 2015). Ultrasound waves also allow for the gas-filled nanobubbles to be viewed in real time.

Nanoliposomes, however, react differently to ultrasound waves. The drug release from nanoliposomes is caused by an increase in temperature due to the liposomes absorbing the ultrasound energy and through photothermal techniques (Zhihao et al, 2017).

Taking theranostics (the use of nano or micro particles in drug delivery and in real-time viewing) into consideration, it becomes clear that nanobubbles have a more desired trait in ocular drug delivery. The ability to oscillate and thus release the drug is more favoured than the ability to release the drug due to changes in temperature. A change in temperature is likely to adversely affect the desired intra-ocular pressure. Thus, one can conclude that the core will play a role in how nanoliposomes and nanobubbles react to ultrasound energy and thus affect drug release. In terms of nanoliposomes and nanobubbles, an important variable is the relationship the drug/protein/nucleic acid has with the drug delivery system. In liposomes the drug is usually entrapped in the aqueous core. The fact that the drug is entrapped also implies that the drug is protected from the outside elements of the human body until it reaches its target site. This implies there is a better likelihood of the drug being released at the target site if the drug is entrapped and the correct polymer combinations are employed. However, if the drug is entrapped in an aqueous core, it means that the drug must be stable in aqueous conditions or should not

be easily dissolved in water or else it will be too diluted once it reaches the target area. If the drug is in fact not stable in aqueous conditions, the formulation of the liposomes then becomes more complicated. This implies that a hydrophobic load/drug/protein may need to be injected or introduced into the core in order to stabilise the drug and to prevent adverse reaction with any hydrophilic components. This also means that the liposomal core needs to be hydrophobic. Another alternative is simply constructing nanoliposomes that are hydrophobic in nature, but all possessing a hydrophilic moiety. These are aptly named nanomicelles.

Nanobubbles, as a consequence of possessing gas core, have effects on the neighbouring environment and drug entrapped. Depending on the chemistry that can be anticipated between the drug and gas, entrapping the drug within may not be a desirable option. Conventionally, the drug is dissolved in the polymer/film and forms part of the bubble film. This poses a challenge because it means that any damage to the nanobubble indicates that the drug will be easily released inappropriately. In nanobubbles, the drug is placed as part of the film, which then causes challenges in gene therapy where the drug of choice may be a protein. Either this protein may interact with the bubble film or will be inappropriately released to the surrounding area if it comes into contact with its substrate elsewhere in the body. This then necessitates a more complex nanobubble film structure when incorporating proteins or nucleic acid loads in drug delivery. The nanobubble may therefore have to be multilayered, with the innermost layer composing of the protein or nucleic acid derivative or gene. If the protein is to be dissolved in a polymer, the properties need to allow for stable protein dissolution and prevent protein denaturation. However, single-layered nanoparticle protein carriers have been used and conducted efficient delivery.

Haidar (2016) has also been able to prove that liposomes are able to carry proteins. These liposomes were composed of a phospholipids film and cholesterol with a spherical morphology and the liposomes were able to deliver osteogenic protein-1(OP-1) via injection and cause no obvious infection or inflammation. An important factor in ensuring a less likely chance of leakage (of the load) was the use of layer-by-layer system. Specifically, alginate derivatives were used and the cationic chitosan was used in the layering. This opposition in charge ensures attractive forces and the diffusion of the polymers into one another as alginate has shorter chains. Du Toit et al. (2010) were able

to corroborate this information, but using nanobubbles filled with the gas poly (butyl-2-cyanoacrylate). Furthermore, these nanobubbles were viewed using ultrasound techniques (see Figure 2.2).

An important aspect of nanobubble and liposomes is their ability to be viewed using ultrasound waves. This makes them useful in theranostic techniques as they assist with diagnosis through viewing and assist by acting as drug vectors. The gas incorporated in these nanobubbles has to be able to facilitate the viewing. The gases mostly used in these instances are usually perfluorocarbons, oxygen and sulphur hexafluoride. In liposomes, the core is aqueous and thus the core is not filled with an echogenic agent, as it is done with the nanobubbles. This can be remedied by using more echogenic agents in the liposome film. However, as mentioned before, liposomes, due to their core, react to ultrasound energy by releasing thermal energy. Thus, liposome usage should be restricted to parts of the body which will not be affected adversely by changes in temperature.

An imperative aspect in nanobubble, liposome viewing and thermodynamic energy is gas leakage. The factor that will affect gas leakage is the molecular weight of the gas and the molecular weight of the bubble film. Another factor is the intramolecular and intermolecular forces of the bubble film. Gas leakage is an important factor to consider because the gas released will change the energy dynamics of the bubble and thus affect kinetic energy and may cause toxicity. Another consideration with regards to the eye, is the intraocular pressure and the change in temperature which may result due to the leakage.

Gas selections are an important feature in nanobubbles as the gas released to neighbouring cells may in fact be toxic to the cells, once the nanobubble bursts. Hwang et al. (2011) showed that perfluorocarbons may have cytotoxic cells on neutrophils. The perfluorocarbons are also able to inhibit the production of reactive oxygen species. Cytotoxicity (caused by perfluorocarbons) is triggered by damaging membrane integrity of cells and causes the leakage of enzymes such as lactate dehydrogenase.

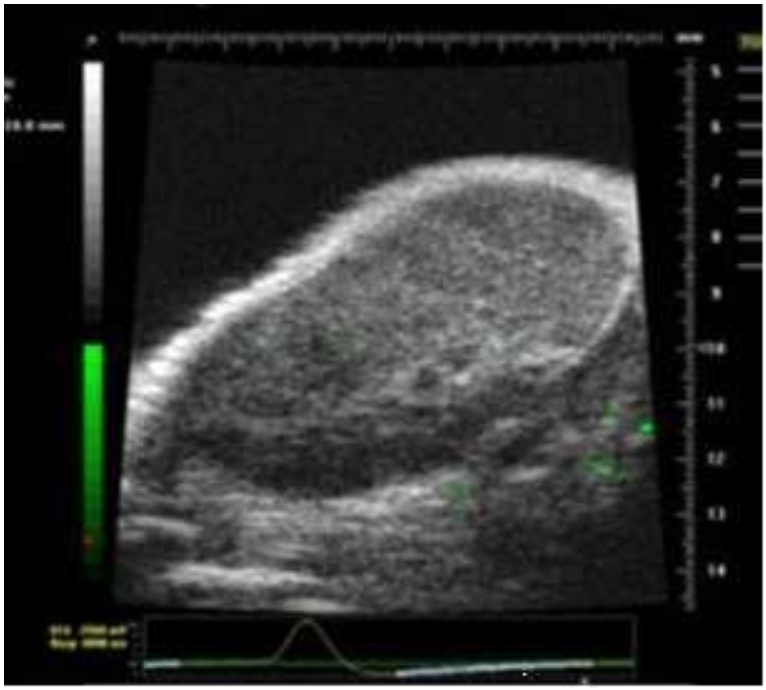

Figure 2.5: Ultrasound image obtained employing the Vevo® microimaging system of nanobubbles (Du Toit et al., 2010). This diagram illustrates earlier work in viewing nanobubbles using ultrasound techniques.

2.6.2. Lipid and phospholipid nanobubbles and viability for ocular drug delivery

Lipid nanobubbles are the simplest of all the nanobubbles. They employ lipid compounds as the film of the nanobubble and introduce a gas as the core. Lipobubbles have been widely used for cancer treatment (Sanlier et al., 2018). Often these nanobubbles have theranostic properties enabling diagnosis and therapeutic treatment dually (Baghbani et al., 2016). They can be referred to as lipobubbles. Wang et al. (2010) were responsible for some of the earlier illustrations of the use of lipobubbles in cancer theranostics. They were also able to demonstrate a simple way of making nanobubbles carrying Coumarin - 6. These nanobubbles make use of soybean lipid and use cholesterol as the film. The core consists of sulphur hexafluoride, a known contrasting agent. These nanobubbles use no other compound to increase the structural integrity, yet these nanobubbles are able to effectively affect drug delivery. Another aspect of these nanobubbles is that they are formulated to be viewed using ultrasound energy. An added advantage is the fact that exposing the nanobubble formulation to ultrasonic emission for a mere 5 minutes can ensure that these nanobubbles can be viewed Thus nanobubbles can still perform two functions for theranostic analysis.

The advantage with these nanobubbles is that they are simple to produce, the process is not time consuming and the lipid polymers are stable enough to effect drug delivery. The process of their production can also be upscaled. An added advantage is the accessibility of the materials used to make the nanoparticles. For example, cholesterol and soybean lipid are simple to obtain and are commonly used in nanobubble formulation. Furthermore, the film, namely cholesterol, is ubiquitous in the body and thus unlikely to be immunogenic. Therefore, the breakdown of any cholesterol nanoparticles will be catalysed and controlled by normal body machinery. The corresponding by-products will not be foreign to the body's machinery and will thus be treated normally. The breakdown will ensure that the drug will reach the desired area and be released in a safe manner.

However, there are disadvantages of using such a simple structure; the single structured composition may cause ineffective gas entrapment and cause gas leakage to neighbouring cells. Furthermore, studies have shown that the larger the size of the nanobubble, the larger the influence of ultrasonic energy will be. Therefore, nanobubble size will have an effect on the resolution of the images. These nanobubbles are able to mimic the phospholipids bilayer of a cell membrane. Nanobubbles make use of Distearoylphosphatidyl choline (DSPC)(Figure 2.6) and distearoylphosphatidyl-ethanolaminepoly (ethylene glycol) (DSPE-compounds)(fig 2.7). DSPE was also used by Thakur et al. (2016) as theirs shells. Generally, the gas used as the core is either oxygen, sulphur hexafluoride (Wang et al., 2010), perfluorocarbons in all nanobubbles. DSPC is a phosphatidylcholine and uses choline in its head group. The use of this compound is favoured because it can be found in substances such as soybeans or egg yolk. This compound can be found on cell membranes and is fairly ubiquitous in nature. The advantage of using nanobubbles that mimic the components of cells is that they are unlikely to be immunogenic. They are also found easily in nature. Other lipids include 1, 2-Dioleoyl-n-Glycero-3-phosphocholine (DOPC) 1, 2-dioleoyltrimethylammonium-propane (DOTAP) lipids (Anderson et al., 2010).

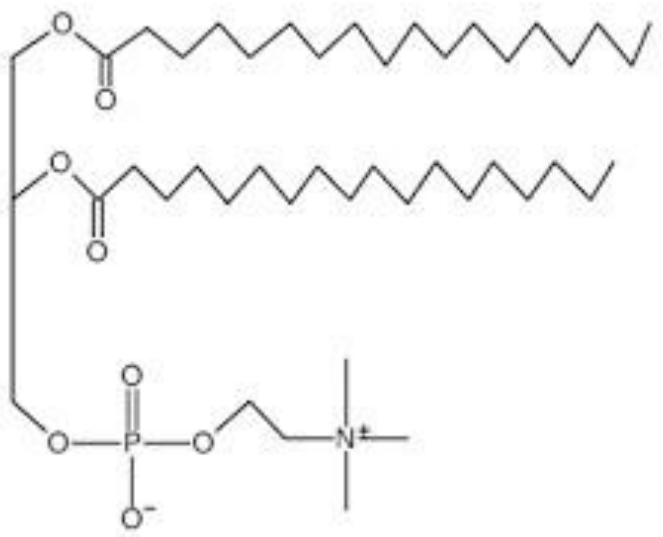

Figure 2.6: Chemical structure of distearoyl phosphatidylcholine. This polymer is widely used in ocular nanoparticulate drug delivery studies

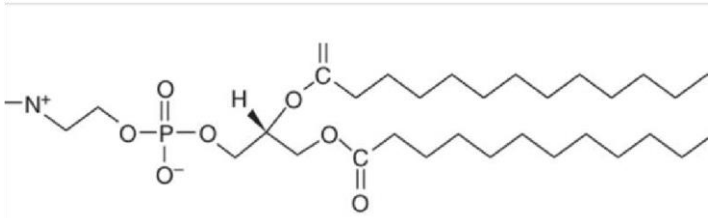

Figure 2.7: Chemical structure of distearoylphosphatidyl-ethanolaminepoly (ethylene glycol). This polymer is widely used in ocular nanoparticulate drug delivery studies

2.6.3. Polymeric nanobubbles and nanostructures

Polymeric-film nanobubbles are usually made from polymers that are bioerodible or biodegradable. These polymers may include alginate derivatives, chitosan, polyethylene glycol and polycaprolactone. Starch derivatives may be used as nanobubble shells because of their easy degradation using ubiquitous enzymes such as amylases. With regards to drug delivery to the posterior segment of the eye, erosion or degradation of the polymer, before passing the barriers could cause inappropriate drug release, and may cause adverse responses to the drug.

A component to consider in choosing from a wide variety of polymers is their charge. Their charges may affect factors such as their ability to adhere to blood vessels and how

they will affect gaseous exchange in lungs. Kamaly et al. (2016) pointed out that charge affects nanoparticulate movement and efficacy and that block polymers such as Poly (lactic-co-glycolic acid) (PLGA) and polyethylene glycol (PEG) are effective in terms of mitigating the effect of charge and managing hydrophilicity. It has been shown by Amrite et al, (2005) that nanoparticles administered subconjunctivally are able to remain at the site of delivery for two months. Amrite et al. (2005), successfully demonstrated that the size of the particles being administered affects their ability to be retained in the posterior eye due to hydrophilic and hydrophobic forces. These particles, composed of polystyrene, produced in varying sizes revealed that particles in the micro range and 200nm showed increased retention in periocular tissues and could be detected 60 days post administration in rats. Amrite et al. (2005) further found that particles as small as 20nm were unable to permeate the sclera, choroid and retinal pigment epithelium.

Some of the most well-liked laboratory-produced polymers include chitosan and polycoglycolide. Chitosan and the synthetic polymer Poly (lactic-co-glycolic acid) (PLGA) are widely used in nanobubble production. Chitosan and (PLGA) are FDA-approved polymers and have been used in nanoparticulate drug delivery systems. Chitosan has been shown to offset and prevent aggregation in poly-co-glycolide nanoparticles (De Campos et al., 2008). Chitosan can also be used to produce other nanostructures, such as nanomicelles. Chitosan-grafted methoxy poly (ethylene glycol)-poly (-caprolactone) nanomicelles have been produced for ocular drug delivery. This nanosuspension ensured increased retention in corneal tissue. This advancement is relevant in posterior drug delivery as the most effective manner for drug delivery is achieving tissue retention (Shi et al., 2015) Nanomicelles are especially effective in the body as they assist with hydrophilic shielding. That means that if a drug is hydrophobic, and is enclosed in a nanomicelle, it can navigate the hydrophilic environment especially in the blood stream, largely unimpeded, until it reaches its target site. However, the hydrophilic moiety has to be shed in order to release the drug.

Chitosan is a widely used polymer in nanotechnology in terms of drug delivery. Its popularity stems from its stability and its mucoadhesive properties. This positively charged polymer is complex and composed of glucosamine (a fairly ubiquitous starch monomer in the body) derivatives. Chitosan is found in nature and more commonly known to be a derivative of chitin (chitosan is produced by the n-deacetylation of chitin),

which serves as exoskeleton to organisms such as grasshoppers and shellfish (Ali et al., 2018). Thus, this polymer is fairly stable, especially in aqueous conditions and can easily be degraded enzymatically by enzymes found in the human body. Chitosan has also been an attractive prospect for ocular drug delivery as it has been proven to be mucoadhesive and could effectively assist with increased bioavailability of the nanoparticles in the eye. Its positive charge has been especially enticing for ocular drug delivery and the cornea and conjunctiva have been found to be negatively charged. This mucoadhesive property allows for the increased residence time for the relevant drug delivery system. Chitosan may also have some additional key benefits to the human body besides in drug delivery technology. Chitosan has been used in a nanoparticulate system consisting of a layer-by-layer system because it is charged and able to be linked to other polymers. It is one of the most commonly used polymers in nanoparticulate systems and can be used in combination with other polymers such as alginic acid because of its stable nature and pH-dependent solubility (Fonseca-Santos et al., 2017).

Chitosan has also proved to be most effective in ocular drug delivery (Ali et al., 2018). Chitosan nanoparticles have been shown to effectively penetrate corneal and conjunctival epithelium. Aggregation is a concern for nanoparticles as they move through bodily tissues. De Campos et al. (2008) observed that chitosan was able to counteract aggregation in poly-epsilon-caprolactone.

Synthetic polymers are used in nanoparticulate systems because naturally found polymers are only available in varying purities (Hans & Lowman, 2002). Natural polymers are also challenging to crosslink because they affect the integrity of the drugs used.

One of the most commonly used synthetic polymers, in producing drug delivery nanoparticulate systems, is polymer (PLGA). It makes use of biocompatible polymers composed of lactic acid and glycolic acid. These polymers are useful because they are biocompatible and can be reabsorbed in natural pathways.

The attraction to this polymer is manipulation of the release rate of drugs, and thus the degradation of polymers. This is accomplished by varying the ratio of glycolic acid units/monomers to lactic acid units/monomers (Hans & Lowman, 2002). PLGA can be polymerized by crystallisation using ethyl acetate (Avgoustakis et al., 2002). This

polymer is stable and has been used to encapsulate doxorubicin and cisplatin nanoparticulate drug delivery systems. These copolymer units are widely used for their ability to release drug for longer periods of time (Dorta et al., 1993). Early on it was discovered that different ratios of lactic acid and glycolic acid had significantly different effects in substances such as lauryl alcohol and stannous octoate (Dorta et al., 1993). PLGA has also been used in nanoparticles with other stable polymers such as PEG (Kamaly et al., 2016).

Synthetically-assembled polymers have shown the significance in producing nanoparticles composed of various polymers and differing methods of assembling the polymers (Hui et al., 2018). PLGA has also been used along with carbomer hydrogels for sustained drug release. This is important in ensuring higher bioavailability and sustained drug release (Abrego et al., 2015). The apparent advantage of using PLGA in ocular drug delivery is the ability to control and manipulate drug release. The multilayered and amphiphilic properties of the eye mean a long journey for nanoparticles to the posterior segment of the eye. The use of PLGA can assist by ensuring that the drug is released at a time-appropriate or pH- appropriate environment.

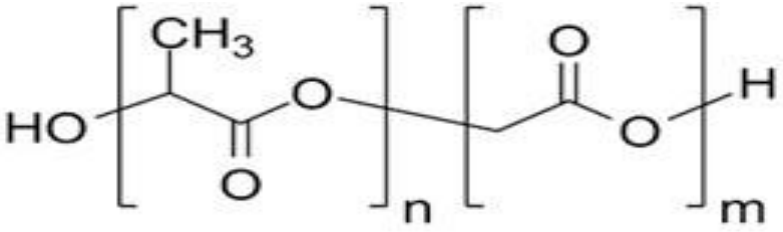

Figure 2.8: Chemical structure of poly (lactic-co-glycolic acid) (PLGA). This polymer is widely used in nanoparticle creation, however can also be used in creating implants in ocular drug deliverty and other areas of drug delivery.

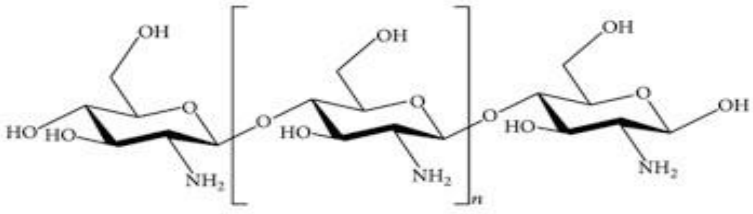

Figure 2.9: Chemical structure of chitosan. This polymer is FDA approved and widely used to create nanosystems for drug delivery

2.6.4. Layer-by-layer nanobubble systems

Layer-by-layer systems are important in ocular drug delivery due to the multiple barriers and the need to protect the drug prior to reaching the intended site of delivery. Layer-by-layer systems are so successful that they have managed to carry sensitive molecules such as DNA and RNA, thereby affecting gene translation (Tan et al., 2018). Layer-by-layer nanobubbles use a combination of the phospholipid/lipid shell and then allow the deposition of polymers (such as alginic acid chitosan) on this film of phospholipid. This technique, which has been demonstrated by du Toit et al. (2010), lists a number of consequences. These consequences include changes in zeta size and zeta potential. One of the changes that may be expected in layer-by-layer systems is that the deposits will cause the zeta size to grow to a large extent. This may not be the case. According to Du Toit et al. (2010), some polymers may diffuse into one another. Charge may play a role in such cases as earlier ascertained. An example is in the case of chitosan which is cationic and alginic acid which is anionic. These two polymers diffuse into one another in a layer-by-layer system. This is due to the attractive forces that exist between them and also due to the variable lengths of the linking chains in each polymer (Du Toit et al., 2010).

The layer-by-layer system aims to ensure that the drug is released at the appropriate time by protecting the drug employing encapsulation technique within the layers. The use of the layer-by-layer system will allow one to dissolve the drug of choice in the innermost layer of the nanobubble, in terms of the emulsion-evaporation method. This indicates that as the drug moves along the bloodstream and collides with tissue, the bubble film is not easily eroded and thus the drug will be intact and will be released at the appropriate time. The layer-by-layer system ensures that the bubble film is eroded when the specificity markers (such as antibodies) are activated. Specificity of the markers is an important aspect in drug delivery in order to avoid cell toxicity and to ensure that the relevant dose is delivered to the desired site. The layer-by-layer system is a system of reinforcement and because of its high stability it is able to allow a wide variety of materials as the loads. These may include growth factors (Haidar et al., 2008), oligonucleotides (Gazori et al., 2009) and drugs.

Some of the polymers used in the layer-by-layer system effectively adsorb to the DSPE and DSPC and may cause thermodynamic instability. The reasons for this are provided by

Sihorkar and Vyas (2001) who suggested that the polysaccharides dislodge easily from the bubble film. Another possibility may be the coagulation of the film (made of DSPE and DSPC) that may cause it to destabilise. The third possibility given by Sihorkar and Vyas (2001) may be that the stoichiometic ligand density is not able to be reproduced.

An important factor in the bubble lifetime with respect to the layer-by-layer technique is that a less negative zeta potential corresponds to a longer bubble lifetime (Du Toit et al., 2010). Du Toit et al. also showed that the self-deposition of charged polymers makes for stronger bubble lifetime. The more anionic the nanobubbles are, the longer their lifetime. Furthermore, these nanobubbles are specially formulated to be multilayered affected gas leakage. The leakage of gas is an important aspect of the viewing and of the rupture of the nanobubble by ultrasound waves. In the event of gas leakage, the gas (which assists in terms of echogenicity) might be toxic to neighbouring cells or lower the resolution with which these nanobubbles could be viewed.

Haidar et al. (2008) have mastered the layer-by-layer method for the purposes of transporting growth factors. Their layer-by-layer method however involved liposomes, and not nanobubbles. The fundamental difference with the nanobubble would be the core. In the Haidar layer-by-layer technique, alginate derivatives and chitosan were used. Thus, their findings can be related to nanobubble production, taking into account that the drug is entrapped and will not form part of the bubble film as it is done in non-layer-by-layer systems.

Understanding the behaviour of polymers when combined is an important component in producing a layer-by-layer drug delivery system. This will affect drug entrapment and drug release efficiency. Haidar and Du Toit, although working on liposomes and nanobubbles respectively, were able to corroborate work by Douglas & Tabrizian (2005) who suggested that in layer-by-layer techniques using chitosan and alginate derivatives, diffusion processes do take place. This study also proved that the shorter alginate chains were able to diffuse into the longer chitosan chains. This accounts for the less than significant change in particle size when these two polymers are used in nanotechnology. The layer-by-layer system might not be effective just for drugs. Haidar (2010) found that the aqueous core can also be useful for gene and protein delivery.

The fact that liposomes and nanobubbles are possibly useful in gene delivery shows that they are stable and firm and still able to protect molecules as fragile as DNA. Karimi et al. (2016) found that nanobubbles are effective in gene delivery. Gene delivery can be an important aspect of cancer therapy because genes can assist in gene silencing or alternatively be able to affect the replication or transcription process.

Layer-by-layer drug delivery bears a special significance in ocular drug delivery as the layering can serve as reinforcement for drug entrapment. The appropriate layer-by-layer systems, using polymers with complementary charge and complementary length of chemical chains, would affect size and allow for a shrinking in size in order to allow for the harsh environment of the eye to be surpassed by these drug delivery systems.

2.6.5. Nanospheres as nanocarriers of therapeutic agents of ocular treatment

Nanospheres have been used in drug delivery to treat diseases such as motor neuron disease and cancer. However, nanospheres are gaining popularity for use in ocular drug delivery (Kang-Mieler et al., 2017). Nanospheres make use of a homogeniser to produce these spheres with an optimal maximum size of 300nm.The use of a homogeniser ensures the mixture of emulsification of compounds that would not naturally mix.

There are a number of methods involved in producing nanospheres. Some recent successes in nanosphere production for ocular drug delivery was by Silva-Abreu et al. (2018), who produced non-irritant PLGA-PEG nanospheres able to be retained in the eye for 14hours. One of the more commonly noted methods is the double emulsion solvent evaporation method. The use of polyelectrolyte complexes is not uncommon practice. The polyelectrolytes used would usually have opposite charges and be allowed to aggregate to form the nanoparticles. A number of factors play a role in the final size and physicochemical properties of the nanospheres. The pH, varying molecular weights, mixing order and mixing ratio determine the final size of the nanospheres (Buchhammer et al., 2003). The ability to manipulate these variables enables one to produce a drug delivery system that is either able to bring about sustained release, or to be pH sensitive or thermosensitive. Polyelectrolyte complex nanoparticles are produced using 2 methods. Primary electrolyte complexes are bound by the electrostatic forces formed due to the varying charge and the secondary stage forms through dispersive interactions. The twostep process is also able to produce hydrophobic nanoparticles, which would allow

for the stability of the nanoparticles under hydrophilic conditions and allow for protection of the drug prior to appropriate release. An example of nanosphere production and the 2-phase step is the use of an organic solvent and primary polymers. Water is added and the mixture is homogenised to form a primary emulsion. This primary emulsion is added dropwise to an external aqueous phase (comprised of appropriate buffer and desired polymer) and emulsification and nanoparticle production takes place through the use of a sonicator. The resultant mixture is centrifuged, washed and lyophilised.

2.6.6. Nanomicelles: Amphipathic structures nano-enabled drug delivery

Nanomicelles have particular advantage in drug delivery as they have dual properties. In producing nanomicelles, molecules with both a polar and non-polar end are selected. Nanomicelles are especially relevant in the drug delivery of hydrophobic drugs. These amphipathic moleculaes may be more effective therapeutically, due to their high bioavailability (Li et al., 2015). A high bioavailability ensures better compatibility for the nanomicelles within the body. Dexamethasone-loaded nanomicelles have also been used in ocular drug delivery (Patel et al., 2015), (see Table 2.1) Thus the nanomicelles are less likely to elicit the immune system and able to deliver the drug to the desired site. Chitosan-grafted methoxy poly (ethylene glycol)-poly (-caprolactone) nanomicelles have been produced for ocular drug delivery. This nanosuspension ensures increased retention in corneal tissue. This advancement is relevant in posterior drug delivery as the most effective manner for drug delivery is achieving tissue retention (Shi et al., 2015). Nanomicelles are especially effective in the body as they assist with hydrophilic shielding. That means that if a drug is hydrophobic, and is enclosed in a nanomicelle, it can navigate the hydrophilic environment especially in the blood stream largely unimpeded, until it reaches its target site. However, the hydrophilic moiety has to be shed in order to release the drug.

2.6.7. Nanoparticles as possible drug delivery vectors for uveitis treatment

Nanoparticles have gained popularity in addressing inflammation and diseases in the posterior segment of the eye. This is due to their size and ability to possibly pass their barriers such as tight junctions (Ruff et al., 2017). However, the use of nanoparticles has

complications. The varying polarity and hydrophobicity may affect movement and may cause aggregation.

In order to address this challenge, modifying the polymeric surface of the drug delivery system has been proposed. Modifying chitosan with the hydrophobic molecule cholesterol has been done by Yuan et al. (2006). These particles were unfortunately unable to reach the posterior segment of the eye. These particles were able to sustainably release cyclosporine. The diameter of the nanoparticles and the critical aggregation concentration decreases with the increasing addition of hydrophobic groups.

Rapamycin-Nanaoparticles were however successful in suppressing the immune system in corneal transplant (Yuan et al., 2008). Nanoparticles have been used in the advancement of the intra-ocular lens. 5-fluoroucil chitosan nanoparticles were produced and introduced into intra-ocular lenses in order to stop the proliferation of human lens epithelial cells (HLECs). This was done in order to prevent posterior capsule opacification.

Modifying nanoparticles to have certain responses to external stimuli can affect drug delivery and ensure that drug delivery is more timeous and targeted – this was achieved by Movahedi et al. (2015).

Nanoparticles mediated with ultrasound have also been used to improve drug delivery to the retina. The nanoparticles were administered intravitreally with the aim to improve the mobility of the nanoparticles in the vitreous and thus ensure drug delivery. This study was done on bovine eye cups. The nanoparticles composed of human serum albumin and coated with hyaluronic acid had improved permeation and diffusibility through the vitreous and retinal pigment epithelium and choroid without damaging the ocular tissues. The eyes were exposed to 1MHz of ultrasound with an intensity of $0.5 W/(cm^2)$ for a duration of 30 seconds.

Table 2.2: Current innovations under investigation for ocular drug delivery (posterior segment disorders)

Administration	Delivery System	Drug	Treatment	Reference
Topical	Nanoparticles			Shi et al., 2015
	Nanospheres	Flurbiprofen	Anterior Inflammation	
	Nanomicelles	Diclofenac		
Implanted	Vitrasert	Ganciclovir	CMV-retinitis	Dhillon et al., 1998
	Retisert	Flucinoloneacetonide		Jaffe et al., 2006
	Donut-shaped Minitablet			Choonara et al., 2009
	Ozurdex	Dexamethasone	Uveitis, macular edema, retinal vein occlusion	
Oral		Kinase inhibitor	retinal neovascularization	Seo et al., 1999
Trans-Tenon Contact Lens	Hydrogel contact lenses	Lidocaine, Timolol Maleate	Glaucoma	Gulsen & Chuhan. 2004, Hiratan & Alvarez-Lorenzo, 2004
Topical	Mini-tabletting	Timolol Maleate	Glaucoma	Chetoni et al., 2009

2.7. NANOBUBBLES AND MICROBUBBLES: A COMPARISON AND VIABILITY AS DRUG DELIVERY VECTORS FOR UVEITIS TREATMENT

Microbubbles and nanobubbles have shown strength in their ability as drug delivery vectors. Both have been used in cancer diagnostics through ultrasound viewing (Jain et al., 2018).

The attraction to microtechnology and nanotechnology is for similar reasons: the size of the devices allows them to pass though certain barriers, or to be implanted in the body without impeding important processes such as blood flow. It is also important to note that the FDA has approved a procedure using microbubbles in echocardiographic evaluation. These bubbles are to be less than 10 micrometres in diameter in order to pass

through the gaseous exchanging capillaries of the lungs. Microbubbles and nanobubbles have shown strength in their ability as drug delivery vectors. Both have been used in cancer diagnostics through ultrasound viewing (Gao et al., 2008) and in combatting neurological diseases (Fan et al., 2017). Both show stability depending on the substance used to form the bubble film. However, the size of the particles, micro or nano, determines whether they cause irritation in the eye or not (Bravo-Osuna et al., 2016) (see Table 2.3).

Furthermore, in microtechnology and nanotechnology, the devices can be modified to achieve high specificity interactions or to allow for real-time tracking and tissue examination. In order to aid theranostic processes in microparticle and nanoparticle polymer production, gas has often been introduced into the core to aid the process. These particles are now aptly named nanobubbles and microbubbles.

Compelling work in microbubbles by Kowalczuk et al. (2011) has shown the ability to transfer genes in an ultrasound-assisted process. These microbubbles were injected in the ciliary muscle in the eye and proved a strong possibility for the use of microbubbles in the treatment of retinal diseases as the intended gene was found in ocular fluids. There was no observable rise in temperature or microscopic damage to the ocular tissues. The studies measured gene levels after 7 days and after 30 days. There was a highly diminished volume of gene level after 30 days. Sonoporation in concert with microbubbles and nanobubbles could assist theranostically or facilitate transient permeability of cells due to the echogenic effect of the polymers used in the formulation (Wamel et al., 2006). Ultrasound targeted microbubble destruction-mediated gene delivery using the green fluorescent protein (GFP) has also corroborated the use of microbubbles to increase permeability of ocular cells to genes and possibly drugs. This ultrasound assisted process is necessary as many drugs are unable to pass through endothelial layers in many tissues. The use of GFP has also been used to show that the transient permeability can cause leakage (Kaddur et al., 2010). This implies that further investigation needs to be conducted to determine the intensity of the sound waves in order to prevent cell damage.

Acoustic properties such as echogenicity, the ability to echo or reflect sound waves is an important aspect in imaging and is strongly related to oscillation of nanobubbles and microbubbles. Echogenicity must be enhanced by the lipid/polymer formulation and

echogenic agents used as part of the formulation, such as Tween®80. The bubbles' prerequisite for effective drug release is to be sizeable enough as a drug vector and small enough to pass through the transient pores in the barriers that need to be surpassed (Suzuki et al., 2011). Oscillation, caused by a sound wave greatly affects drug delivery (Guvener et al., 2017) and will need to be enhanced, as it affects membrane permeability. Consequently, the polymer chosen to coat the nanobubble must facilitate effective oscillation. Echogenicity, the ability to echo or reflect sound waves is an important aspect in the imaging and is strongly related to oscillation of nanobubbles. Echogenicity has to be enhanced by the lipid formulation and echogenic agents used as part of the formulation. Oscillation, caused by a sound wave greatly affects drug delivery (Ferrara et al., 2008) and will need to be enhanced, as it affects membrane permeability. Consequently, the polymer chosen to coat the nanobubble must facilitate effective oscillation.

Microbubbles have in the past been preferable because studies had shown that the larger the echogenic object, the more influence the ultrasound will have on the drug delivery vector (Ferrera et al., 2009). Although this is very true, nanobubbles have a distinct advantage because of their size. Instances where nanobubbles are required to navigate through dense tissue before bursting to a smaller size should prove to be advantageous. Using specialised contrast agents can assist in the viewing process in theranostic processes (Perera et al., 2015).

In nanobubble and microbubble production, the most common method used in producing these drug delivery vectors is by using the emulsion evaporation method. This is either with phospholipids, polymers, lipids or combinations. They are then sonicated or homogenised to produce the nanostructures or microstructures. The transition from microbubble to nanobubble is relatively easy. To decrease the size of microbubbles one can freeze and thaw the microbubble multiple times in order for them to be in the nanometre range. Most microbubbles and nanobubbles are generally made in the same manner. Thus, size can be manipulated taking into account, a number of factors.

Freezing and thawing was done by Du Toit et al. (2010). Furthermore, the method used to produce the microbubbles and nanobubbles can affect size. The more common ways to produce these structures is by using a homogeniser or sonicator. The time of sonication

and homogenisation has been proven to account for the size of the micro or nano structures. The number of revolutions per second would affect size when using a homogeniser to agitate and mix the aqueous phase and organic phase to produce the micro or nanoparticles. Another factor is the intensity of the sound waves that the sonicator releases. The rotar-evaporator, which makes use of heat and vaporisation pressure may also play a role in the sizing dynamics of microparticles and nanoparticles. The use of the organic solvent in the emulsion-evaporation process may also affect size.

Microbubbles and nanobubbles have been used in cancer therapies and for viewing in real time, any changes in the tumour. Huang et al. (2010) have demonstrated how heat-sensitive microbubbles that are biodegradable have been effective in drug delivery. These nanobubbles were able to be transported to the target site either by injection or systemic administration.

Microbubbles and nanobubbles have presented opportunities in drug delivery to the posterior segment of the eye with microbubbles also possessing the ability to improve permeability of certain cells through sonoporation (Sennoga et al., 2017) and ultrasound assisted destruction (Liu et al., 2017).

However, in the quest to ensure auto passage through barriers without aid, nanotechnology may be of best use in drug delivery to the posterior segment of the eye. Furthermore, there have been no conclusive studies demonstrating topical use of microparticles to the posterior segment of the eye. In the attempt to produce a topical formulation, nanosized bubbles may be a more apt use in this case.

Table 2.3: Differences between nanoparticles and microparticles with regard to ocular drug delivery

Aspect	Microparticles	Nanoparticles
Size	Larger that 10μm causes ocular irritation (Bravo-Osuna et al., 2016)	Less associated with irritation in ocular tissues
Drug	Enhanced drug delivery of tetracaine as comparted with ophthalmic solution	Charge a factor adhesion to ocular tissue (Chonker et al., 2015)
Delivery efficacy	Charge less of a factor in mucoadhesivity and Bioavailability	

2.8. NANOTECHNOLOGY AS AN EFFECTIVE TOOL FOR TOPICAL ADMINISTRATION TO THE POSTERIOR SEGMENT OF THE EYE: CONSIDERATIONS AND POSSIBLE ROUTES

Nanotechnology has distinct advantages in controlled drug release because of the enlarged surface area afforded through the drug encapsulation by minute nanostructures and obvious advantages with regard to the surface area and volume. An enlarged surface area corresponds to an increased likelihood for therapeutic dose. Nanotechnology also has advantages in improving solubility and bioavailability in microemulsions (Lidich et al., 2019).

The use of polymeric nanoparticles may enhance current technologies in sustained or environmentally-controlled drug release. The use of relevant polymers may retard unwanted burst release in certain medications, decreasing the need for increased re-administration of the medication. Burst release contributes to the inappropriate dosage of the drug released and there have been advances to impede burst release to ensure appropriate and sustained drug release using PLGA nanoparticles (Pakulska et al., 2016). Burst release may contribute to cell cytotoxicity as it may release concentrations above the therapeutic dose, or release to neighbouring tissue.

The key challenge in drug delivery to the posterior segment of the eye is the complex barrier that needs to be surpassed by the drugs. The current gold standard in therapeutic treatment, intravitreally implanted medications is associated with more complications. The most patient-friendly mode of administration is a topical formulation that can reach the posterior segment of the eye. This innovative formulation would allow for treatment at home, significantly diminishing loads at healthcare facilities and due to the ease, increase compliance.

Key essentialities in such a formulation are that it is not cytotoxic and does not elicit the immune system as it passes through the numerous barriers. The formulation needs adequate ability to contain the drug as it passes through the barriers and only releases the drug appropriately. This is necessary so that the appropriate dose is able to reach the affected area. The Fickian kinetics needs to be examined in order to avoid widespread cytotoxicity due to drug leakage.

The route of the movement of a nanoparticulate drug delivery system needs to be considered as it may affect timing of the drug release. Depending on the route, there may be instances of mechanical stress and forces exerted and this may affect the properties of the polymer in use. In terms of administration in the cul-de-sac of the eye, movement around the eyeball followed by movement through the sclera is the likely movement. In a topical formulation, research has shown that the cornea has varying permeability to drugs. Drugs are impermeable to the lens, thus they may move through the ciliary body and into the vitreous humour as there is no path past the lens.

2.9. DRUG TREATMENT IN THE EYE

2.9.1. Indomethacin as a therapeutic possibility in treating uveitis

Indomethacin has been known to lower blood pressure as a nonsSteroidal anti inflammatory drug (NSAID). Indomethacin is effective in treating inflammation and due to its ability to lower blood pressure could not adversely affect intra ocular pressure. Some of the other symptoms which are rare are blurred vision. However, Indomethacin has been used in concert with other drugs in the treatment of high blood pressure (Conlin et al., 2000). Although best used with solubilisation enhancers, indomethacin has been used in the treatment of ocular disease (Mandal et al., 2017). Systemic high blood pressure has been proven to be directly correlated to intraocular pressure (Klein et al., 2005). A drug able to have the dual function of stabilising blood pressure and intraocular could be ideal for treating uveitis as intraocular pressure can be managed, while inflammation is also being treated. Thus indomethacin may be an ideal drug in terms of its efficacy in the eye.

2.9.2. Fluocinolone acetonide as a possible drug of choice for treating uveitis

Fluocinolone acetonide has been used to treat uveitis in many instances. Retisertfi is an implant to treat non-infectious posterior uveitis (Leinonen et al, 2018). Fluoconolone acetonide is a corticosteroid with a synthetic hydrocortisone derivative. Fluocinolone acetonide has anti-inflammatory properties through its ability to prevent synthesis of prostaglandins and leukotrienes. Prostaglandins assist to maintain homeostasis and are mediate pathogenic mechanisms. Prostaglandins are generated from arachidonate by the action of cyclooxygenase isoenzymes (Ricciotti & Fitizgerald, 2011).

2.10. CONCLUDING REMARKS

Treating diseases and inflammation in the posterior segment of the eye is a challenging feat. The current approaches to treating these diseases and inflammation is treatment that is invasive and surgical in nature. Unfortunately, these interventions result in further damage to the eye in the long term. Implants that may treat the diseases are able to control dose and controlled release but are associated with the surgical complications and may migrate and cause damage to the eye. Intraocular injections cause damage to the eye and their administration needs to be repeated. The most optimal type of treatment would be to be able to surpass the barriers in the eye and to treat the inflammation or disease with high specificity. Nanoparticulate systems offer the ability to surpass the barriers in the eye while ensuring more specified and controlled release of the drug.

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CHAPTER 3
PREFOMULATION STUDIES AND DESIGN FOR PRODUCTION OF OPTIMISED INTRAOCULAR NANOBUBBLE SYSTEM

3.1. INTRODUCTION

Targeted drug delivery to the posterior segment of the eye has proven to be a challenge due to barriers such as the blood-aqueous barrier and the blood-retinal barrier. These form the blood-ocular barriers. The blood-aqueous barrier controls and regulates the flow of blood and intraocular fluid in the anterior segment of the eye. The main regions affected and controlled by the flow of blood and intraocular fluid comprise the ciliary body, trabecular meshwork.

The blood-retinal barrier is responsible for the control of metabolic substances flowing in and out of the eye and into the ocular bloodstream. The retinal pigment epithelium forms the surface of the blood-retinal barrier (Huang et al., 2018).

Anterior to the retina is the vitreous humour. The vitreous humour of the eye contains proteins, salts, certain carbohydrate derivatives and is gel-like in nature. This gel-like structure allows for the eye to maintain its shape. The multilayered nature of the eye renders ocular drug delivery, especially to the posterior eye, near impossible. Intraocular injections have long been seen as the best practice for treating inflammation of the posterior segment of the eye. This procedure involves using an injection to administer the drug to the posterior segment of the eye. Topical route of drug delivery to the posterior segment of the eye remains a challenging feat due to factors such as the tearing effect. The current status quo with regard to the posterior segment of the eye is the use of an intraocular injection and implants. The procedures are however time consuming to complete as it has to be done through anaesthesia. This procedure has also been associated with cataracts. A further complication with procedures such as intraocular injections is the possible neighbouring cell toxicity to the drug. Moments after the relevant drug has been injected, drug levels may be high and thus cause toxicity to the neighbouring cells. Furthermore, as time passes, the drug levels become ineffective as a

very low dose of the drug is found in the affected area just before inoculation of a renewed dose of drug (Weiner, 2008).

Treating disorders of the posterior segment of the eye has ushered in many novel and effective treatments. However, though deemed effective, these procedures are invasive. Driot et al. (2004) reported that Retisertfi, their novel intravitreal implant, was inserted first by sclera incision using a micro-vitreoretinal blade. This procedure was performed on rabbits initially. Though this procedure was invasive, they were able to ensure that Retisertfi was sustainably released over a year. The drug fluocinoloneacetonide was found in constant levels over the year, in the vitreous, the retina, retina pigment epithelium and choroid. This drug was not found in urine which suggests that the implant effectively delivered the drug to the relevant areas, thus ensuring there was no cytotoxicity. Like any ocular invasive procedures, cataract surgery was needed within 2 years in most patients. The drawback to this procedure is complications such as retinal tears.

Nanoscale drug delivery systems have received much attention in ophthalmic therapeutics. Nanoparticles conventionally used in the eye are between 100-1000nm, and composed of polymers. These polymers have properties that ensure that the desired drug is effectively entrapped or encapsulated, and furthermore released sustainably.

Nanostructures have been regarded as a viable drug delivery system due to their size. Their size allows for them to be able to travel through barriers within the eye. As mentioned, drug delivery to the posterior segment of the eye presents many challenges. Most importantly are the multi-drug resistant proteins that block drug entry through efflux pumps (Kansara et al., 2007). Nanoscale drug delivery systems such as nanoliposomes, nanoparticles and nanobubbles have a distinct advantage because they increase the surface area of the drug delivery system. This allows for an effective dose to be released over a larger area of any affected area. This effective release could help treat diseases and disorders with speed and effectiveness.

Ocular gene therapy has been necessary for treating ocular disorders such as retinal dystrophy and retinitis pigmentosa. Non-viral gene transfer to treat ocular disorders was proved to be a possibility. The nanoparticles were delivered by intravitreal injection.

The site of injection was the subretinal (Farjo et al., 2006).

Theranostics is defined as the dual ability to unite diagnosis, therapy and confirmation of the effectiveness of the therapy (Wagner et al., 2010). Thus, when using nanoparticles, it is ideal that they be tracked in real time. Magnetic resonance imaging (MRI) and ultrasound make real-time imaging a possibility. The use of echogenic materials allows for real-time viewing using ultrasound. Ultrasound is a widely-used technique in which soundwaves of about 100kHZ are transmitted to a target area. As the soundwaves encounter a barrier, the waves are reflected back at frequencies that allow for a pattern to be formed. This pattern allows for viewing in real time. Drug-loaded nanoparticles have been tracked by ultrasound in many instances. Clarity is important for the viewing of such minute materials. It has been shown that temperature does affect the movement of ultrasound waves in ocular tissues of rabbits, pigs and dogs (Gorig et al., 2006). Echogenic substances allow for viewing of nanoparticles. Echogenicity, the ability to echo or reflect sound waves is an important aspect in imaging and is strongly related to oscillation of nanobubbles. Echogenicity has to be enhanced by the lipid formulation and echogenic agents used as part of the formulation. Substances such as Tween®80 are considered to be echogenic and have been used in viewing nanobubbles (Wang et al., 2010). These nanobubbles as characterised in Wang et al. (2010) were able to travel intracellularly. Ubiquitous polymers cholesterol and soybean oil were used as the bubble film. Nanobubbles have a gas-filled core, which also facilitates ultrasound viewing of nanobubbles). Oxygen or gas delivery can be enhanced using factors such as frequency and sonication (Cavalli et al., 2009). Gases such as sulphur-hexafluoride, perfluorocarbons are able to enhance viewing. It is thus plausible that in order to have stable and sustainable ultrasound viewing, nanobubbles are best. The gas is able to affect the nanobubble half-life and size (Brenner & Lohse, 2008). The gas to be used must therefore be considered carefully. In the case of gas leakage, the gas should not cause cellular damage. Gas leakage must also not cause mechanical stress on the neighbouring cells as that would also cause considerable damage. Hwang et al. (2011) have shown that perfluorocarbons may have cytotoxic cells on neutrophils.

Layer-by-layer nanoparticulate systems have become an exploratory path in drug delivery. Layer-by-layer systems are viable as they assist in preventing drug leakage of the drug entrapped in the nanosystems. New chemical bonds may be formed that will

reinforce the nanostructure. Cholesterol, chitosan and polyethylene glycol may be employed in a layer-by-layer system.

Chitosan is a chitin-derivative and has proven to be useful in the production of drug delivery systems. The positively-charged polymer is useful as it is mucoadhesive and will be able to adhere to the negatively-charged cornea. Chitosan has also been shown to have antioxidant properties (Anraku et al., 2011). Its low solubility in water allows for it to be stable in human body, a largely aqueous environment. Chitosan is largely biodegradable and thus susceptible to enzymatic digestion in the human body. Chitosan has also proven to improve membrane permeability. Chitosan is FDA approved and widely used in drug delivery. Chitosan is found in nature and more commonly to be a derivative of chitin (chitosan is produced by the n-deacetylation of chitin), which serves as exoskeleton to organisms such a grasshoppers and shellfish (Khor & lim, 2003). Cholesterol is found in the human body and is therefore susceptible to human enzymatic digestion. Cholesterol has been used in drug delivery and nanoparticles. Furthermore, it has also proven effective in drug delivery.

Polyethylene glycol is also widely used and has been proven to be an effective drug delivery vector. PEG has also been used in conjunction with chitosan in drug delivery through the blood-brain barrier.

Polyethylene glycol has been used widely because of its biocompatibility. Polyethylene glycol ensures easy movement in the blood stream without eliciting macrophages. The use of polyethylene glycol increases the nanosystem's half-life in the blood stream and polyethylene glycol is used as it is able to be conjugated onto drugs without altering the therapeutic hydrophilic ability of the drug. Ci et al. (2013) have shown that adding poly ethylene glycol is able to enhance the fraction of active component of the drug. The conjugation allowed for the drug to be forming amphiphilic phospholipid-mimicking prodrugs forming amphiphilic phospholipid-mimicking prodrugs. This procedure allowed for the prevention of a burst release in an aqueous environment due to the hydrophilic component.

The disadvantage of using polyethylene glycol as a carrier is that it reduces cellular uptake of nanocarriers. It further acts as a barrier of drug diffusion due to its hydrophilic shielding effect (Wen et al., 2011).

The focus of this chapter is on the preformulation and subsequent optimisation of a nanobubble system via a Box-Behnken experimental design approach using indomethacin as the model drug.

3.2. PREFORMULATION CONSIDERATIONS IN PRODUCING A NANOLIPOSOMAL FORMULATION

There have been preformulation considerations according to the anatomical dimensions of the eye and the ability of the polymers to be introduced to the eye, without causing immunogenic responses. The polymers used were Methacrylic acid, also named Eudragit (RL 100 and RS 100), soybean oil and cholesterol.

These Eudragit® polymers assist with time-controlled drug release. RL 100 and RS 100 are granular (Evonik, Germany). These Eudragit® types are pH independent. Eudragit® RL 100 allows for its release to be customised and controlled by adding different rations of Eudragit® RS 100. Eudragit® RS 100 has also been proven to improve corneal penetration (Patra et al., 2017).

RL 100 is able to increase bioavailability (Singh & Pai, 2016), an important aspect that allows for the nanostructures to be absorbed by the biological tissues. RL 100 is composed of ethyl actylate, methyl methacrylate and a low concentration of methacrylic acid ester with quaternary ammonium groups. The ammonium groups assist with permeability. Native RL 100 has a glass transition temperature of $70^{o}C$ at 32 000g/mol. RL 100 has been used for ophthalmic inserts.

Producing a layer-by-layer nanoliposomal system able to effectively deliver indomethacin to the posterior eye, and able to be viewed in real time using ultrasound techniques, requires polymers that are able to entrap the drug effectively without altering the chemical structure of indomethacin. An effective contrasting agent, such as Tween®80 would be required in order to observe in real time using ultrasound.

3.2.1. Preformulation studies – Factors affecting stability of the nanoparticles

The process of producing nanoliposomes which are to be viewed in real time involved the release of sound waves to ensure the particles have ultrasonic properties, and this is done with a sonicator. However, preformulation studies were important in order to

investigate the variables governing the size and stability of the nanobubbles. Also, nanobubble size is an important factor determining if they may penetrate ocular barriers. The important considerations amongst others were the polymer combinations, the maximum optimal drug entrapment, and the drug release profile. All these factors were affected by the production process. The variables discovered to have the most effect on the stability the nanoparticles were the time of sonication, number of freeze-thaw cycles, and the composition of the innermost layer.

3.2.1.1. Amount of soybean oil as factor determining nanoparticulate size and efficacy

The two polymers used in the production of these particles are ubiquitous and this would support mass production if necessary. However, the amount of polymer in the formulation would affect how much drug would be incorporated and whether uniformly shaped nanoliposomes can be produced. The drug of choice, indomethacin, is practically insoluble in water, thus largely non-polar. The compounds found in soybean oil are also largely non polar. Thus the drug would be able to dissolve into the soybean oil and be able to possibly retain its chemical stability. Soybean oil also contributes to the creation of the emulsion system, and thus ensures that the system is an oil in water emulsion system. Tween®80, as a surfactant, will have an effect on the stability of the suspension.

3.2.1.2. Time of sonication and exposure to ultrasonic energy as a determinant of size

The sonicator releases ultrasonic waves and allows for the nanoparticles to be viewed in real time using ultrasound. Exposure of the organic phase and the aqueous phase to the waves causes agitation between the two phases and facilitates in the production of the nanoparticles. The addition of the Tween®80 allowed for the real-time viewing as it acts as a contrasting agent (Wang et al., 2010). The time of sonication causes the agitation of the polymeric emulsion of the polymers and buffers. The tome of the exposure to the sonicator will determine size and its contrast qualities.

Sonication involved exposing the nanoliposomes to ultrasonic energy. Ultrasonic energy has been used in drug delivery of insulin transdermally in mice (Tachibana & Tachibana, 1991) and ultrasound has been used experimentally in the treatment of glaucoma (Coleman et al., 1985). The use of high intensity ultrasound in treating glaucoma was due

to its ability to thin out intraocular collagen and the damage caused on the ciliary epithelium was able to lower the intraocular pressure for at least 3 months. Thus, the effects of the ultrasound need to be monitored.

Transdermal drug delivery of insulin has demonstrated that ultrasound can be used to penetrate multi-layered bodies or organs in the body. The skin consists of an epidermis, dermis and hypodermis (a fat layer). (Malkinson & Keane, 1981). The epidermis is the outermost later of the skin and regulates water loss to the environment and protects the body from pathogens. The epidermis is composed of flattened cells and columnar epithelial cells. The dermis consists of dense connective tissue and is composed of 2 layers, namely a papillary region and a reticular dermis. The hypodermis is mainly for fat storage and contains adipose cells, fibroblasts and macrophages. The sclera layers, in contrast, consist of 4 layers – the episclera, stroma, lamina fusca, and endothelium. Both the skin and sclera are multilayered and each layer has varying traits in terms of thickness, permeability to water, and presence of proteins. If ultrasound techniques could be used to achieve transdermal drug delivery, a hypothesis that transscleral drug delivery can be achieved is fair and worthy of investigation.

3.2.1.3. Number of freeze-thaw cycles as a determinant of nanoliposomes size

The number of freeze-thaw cycles had an effect on converting the vesicle from a multilamellar form to a unilamellar form of the nanostructures produced. This was important considering that the nanoliposomes are multilayered. Thus, the surface of the particles affected would affect the stability of the layering.

3.2.1.4. Tween®80, a contrasting agent able to aid real-time viewing of nanoliposomes

Tween was an important part of the nanoliposomal system as it served as a contrast agent that will assist in ensuring the system can be viewed in real time. Tween®80 is a non-ionic emulsifier and is polar in nature. Tween, as an emulsifier would have a kinetic stabilising effect on the nanoliposomal suspension. Its viscous nature may also play a role in how the eye reacts to the nanosystem. Tween®80, when used as a coating agent on polybutylcyanoacrylate nanoliposomes, has aided in complete desorption of peptides (Olivier et al., 1999). This has also led to prolonged pain relief. Thus, Tween®80 can assist

in the drug delivery process. Tween®80 was used by Olivier et. al. (1999) to assist in ensuring a peptide could pass the blood brain barrier. Tween®80 is a well-known surfactant and is able to assist in stabilising water in oil systems and oil in water systems.

3.2.1.5. Incorporation of sulphur hexafluoride to produce nanobubbles

Sulphur hexafluoride is a gas which acts as a photosensitiser. Photosensitisers act by absorbing light and then transferring them to nearby molecules. Sulphur hexafluoride would be the gas introduced into the nanoliposomes to produce nanobubbles. The sulphur hexafluoride would increase the resolution of the nanobubbles when ultrasound is used for real-time viewing.

Table 3.1: The 3 main variables affecting optimal drug delivery to the posterior segment of the eye

Variable	Variable effect
Time of sonication	Longer agitation of the aqueous phase and organic phase will improve probability of smaller and larger volume of nanoparticles [t]
Amount of soybean	The use of a polymer absorbable and digestible in the human body increases probability of absorption and facilitates proper layering
Number of freeze-thaw Cycles	Assists in forming unilamellar nanoparticles (Du Toit., 2010)

3.3. PREFORMULATION STUDIES: MATERIALS AND METHODS

3.3.1. Materials

Sodium hydroxide pearls (NAOH, Mw = 40 g/mol) acetic acid, potassium dihydrogen orthophosphate (Mw =136.09 g/mol), soybean oil (Sigma-Aldrich Corp. St Louis. MO.), Tween®80 (Sigma-Aldrich Corp. St Louis. MO.), solid cholesterol granules (Sigma-Aldrich Corp. St Louis. MO.), chitosan (Sigma-Aldrich Corp. St Louis. MO.), polyethylene glycol 400 (Sigma-Aldrich Corp. St Louis. MO.), double deionised water (Milli-Q Gradient, Millipore, MA, USA, electrical conductivity 18.2 Ohm/cm at 2525^{o}C), sodium decanoate (Mw 194.25 g/mol), methanol (32.04 g/mol), potassium dihydrogen orthophosphate (Mw 32.04

g/mol), (136.086 g/mol), phosphate buffered saline (Mw 411.029), dialysis tubing (Mw 10 000), chloroform (Sigma-Aldrich Corp. St Louis. MO.), and indomethacin (Sigma-Aldrich Corp. St Louis. MO.). Chemicals were purchased from Sigma-Aldrich. All other solvents and chemicals were of analytical grade.

3.3.2. Preparation of layer-by-layer nanoparticles

Soybean oil, indomethacin, sodium decanoate were dissolved in chloroform and a pH7.4 buffer (sodium hydroxide and Potassium dihydrogen orthophosphate dissolved in deionised water, and titrated to pH 7.4) as the emulsion evaporation method. This mixture was sonicated (6mm probe, 20 kHz, 50 W) for a minimum of 10 minutes to produce a milky homogenous mixture.

The chloroform was then evaporated during agitation with a magnetic stirrer. The solution underwent 3-4 freeze-thaw cycles in order to produce a uniform shape of the nanoparticles. The freeze-thawing process involves freezing the formulation for 2 hours, and then allowing the formulation to thaw for at least 3 hours. A 1% solution of chitosan was added at a 1:1 ratio and allowed to settle for 12 hours. Following that a 5% solution of PEG 400 was added at a ratio of 1:0.4 and left for 4 hours after addition.

3.3.3. Size and stability analysis of the nanoliposomes

Zetasizing studies were undertaken on the formulations in order to ascertain that the nanoparticles were the optimal size within the nano range and to determine the size distribution profiles and polydispersity index. This study was determined using a Zetasizer NanoZs (Malvern Instruments, Malvern UK). Zeta potential was also examined using a Zetasizer NanoZs. The Zetasizer makes use of dynamic light scattering and one of the factors in determining size is through measurement of the Brownian movement of the particles.

Table 3.2: A summary of the preformulation nanobubble combinations

Formulation	Layer 1	Layer 2	Layer 3	Tween®80 Present
1	Cholesterol	Chitosan	1:2 Eudragit® RL100:RS100	No
2	Soybean Oil	Chitosan	1:2 Eudragit® RL100:RS100	Yes
3	Cholesterol & Soybean Oil	Chitosan	1:2 Eudragit® RL100:RS100	No
4	Cholesterol	Chitosan	1:2 Eudragit® RL100:RS100	Yes
5	Soybean Oil	Chitosan	1:2 Eudragit® RL100:RS100	No
6	Cholesterol & Soybean Oil	Chitosan	1:2 Eudragit® RL100:RS100	Yes
7	Cholesterol	Chitosan	PEG	No
8	Soybean Oil	Chitosan	PEG	Yes
9	Cholesterol & Soybean Oil	Chitosan	PEG	No
10	Cholesterol	Chitosan	PEG	Yes
11	Soybean Oil	Chitosan	PEG	No
12	Cholesterol & Soybean Oil	Chitosan	PEG	Yes

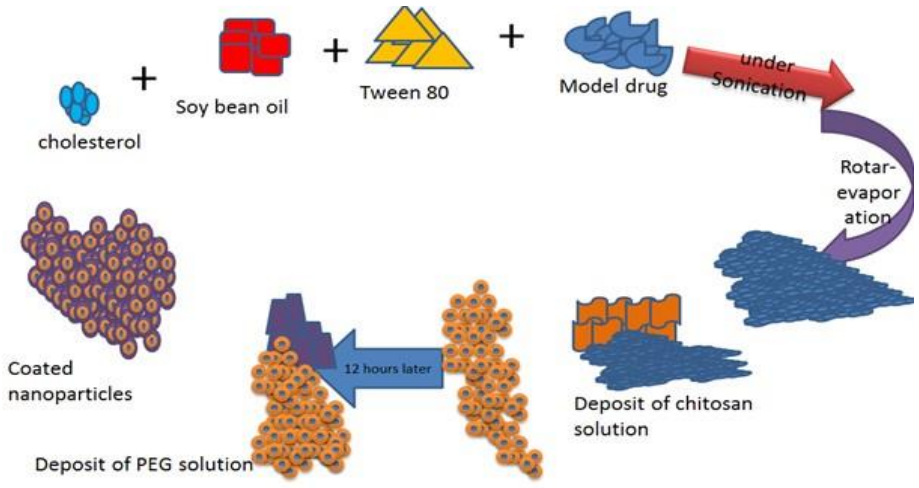


Figure 3.1: Schematic diagram illustrating the production of the nanoparticles

3.4. PREFORMULATION STUDIES: RESULTS AND DISCUSSION

3.4.1. Zeta size analysis

Formulations 1-6 had used Eudragit® as a final layer and showed varying sizes and zeta potential. This could be due to the presence of Tween®80 and its reaction to the Eudragit® polymers. RL 100(positively charged) and Eudragit® RS100 were used at a 1:1 ratio and were used because of their properties. These properties include pH-independent swelling and insoluble. RS 100 is insoluble, pH independent swelling. These are also ideal for controlled-drug release (Salatin et al., 2017). RS 100 and RL 100 allow for customisable drug release; however, the swelling may affect the movement of the nanobubbles in the eye. The eye contains many barriers and the swelling may cause adverse effects.

A number of formulations produced poor results especially in zeta sizing and zeta potential analyses. Dialysis tubing in some aspects has assisted in producing better results. The formulations that produced the best results had no Tween®80 and seemed more stable in that form. However, in that form, these nanoliposomes will not be echogenic and thus were not viable for this investigation.

Formulation 2 produced nanoliposomes that were 63.26nm in diameter with a polydispesity index of 0.483. The size is small and may indicate a less than optimal drug entrapment efficiency. The general results of batch 1 run were poor. Formulation 2 produced a less than satisfactory zeta potential. The zeta potential; was on average 0.333. This formulation used soybean oil as an inner layer and Tween®80 was added in the formulation. Formulation 3 produced nanoliposomes whose sizes were slightly larger than of formulation 2. The Polydispersity index was 0.307. This formulation had no Tween®80 added to it. The zeta potential is 16. Formulation 4 produced poor results too. The nanoliposomes produced were 39.7 nm in size. The pdi was 0.559, which is above the desired threshold for pdi. A second run was attempted and its size was 53.19. However, its pdi was below the threshold, but yielded poor results. The zeta potential was 0.460. Formulation 5 yielded better results. The nanoliposomes yielded had a size of 120.8 nm with a pdi of 0.294. The zeta potential was 26.6. Formulation 6 yielded a poor run and the zeta potential. Formulation 4 and Formulation 6 were both poor runs and

both had Tween®80 added. Thus, it is possible that this formulation along with the addition of the Tween®80 may not yield stable nanoliposomes.

3.4.2. Formulation 6-12

Batch 2 consisted of a final layer of PEG 400, which is in liquid form. Formulation 7 had an initial layer of cholesterol, with a middle layer of chitosan. The outermost layer was PEG. This produced nanoliposomes sized 332 nm. The pdi was 0.521. The resultant zeta potential was 35.8. Formulation 8 was composed of soybean oil only. Chitosan and PEG. This formulation had Tween®80 incorporated into the innermost layer. The formulation was 265 nm and with a pdi of 0.37. This formulation was in overall a stable formulation with size homogeneity. The zeta potential was 18.2, a strong positive reading. Formulation 9 produced nanoliposomes with a size of 221.7 nm and a pdi of 0.25. This formulation contained both cholesterol and soybean oil. This formulation had no soybean oil, meaning it would not have echogenic properties. Formulation 10 produced a formulation that was 372 nm and a pdi of 0.287. This formulation was a not a viable formulation due to size.

3.5. EXPERIMENTAL PARAMETERS AND EXPERIMENTAL DESIGN

As discussed, the variables chosen as part of the experimental design are the amount of soybean oil polymer, the time of sonication and the number of freeze-thaw cycles. These 3 inputs are instrumental in ensuring that the nanoliposomes which are in the desired size range, with effective drug entrapment, and which are circular and unilamellar are produced. These 3 inputs were organised and ordered in different combinations, using upper and lower limits to produce 15 formulations, and ultimately the optimised formulations were determined.

Table 3.3: The upper and lower limits identified for generation of the experimental design

	Time of	Freeze thaw cycles	Amount of soybean oil sonication
Lower limit	10 minutes	1	0.2mL
Upper Limit	20 minutes	5	1mL

Table 3.4: The 15 formulations generated via the Box-Behnken experimental design

Formulation	Sonication time(X1)	Freeze-thaw cycles(X2)	Soybean oil(X3)
1	10	3	0.2
2	15	1	0.2
3	15	5	1
4	15	3	0.6
5	10	3	1
6	20	3	1
7	15	3	0.6
8	15	5	0.2
9	20	5	0.6
10	20	1	0.6
11	10	5	0.6
12	20	3	0.2
13	15	3	0.6
14	10	1	0.6
15	15	1	1

Table 3.5: Output measurements of the Box-Behnken

Output	Parameter	Desired Output
Y1	Size	≤ 200nm
Y2	Drug Entrapment	Maximise
Y3	Mean dissolution time (MDT)	Higher

3.6. CHARACTERISATION OF THE NANOLIPOSOME FORMULATIONS

3.6.1. Size and stability analysis of the nanoliposomes

Zetasizing studies were done on the formulations in order to ascertain that the nanoliposomes were the optimal size within the nano range and to determine the size distribution profiles and polydispersity index.

3.6.2. Drug entrapment studies

In order to determine the amount of drug entrapped in each formulation, each lyophilized formulation was suspended in 100ml buffer and centrifuged (Model TG16-WS, Shanghai Luxiangyi Centrifuge Instrument Co., Ltd., Shanghai, China) at ultra-high speed for 30min. This caused the disintegration of the polymers, allowing them to become sediment at the bottom of the tube and the drug to be suspended in the buffer in the supernatant. The supernatant was then extracted. With the use of Ultraviolet spectroscopy (Perkin Elmer Life and Analytical Sciences Inc., Shelton, CT, USA) and the drug calibration curve constructed, drug entrapment in each formulation was determined.

3.6.3. Drug release analysis from the nanoliposomal system

A drug release profile was determined using a shaker bath set at $37^{o}C$ The drug release profile of each formulation was performed in simulated vitreous humour for over 48 hours. A known amount was suspending in 3mL simulated vitreous humour within dialysis tubing. This tubing was then suspended in 2mL simulated vitreous humour. At certain time points, namely 30 min, 1 hr, 1.5hr, 2hr, 2.5hr, 3hrs, 6hrs,12hrs, 24hrs, 48hrs, simulated vitreous humour (SVH) (1mL) was extracted and replaced with 1mL fresh SVH. The drug concentration was determined using ultraviolet spectroscopy(Lambda 25 UV/Vis Spectroscopy, Perkin-Elmer, Shelton, CT, U.S.). programmed to measure absorbance at a wavelength 318nm, the wavelength of determination of indomethacin.

3.7. CHARACTERISATION AND DETERMINATION OF OPTIMAL FORMULATION: FURTHER INVESTIGATIONS

Taking into consideration the 3 outputs to measure, an optimised formulation was determined based on its ability to remain within the parameters of the desired outputs. This was obtained with the use of statistical software. Following determination of the

optimised formulation, further characterisation studies were performed on this optimised formulation as presented in the ensuing chapters: determination of molecular structure of the optimised nanoliposomes was determined using Fourier transform InfraRed (FTIR) spectroscopy, changes in enthalpy and glass transition temperature was determined using differential scanning calorimetry (DSC). The optimised formulation was furthermore suspended in a solution of Pluronic F147 and Pluronic F68. This suspension was to be used in *in vivo* animal studies with 60 New Zealand white rabbits. Tissue analysis experiments were performed to examine inflammatory responses and necrosis of cells. Furthermore, real-time viewing of the drug delivery system in the rabbit eye was done to indeed prove that the drug delivery system possesses theranostic capabilities.

3.8. INCORPORATION OF GAS INTO OPTIMISED FORMULATION OF NANOLIPOSES TO PRODUCE NANOBUBBLES

In order to aid the aims of theranostic capability, a suitable echogenic contrast agent was required to be added to the optimised formulation of the nanoliposomes.

Perfluorocarbons have been noted to be suitable contrast agents and aid in better resolution in ultrasound viewing. In this case, sulphur hexafluoride was the chosen gas. In order to introduce this gas, the nanoliposomes were sonicated in a sealed beaker containing sulphur hexafluoride, as shown in Figure 3.2.

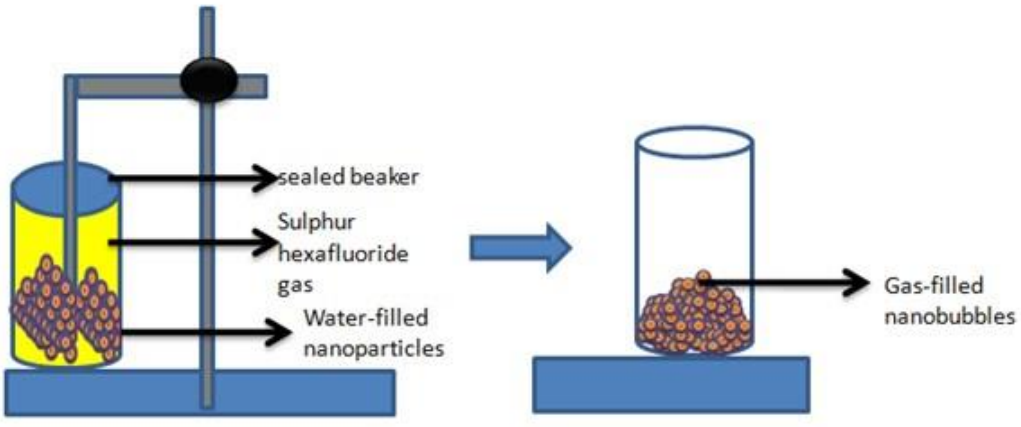


Figure 3.2: Production of gas-filled nanobubbles from a nanoliposome solution

3.9. RESULTS AND DISCUSSION

The Box-Behnken design produced 15 formulations. The majority of formulations were within 200nm, a suitable size for nanoliposomes to pass through ocular barriers. It has been proven that particles at that size, and larger are able to be retained subconjuctivally for atleast 2 months or more (Amrite et al., 2005). Motwani et al. have produced nanoliposomes as large at 572nm for ocular drug delivery. They observed an inverse relationship between the particle size and drug encapsulation. The morphology of the layer-by-layer system revealed effective layer in the nanosystems. The images were done by TEM and AFM. TEM showed a thickened film revealing effective layering. Du toit et al., has spoken of how in layer-by-layer systems, shorter chains may deposit into the longer chains. The intermolecular forces that will govern the interactions between the longer and shorter chains may cause a reduction in size. Du Toit et al., had that in the layer-by-layer polymeric systems, the lowered nanobubble size was caused by long and short chain interaction and the positive and negative charges found in chitosan (positively charged) and in alginate(negatively).

3.9.1. Zetasizing

Zetasizing studies revealed interesting trends with regards to the layering system. Comparisons of the innermost layers and the final sizing analysing reveals an increase in size due to the layering. This shows that the chitosan and polyethylene glycol layers are successfully deposited onto the lipid nanobubble. The most common amount of size increase was within 35nm. The biggest increases were over 100nm (formulation number 9). Over 65% of the formulations revealed sizes within 110nm for the innermost layer and an approximate increase of 35nm. For formulations mostly producing nanoliposomes of above 110nm for the innermost layer and with a general increase in size above 35nm due to layering, the sonication times was 20 minutes. Thus this reveals a larger particle size for a longer sonication time (with no direct correlation to the amount of soybean oil and number of freeze-thaw cycles). A longer sonication time correlates to longer agitation between the aqueous and organic phases, and corresponds to longer exposure to ultrasonic energy. Shorter sonication time corresponded to the smallest particles generally for the innermost layer. This demonstrates that sonication time was an apt variable to consider in producing nanoliposomes within the 200nm range. An

increase in particle size due to longer sonication time implies the extra ultrasonic energy facilitates a higher layering capacity on the lipid nanoliposome.

3.9.2. Drug entrapment

A drug entrapment efficiency of 25%-40% was obtained for the formulations. In our Box-Behnken design, the optimised formulation required only 0.2mL of soybean oil, 10 minutes sonication time and 3 freeze-thaw cycles. This optimised formulation had a final diameter of 100nm. 100nm is an improvement on the previously recorded 572nm.

3.9.3. Drug release profile

The drug release profile of all 15 formulations indicate over 10% fractional release in each formulation within an hour. All formulations release the drug fully within 24 hours. Thus, this formulation may likely require at most, twice daily application, also taking into account the half-life of indomethacin, depending on the drug concentration found in the suspension. In all the formulations, within 3 hours, over 50% of the drug is released. Thus, depending on the time taken to reach the affected area, therapeutic relief should be within that timeframe. The optimised formulation required 0.2ml of soybean oil. The drug release profile of the formulations 1-5 shows an 80% release after approximately 10 hours, meaning that if any of these formulations were the most optimised, this formulation would have to be administered twice a day (Figure 3.3).

Formulations 6-10 have no general trend, but most striking is formulation 9 which releases almost all the drug in 5 hours (Figure 3.4). Formulations 11-16 have a similar drug release pattern with a burst-release phase, in which approximately 65% of the drug is released within 5 hours (Figure 3.5).

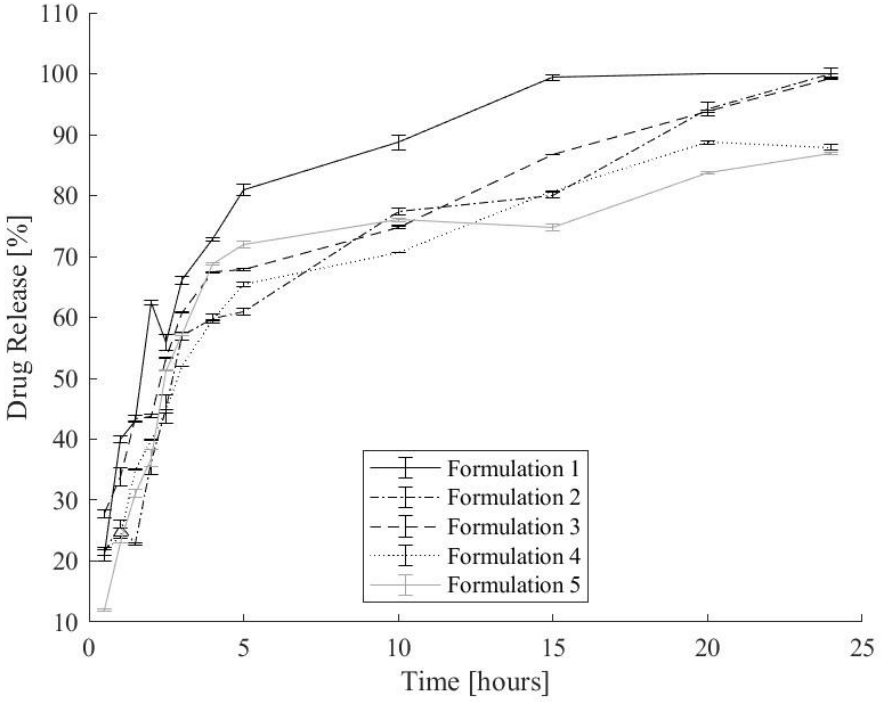


Figure 3.3: Drug release profile of the first 5 formulations.

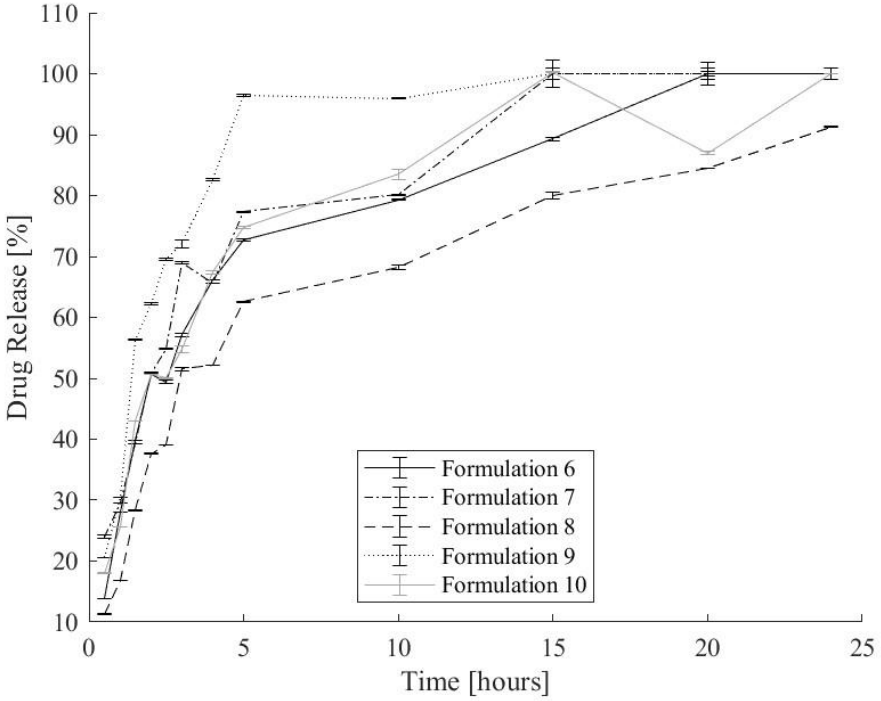


Figure 3.4: Drug release profile of the formulations 6-10

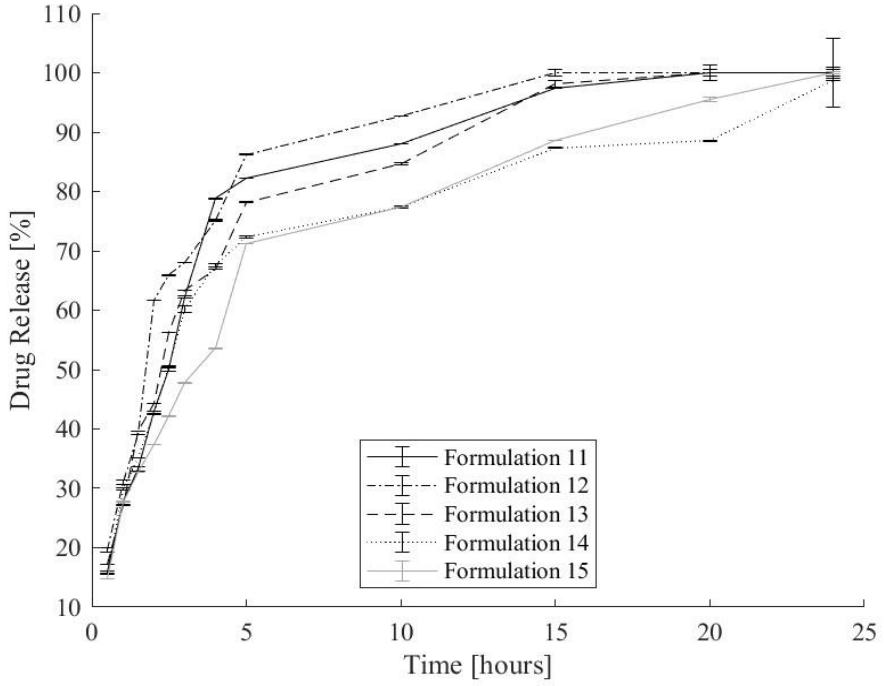


Figure 3.5: Drug release profile of the formulations 11-15

Table 3.6: Characterisation of the 15 Box-Behnken formulations in terms of size, entrapment and MDT

Formulation	Final size (nm)	Drug entrapment (mg)	Drug entrapment Efficiency (%)	MDT
1	100	1.426	33.157	3.333
2	72	1.291	30.020	6.277
3	117	1.084	25.2163	5.406
4	107	1.348	31.349	4.562
5	137	1.404	32.643	4.333
6	144	1.171	27.237	4.892
7	31	1.194	27.769	4.011
8	167	1.597	37.146	5.939
9	221	1.514	35.213	2.247
10	307	0.726	16.885	4.637
11	139	1.420	33.033	3.654
12	244	1.706	39.680	3.943
13	148	1.566	36.419	5.397
14	168	1.533	35.657	5.510
15	118	1.718	39.946	3.486

3.10. INTRODUCTION: MATHEMATICAL ANALYSIS TO DETERMINE FACTORS AFFECTING NANOBUBBLE SIZE AND DRUG ENTRAPMENT EFFICIENCY

The Box-Behnken design analysis allowed for the optimal formulation to be identified (Ranch et al., 2019). There are 3 inputs which have upper and lower limits, and 15 possible formulations were generated using this model. However, this model allowed one to examine the outputs and thus be able to determine the factors that determine the best formulation that will produce the desired outcome. In this instance the best formulation would have the best drug entrapment efficiency and a size of less than 200nm.

The output of the Box-Behnken designs also demonstrate the behaviour of the formulation with respect to the upper and lower limits set. This aspect demonstrated the manner in which sonication time, freeze-thaw cycles and the amount of freeze-thaw cycles affected the stability of the nanoliposomal structures.

3.11. MATERIALS AND METHODS

MATLAB® was used for numerical analysis and to graphically represents trends. Regression analysis was used to determine correlations in nanoparticle size and drug entrapment efficiency size and generated graphs. Regression analysis follows the general formula:

$$Y = \beta_0 + \beta_1 X_1 m + \beta_2 X_2$$

Where,

Y is the response, β_0 represents the intercept of the plane, β_1 and β_2 are partial regression coefficients, X_1 and X_2 are responses per unit change (Dast et al., 2010).

Regression analysis draws a trend between data sets by drawing a trend-line between the data sets. Thus, regression analysis allows for interpolation of data and understanding what factors may be at play if nanoliposomal production had to be significantly upscaled. Regression analysis was conducted and graphs were plotted examining the relationship between particle size with amount of soybean oil and freeze-thaw cycles, which were the inputs of the Box- Behnken design study.

3.12. RESULTS

3.12.1. Percentage change in size and in drug entrapment efficiency

Analysis of the inputs and corresponding outputs demonstrated relationships between the 3 factors. Using the equation:

$$incr(decr)\% = \frac{formulationB - formulationA}{formulationA} \times 100$$

Changes from formula to be examined with respect to the changes made in the 3 factors.

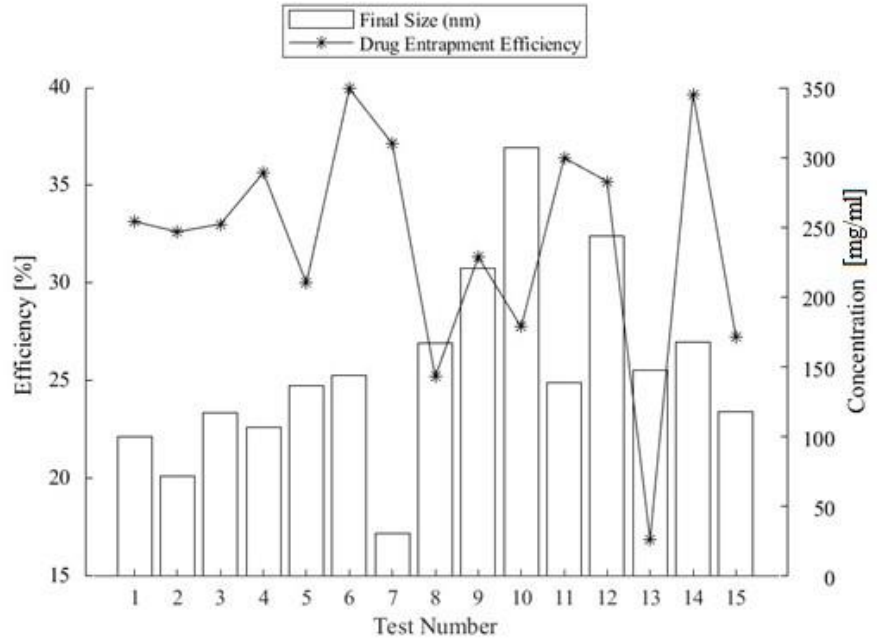


Figure 3.6: Important outputs size, drug entrapment efficiency in comparison to inputs (sonication time, soybean oil, freeze-thaw cycles) for all formulations in Box-Behnken design

The general trend was seen with formulations with similar variables. When freeze-thaw cycles and time of sonication were kept constant, a higher number of freeze-thaw cycles resulted in a larger bubble size and larger entrapment efficiency. An example of this was in formulation 1 and 5. Formulation 1 and formulation 5 had the same number of freeze-thaw cycles and the same time of sonication. However, the amount of soybean oil was 5 times as much. With a larger amount of soybean oil, the size is 37% bigger, however the drug entrapment efficiency was smaller.

Keeping the amount of soybean oil low affected drug entrapment efficiency and size. Regarding formulation 2 and 8, formulation 8 was 131% larger than formulation 2. Thus a high sonication time and perhaps a lower amount of soybean oil, with a higher freeze-thaw cycles affected the size. Formulation 2 and formulation 15 both had 15 minutes of sonication times, both 1 freeze-thaw cycles. Formulation 2 had 0.2mL and formulation 15 had 1mL. Formulation 15 was 64% bigger than formulation 2, with a 33% higher drug entrapment efficiency. Formulation 2 and formulation 8 both had 15 minutes of sonication, the same amount of soybean oil (0.2mL). However, formulation 8 had 5 freeze-thaw cycles, and formulation 2 had only 1 freeze-thaw cycle. Formulation 8 was 131% bigger, but only with a 23% better drug entrapment efficiency. Thus a high sonication time and perhaps a lower amount of soybean oil, with a higher freeze-thaw cycles affects the size.

When sonication time and the amount of soybean oil and sonication time were kept constant, the highest number of freeze-thaw cycles was increased in nanoliposome size by approx. 20% and better drug entrapment efficiency increased by 7%.

Formulation 11 and 14 had the same sonication time and amount soybean oil and the number of freeze-thaw. Formulation 11 had 5 freeze-thaw cycles and 1 freeze-thaw cycle for formulation 14. With this change, formulation 11 was 20% bigger in size, with only a 7% increase in drug entrapment efficiency. Thus, generally freeze-thaw cycles increased and an increase in drug entrapment efficiency.

Formulation 3 and formulation 8 both had 15 minutes of freeze-thaws cycles and the same number of freeze-thaw cycles, 5, Formulation 8 had 0.2ml of soybean oil, and formulation 3 had 1 ml of soybean oil. Formulation 3 is 23% bigger in size, and with 32% more drug entrapment efficiency. Doubling sonication time, from 10 minutes to 20 minutes while having kept freeze-thaw and cycles (at 3) and amount of soybean oil (0.2mL), as in formulation 1 and 12, produced a larger nanoliposome, by over 100%, but did not significantly improve drug entrapment efficiency .

Doubling sonication time, from 10 minutes to 20 minutes while having kept freeze-thaw and cycles (at 3) and amount of soybean oil(1mL), as in formulation 5 and 16, produced a nanoliposome that is only 5% larger, with only a 16% larger drug entrapment efficiency.

Thus a high volume of soy bean oil did not favour a larger nanoliposome or better drug entrapment efficiency.

Doubling sonication time, from 10 minutes to 20 minutes while keeping freeze-thaw and cycles (at 5) and amount of soybean oil(0.6mL), as in formulation 11 and 9, produced a bubble that is almost 60% larger and with a 66% better drug entrapment efficiency.

Doubling sonication time, from 10 minutes to 20 minutes while keeping freeze-thaw and cycles (at 1) and amount of soybean oil (0.6mL), as in formulation 14 and 10, produced an 85% larger nanoliposome, and a 52% improved drug entrapment efficiency.

3.12.2. Examining effect of soybean oil and freeze-thaw cycles on sonication time

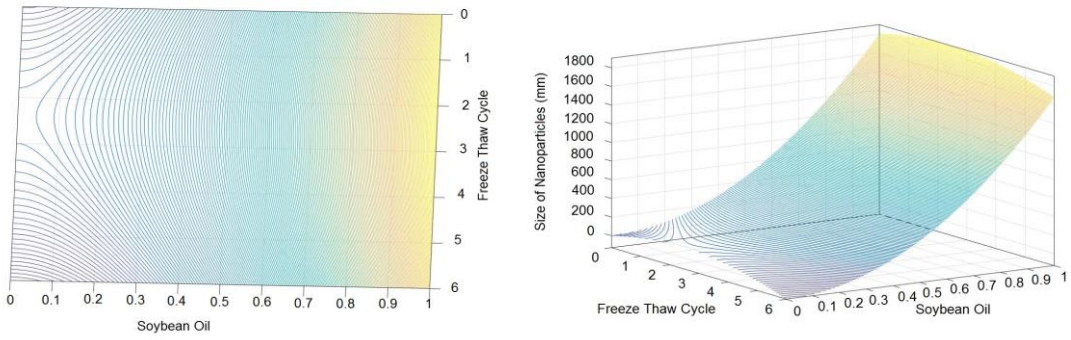
Sonication of the formulation is the main producer of the nanoliposomes as the release of the ultrasonic waves causes agitation of the molecules under high pressure and high speed, thus producing the nanoliposomes. The stability of these nanoliposomes was affected by the polymers used in the innermost layer (soybean oil being one of the polymers) and freeze-thaw cycles, as it assisted in ensuring the surface was unilamellar.

When the sonication time was kept constant at 20min, the amount of soybean oil played a larger role in the final nanoliposomal size. The freeze-thaw cycles played a lesser role in determining the final particle size. The surface plot, Figure 3.8, showed an increase in nanoliposome size and the amount of soybean oil increases. The freeze-thaw axis had little change in gradient.

Regression analysis was also used to examine the trends that occurred when sonication time was kept as a constant in regression analysis. This aspect was important in demonstrating the factors that contribute to nanoliposome size and drug entrapment efficiency. When the sonication time was kept constant at 15minutes, while the freeze-thaw cycles and amount of soybean varied, the following was discovered: Per the surface plot, Figure 3.9, when freeze-thaw cycles exceeded 2 cycles, the size of the nanoliposomes increased. Furthermore, at 2 freeze-thaw cycles, the contour lines of the surface plot were less densely packed, which was an indication of less change, and a lesser change in gradient. This could indicate that more than 2 freeze-thaw cycles contributed to a lesser change in the nanoliposomes when sonication time was 15min. Examining the trends of

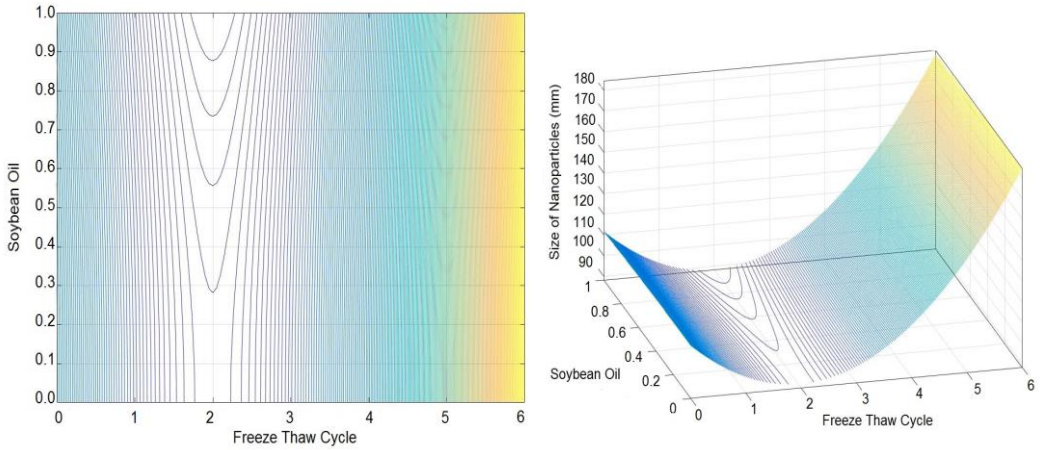
the graph from 2 thaw cycles, an increase in soybean oil also corresponded to an increase in particle size.

When the sonication time was kept at a constant 10 minutes, a slightly different trend was observed. A look at the contour lines, demonstrated that at 1-4 freeze-thaw cycles, with lower amount of soybean oil, there was less change in size. An increase in soybean oil corresponded to a constant increase in size. The highest point of density is at the circular point per the graph (Figure 3.10), which corresponds to 160nm. This corresponds to soybean oil amount of 0.65mL and 3 freeze-thaw cycles.



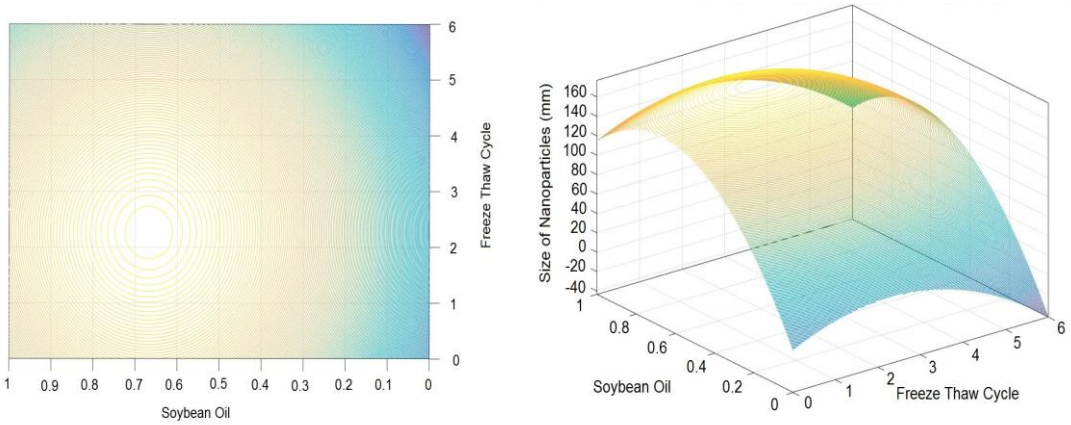
(a) (b)

Figure 3.8: Graphs illustrating the effect of freeze-thaw cycles and soybean oil levels on nanoliposome growth when sonication time is kept constant at 20min. (a) is a surface plot and (b) is the full 3 axis graph.



(a) (b)

Figure 3.9: Graphs illustrating the effect of freeze-thaw cycles and soybean oil levels on nanoliposomes growth when sonication time is kept constant at 15min. (a) is a surface plot and (b) is the full 3 axis graph



(a) (b)

Figure 3.10: Graphs illustrating the trend of nanoliposomes growth when sonication time is kept constantly at 10min. (a) is a surface plot and (b) is the full 3 axis graph.

3.13. OTHER NANOPARTICULATE STUDIES FOR OCULAR DRUG DELIVERY

Other studies used higher particle size in ocular drug delivery. However, these particles were not multi-layered. Wang et al. (2018) produced nanoparticles for ocular drug delivery and optimised the formulation using the Box-Behnken design method. The particles were produced by drop-wise addition of the organic phase under vigorous magnetic stirring conditions. The optimised formulation was 247.7nm with a 58% drug entrapment efficiency. This formulation was composed of chitosan coated solid nanoparticles. The drug used was methazolamide. Ameeduzzafar et al. (2020) have

produced nanoparticulate niosomes using a sonicator and also using cholesterol as an input. The drug of choice was ciprofloxacin. The niosomes were sized at 180nm, 78% drug entrapment efficiency. Stearic acid nanoparticles using levofloxacin produced optimised nanoparticles with a 78.71% drug entrapment efficiency and a size of 237.83nm (Baig et al., 2016). In comparison to the formulations produced per Table 3.6 above, the highest efficiency was 39.9%. This may mean that larger nanoparticles may support a better drug entrapment efficiency; however, a size of 200nm or less will be optimal for passing though ocular barriers.

Pandit et al., 2017 have produced nanoparticles for ocular drug delivery to the retina. These nanoparticles were composed of poly (lactide-co-glycolic acid) (PLGA), but coated with chitosan. Box-Behnken design was used to find an optimised product. In that study the independent variables were amount of chitosan and polyvinyl alcohol(PA),sonication time and the amount of PLGA. The size range is 210nm- 420nm. This reinforces general observations that nanoparticles around the 200nm might be more optimal.

Surfactants as important components in creating stable nanoparticulate formulations are also highlighted in various other studies. It is an important component in stabilising the nanoparticulate formulation. They are an important factor in the creation of nanoparticles for treating conjunctivitis using the drug levofloxacin (Baig et al, 2016). In this study, the 2 surfactants were used as independent factors/variables along with the amount of lipid polymer. Particle size and drug entrapment efficiency were the measured outputs. The optimised formulation had a final size of 237.82 nm with 78.1% drug entrapment efficiency. Polyethlyene glycol as a co-polymer of choice has also been used in other ocular drug formulations. Patil et al., 2018 used polyethylene glycosylated lipids as the drug delivery carrier. This formulation had an optimised formulation of less than 300nm.

In observing other Box-Behnken designs for ocular drug delivery, using solid lipid (X1), surfactant (X2), and drug/lipid ratio (X3) level as independent factors (Hao et al., 2011) are common. In the study here cited, these nanoparticles had an average size of 248nm. 83.29% was the drug entrapment efficiency. Full drug release was within 6 hours. The surfactant in this study would play a role in ensuring the effective mixing of drug, chloramphenicol and lipid, glycerol monostearate. This formulation was mono-layered.

The nanobubbles for this study used a surfactant, Tween 80. Full drug release in 6 hours is not best as that means the formulation would need to be taken at least 3 times a day. It is not an improvement on current formulations in the market.

However the study undertaken used Tween®80, both as a surfactant, but also as a contrasting agent to ensure that once ultrasound techniques were used for real-time viewing, which is what makes the study novel.

3.14. CONCLUDING REMARKS

The 3 factor Box-Behnken design assisted in formulating the preferred formulation to entrap the maximum amount of drug. The initial polymers used were Eudragit polymers in pre-formulation studies. However, soybean oil and cholesterol were selected to be the polymers to be used. In producing the most optimal formulation, the best possible size nanoparticles would be under 200 nm. When using soybean oil and cholesterol as part of the innermost layers, the Box-Behnken design revealed that all formulations were able to uptake the drug, Indomethacin. The formulations were also tested and the corresponding outputs were analysed using regression analysis to examine the trends in the outputs.

In the 3 factor Box-Behnken conducted, an extended sonication time generally corresponded to a larger particle size but not necessarily to a higher drug entrapment efficiency. The formulation yielding a smaller particle size, but with a higher drug entrapment efficiency, had a 15 minute sonication time and higher volume of soybean oil. The higher volume of soybean oil allows for more volume to mix between the organic solvent and aqueous phase in the longer 15 minute timeframe. Thus a longer sonication allowed for a more stably produced emulsion and the soybean oil being a main layer is instrumental in producing smaller nanoparticles with a higher drug entrapment efficiency.

Many other nanostructures engineered for ocular drug delivery, had a lipid in their formulation, were single layered and the nanostructures large than those produced in the current study. This study also found that a generally larger particle size had a higher drug entrapment. The formulations in this study also had a lower drug entrapment efficiency. The similarity with this study and other studies was that the amount of lipid layer was

considered as an independent variable, which demonstrates that concentration of lipid polymer is important in creating stable nanoparticles. One of the factors that were tracked in the Box-Behnken design for this study was the number of freeze-thaw cycles. This was important as it ensure the stability of the layered, possibly contributing to the sizing. This was not done in other studies as layering was not applied.

The independent factors measured for Box-Behnken need to take into account not only entrapment and release, but also the other possible uses for the nanobubbles, which will also be real-time viewing of the movement of these bubbles.Furthermore, Prolonged drug release was also preferable for this study in order to ensure that fewer doses need to be taken over the day. It would be preferred that people need to take 1 dosage a day.

As a general trend, nano-enabled formulations for ocular drug delivery seem to single layer and not geared toward theranostic capability. However, the formulation produced also hoped to achieve the ability for real time viewing, and was multi-layered in order ensure optimal drug entrapment.

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CHAPTER 4

CHARACTERISATION OF THE OPTIMISED LAYER-BY-LAYER IONBS

4.1. INTRODUCTION

A Box-Behnken 3 factor design in which 15 possible formulations were tested revealed an optimised formulation using statistical optimisation (see Figure 4.1). Further studies were conducted to elucidate the qualitative character of the optimised formulation. Much of the studies were conducted layer-by-layer in order to demonstrate the effects of the layering on the chemical character of the optimised product.

The optimised product in terms of zeta potential is stable and is within the 300nm range. This product was stable when the time of sonication was indicated as 10 minutes with 3 freeze-thaw cycles and 0.2mL of soybean oil. Its stability was also demonstrated by the fact that it produced a free-flowing powder once lyophilized.

This chapter focuses on the character of the optimised formulation. The studies conducted examined the nanoliposomes on a layer-by-layer basis and demonstrate how the layering encloses the drug and assists with the stability of the nanosystem for optimal drug delivery. In line with the desired objectives, formulation 1 was within the required parameters to achieve the objectives of appropriate size, effective entrapment and a suitable mean dissolution time. The use of the statistical software assisted in obtaining an optimised formulation. This was achieved through the use of a statistical optimisation tool. Pharmacokinetic software, WinNonLin Version 5.1 (Pharsight software, Mountain View, CA, USA), was used to model release data.

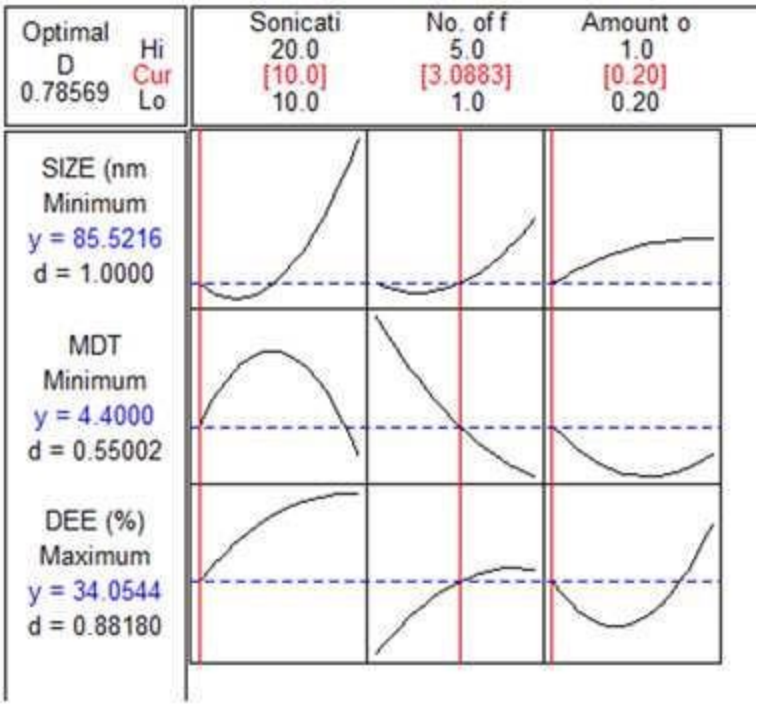


Figure 4.1: Optimisation plots displaying factorial levels and desirability values for the chosen optimised formulation

4.2. METHODS

4.2.1. Investigation of size of the optimised nanosystem using atomic force microscopy and zetasize studies

Atomic force microscopy was used to confirm that the nanoparticles were in the desired size range. The options for viewing for atomic force microscopy is contact mode, noncontact mode and tapping mode. In contact mode microscopy, the cantilever drags through the sample and thus may affect sample integrity, while in non-contact mode the cantilevers hovers above the sample producing Van Der Waals (Jalali et al., 2004). Tapping mode was used in this instance, and is widely considered to be the most advanced form of atomic force microscopy. A dry sample was dissolved in ultrapure water in the preparative process. Atomic force microscope is a high resolution microscopic technique which uses a cantilever that vibrates over the surface of the sample, brings about forces between the surface of the sample and the cantilever, and once this is computed, produces visual data.

Size analysis was used to quantify the average particle size. In order to ascertain that the nanoliposedes were within the expected size range of less than 200nm, the Zetasize NanoZS (Malvern Instruments Ltd, Malvern, United Kingdom) was utilised.

4.2.2. Heat signature studies using thermo-gravimetric analysis on layer-by-layer optimised nanosystem

Responses to heat were determined by Thermogravimetric analysis. Thermogravimteric analysis was done using a PerkinElmer TGA 4000 instrument (Llantrisant, Wales, UK). Heating commenced from 30^oC and was set to increase at 10^oC per minute, with heat increase terminating at 900^oC under continuous flow of nitrogen. Thermograms were produced indicating percentage mass changes on the samples as a result of the heat increase. PyrisTM software (PerkinElmer, Llantrisant, Wales, UK) was used to analyse the thermograms. Thermoigravimetric analysis was performed in order to assess heat flow and mass change of the sample in relation to temperature increase.

4.2.3. Differential scanning calorimetry on layer-by-layer optimised nanosystem to investigate thermal transitions

Thermal stability, endothermic and exothermic changes and the determination of the glass transition temperature were elucidated using differential scanning calorimetry (Mettler Toledo, Schwerzernback, Switzerland) for the optimised formulation. The instrument was configured to heat the samples to a maximum of 150^oC. The samples were heated at a rate of 10^oC per minute. The sample was analysed on a layer-by-layer basis to ensure determination of the polymers responsible for significant endothermic and exothermic changes. The heating also allowed for further investigation of endothermic and exothermic changes due to the layering. Information in this regard led to understanding of whether new bonds were formed.

4.2.4. Fourier-transform infra-red analysis on the layer-by-layer optimised nanosystem

FTIR spectroscopy (Perkin-Elmer, Beaconsfields, BUCKS, UK) was used to determine possible chemical changes in the formulation due to potential interactions between the polymers employed to produce the nanoparticles during formulation and the layer-by-layer technique. The samples were analysed over 25 scans over a range of 4000-550cm-

1. FTIR was performed on the compounds singularly and on the nanoparticles on a layerby-layer basis in order to examine possible chemical changes.

4.2.5. X-Ray crystallography analysis for determination of the degree of crystallinity of the optimised nanosystem

The configuration and arrangement of the polymers can be examined through XRay crystallography (MiniFlex 600, Rigaku, Japan). The mixing of the polymers may cause varying arrangements: these being crystalline, semi-semi-crystalline and microcrystalline. Defining these configurations is important as the arrangement affects drug delivery. X-Ray results of the native polymers and of the nanobubbles were performed at a scanning rate of 5^o/min with a scope of scanning set at 2θ ranging from 5^oC to 90^oC

4.2.6. Rheological studies for determination of properties of IONBS formulation

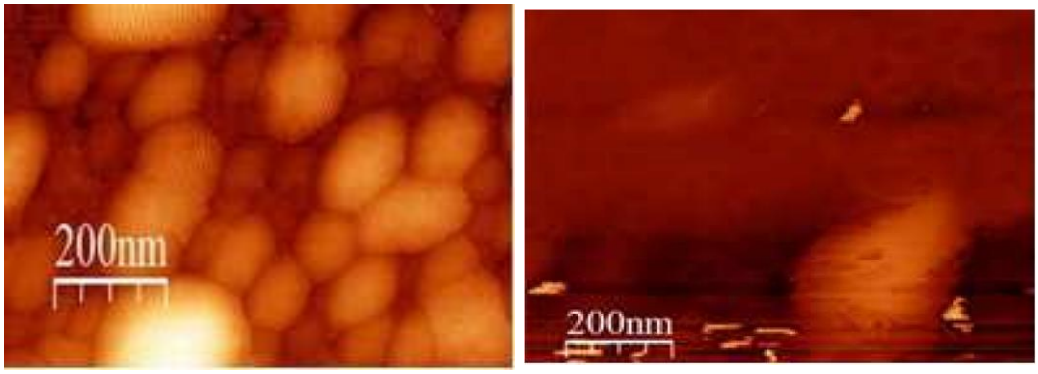
The IONBS was to be suspended in a solution that will ensure the system is able to remain stable, and once in contact with the eye, be able to release the drug entrapped system. The rheological behaviour of the formulation was examined using a Haake Mars Rheometer (Waltham Massachusetts, USA) Poloxamers have been found to be mucoadhesive (Ludwig, 2005) and thus have the potential to pass through some of the barriers in the eye. In this system, Pluronic F127 and Pluronic F68 were used at a 1:1 ratio.

4.3. RESULTS AND DISCUSSION

4.3.1. Investigation of size of the optimised nanosystem using atomic force microscopy and zetasizing

Atomic force microscopy reveals nanoliposomes within the 200nm range. Atomic form microscopy has also shown effective layering of the chitosan and polyethylene glycol. A 2 dimensional image displays effective layering and a thickened shell (see Figure 4.2).

The nanoliposomes are also rounded in shape and the sizes are mostly uniform.



(a) (b)

Figure 4.2: Atomic force microscopy image of optimised nanoparticles. (a) General morphology of the nanoparticles. (b) Particles within the 200nm range with demonstration of the thick layering

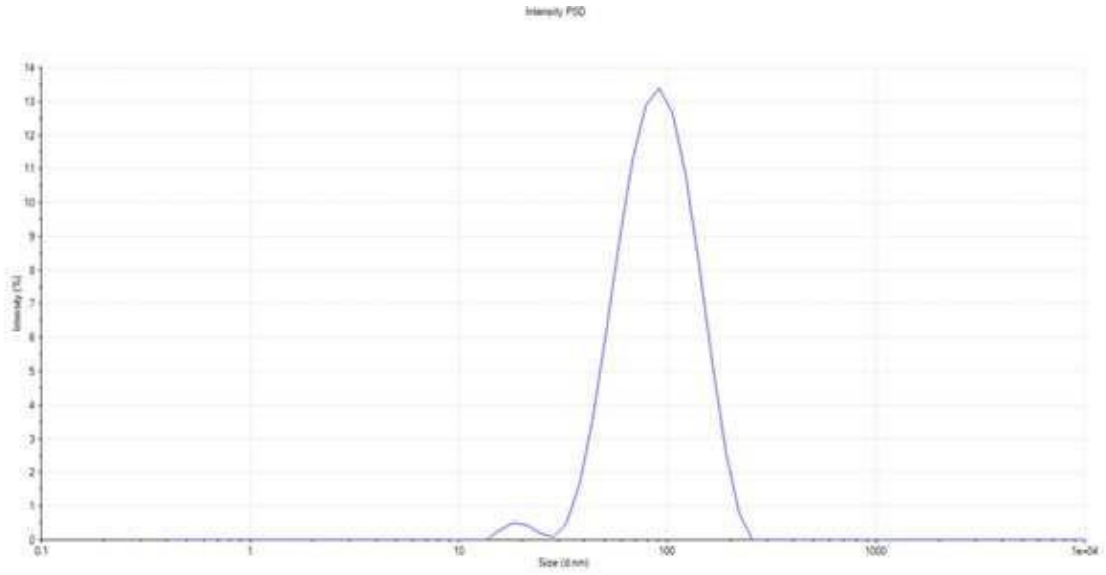


Figure 4.3: Zetasize analysis of the optimised formulation with a final size of 79.64nm

4.3.2. Heat signature studies using thermo-gravimetric analysis on layer-by-layer optimised nanosystem

Thermogravimetric analysis, shown in Figure 4.4, has elucidated that the layer-by-layer nanoparticles are stable in terms of mass percentage loss up to $100^{o}C$. Ten percent of the weight is lost within the initial minutes of the run. This could safely be concluded to be water vapour settled on the sample. From 100”C to $250^{o}C$ a loss of 5% in weight is lost. This negligible loss over $150^{o}C$ corresponded to high stability of the nanoparticles within

the 250^oC range. This corresponded to stable and strong possible covalent bonds. However, much change was observed past this point: i.e. over 50% loss of weight of the nanoparticles took place between 250^oC to 350^oC. This was the most significant loss.

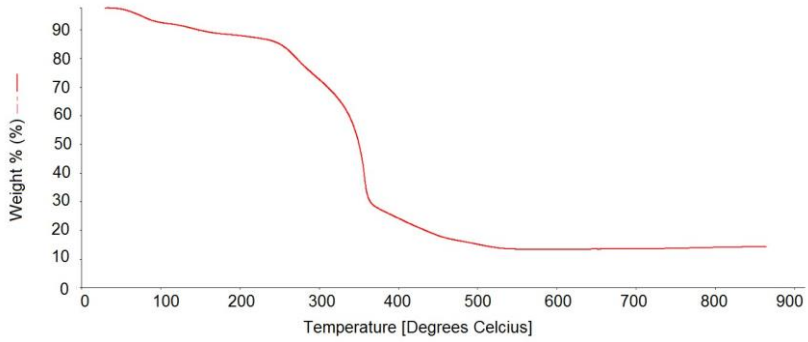
From 350^oC to 550^oC, a further loss was observed and 20% weight was further lost until just below 15% of the sample weight is left. Heating from 550^oC to 900^oC revealed no further loss of weight of the nanoparticle samples.

From the thermogravimetric analysis of the placebo, shown in Figure 4.4, nanoparticulate system revealed a similar heat signature to the optimised nanosystem. The placebo, like the drug-loaded particles, displayed a loss of weight within the temperatures of 100^oC due to the loss of water vapour. More of the samples weight was further lost between 350^oC to 550^oC, as with the drug-loaded formulation. The difference came after 500^oC. All the sample weight was lost at 500^oC in the placebo, whereas this did not occur with the drug-loaded formulation. This meant that the addition of the drug produced a stronger nanoparticulate complex and thus required more energy to be added to the sample to affect more loss in mass. The reactive aromatic rings and double bonded oxygens within the carboxylic acid functional groups may be responsible for the changes. Aromatic rings and double bonded required energy to break the bonds and thus in a heat signature, mass was lost if a high amount of heat was applied.

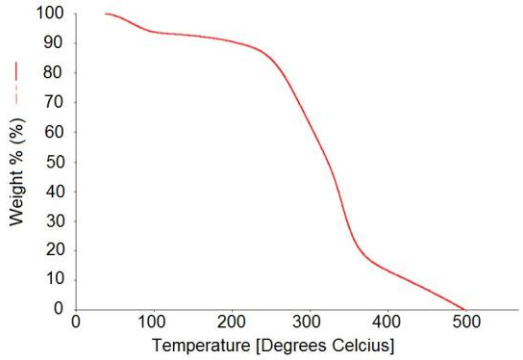
It was proposed that complexation of the polymers did not occur on nanoliposomal formation as the heat signatures of native polymers show a different pattern to that of the nanosystem. Native cholesterol lost the majority of its weight at about 350^oC. Chitosan lost 40% of its total weight at about 300"C. A further 50% weight loss was experienced in a linear fashion, from 300^oC to 620^oC. From 620^oC, only 10% of the total sample remained with no further weight loss. Native Peg 4000 was the most stable polymer at the higher temperatures. It lost 60% weight until 300^oC, and thereafter no further weight loss occurred until the sample reached 900^oC. However, the final optimised formulation with PEG 4000 as the final layer showed further weight loss with temperatures over 600^oC, indicating a change in the native structure of the PEG. Chitosan lost most of its weight between 300^oC and 600^oC, with general stability before 300^oC. The final optimised structure ceased to lose weight after 500^oC. This change could be attributed to possible

bonds formed between the two layers. This is further explained by the FTIR data presented in a subsequent section.

TGA showed that the nanoliposomal complex was stable. The placebo profile and drug-loaded complex showed a similar profile meaning that the drug was fairly unchanged and thus able to perform its therapeutic function.



(a)



(b)

Figure 4.4: Thermogravimetric analysis illustrating heat signatures of different formulations. (a) Image illustrating heat-weight signature of optimised formulation; (b) Image illustrating heat-weight signature of the drug-free (placebo) optimised formulation

4.3.3. Differential scanning calorimetry on layer-by-layer optimized nanoliposomes to investigate thermal transitions

A layer-by-layer nanoparticulate system is necessary as it ensures the drug is enclosed and that the gas is entrapped on nanobubble formation. Differential scanning calorimtery (DSC) elucidates a heat signature for the nanoparticles. The individual components also underwent DSC, as shown in Figure 4.5. Superimposition of endothermic graphs displays a change as the layers are added on the nanoparticles. Previous authors have shown that

chitosan-polyethylene glycol interactions involved a packing motion over one another with no significant new bonds formed (Aktaset al., 2005). However, a significant endothermic peak was observed in the multilayered optimised formulation. This peak may be due to new intermolecular forces formed by hydrogen bonding, the strongest of intermolecular forces and may account for the peak. The hydrogen bonding would likely occur between the hydrogen in carboxylic acid in the terminal glucosamine ring (of the chitosan) and the highly reactive oxygens double-bonded to the carbon atom in the polyethylene glycol.

The single layer and innermost layer, consisting only of soybean oil and cholesterol, displays a largely heat stable combination. The lowered temperature corresponds to the loss of water vapour. The peak noted in the initial phase of the heating is likely due to unreacted soybean oil or cholesterol.

The single and double layer combination (soybean oil and cholesterol, layered with a chitosan solution) displayed a constant lowering of the graph and a peaking of the graph. There are no distinct peaks in this sample. This reveals that there may be new bonds or new forces that develop between the first two layers.

The optimised and fully layered drug delivery system showed a significant difference from the previous two layers. Chitosan and PEG do not form significant new bonds by being combined together in formulation and this is seen in the profile of the optimised later. Thus we could conclude that the addition of PEG may correspond to strong intermolecular forces being formed, likely to be hydrogen bonding. PEG as a chemical structure has numerous double bonds and oxygen molecules able to form hydrogen bonds with neighbouring molecules. Thus, when adding the chitosan and polyethylene glycol, strong intermolecular bonds can be formed between the polymers used in the formulation.

In our case, DSC (the heating of the drug delivery system to 150^{o}C) suggested that there may have been formation of new bonds. Three layers would suggest that one would suspects 3 peaks; however, 2 peaks suggested that either certain polymers have the same peaks or that chemical bonds formed between certain polymers and allowed for the formation of a new peak. In the study where PEG was used, soybean oil, cholesterol, and Tween® formed the major components of the first layer, the second was chitosan, and the

third was PEG 4000. Both graphs had two peaks but at different temperatures. This means that the polymers used after being cooled did not return to their natural state. Hence, the number of peaks stayed the same, but the melting temperatures differed because the polymers may have been chemically modified with the addition of heat up to 150^{o}C.

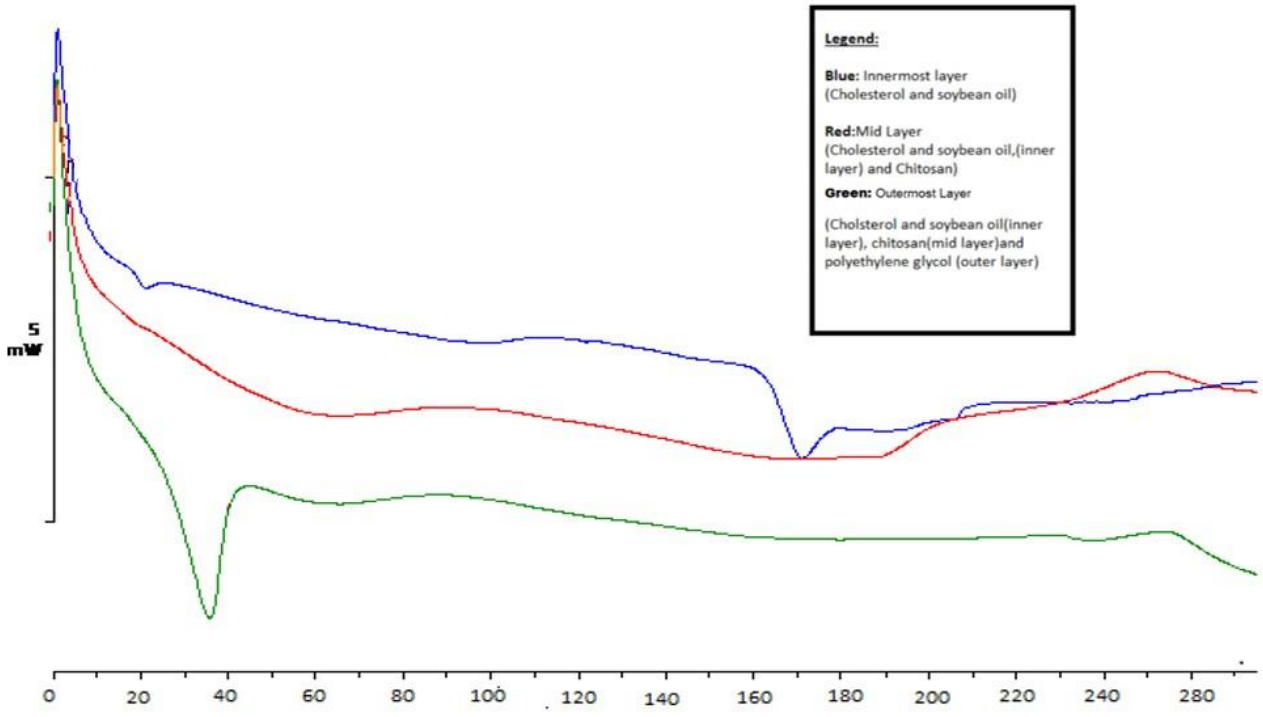


Figure 4.5: DSC thermogram illustrating the heat-reaction of multilayered drug delivery system, layer-by-layer

4.3.4. Results: Fourier-transform infra-red analysis on the layer-by-layer optimised nanoliposomes

FTIR studies were conducted on individual components of the nanoparticles and on the nanoliposomes. The innermost layer, composing of soybean oil and cholesterol, revealed a number of chemical changes due to the mixing of these lipids. Soybean oil consists of oleic acid, linoleic acid (9,12 cis, cis and linoleic acid [9,12,15, cis, cis, cis]). These long hydrocarbon chain molecules possess carboxylic acid functional groups, prone to reaction with other polar functional groups found in cholesterol. A mix of the two polymers reveals a disappearance of a hydroxyl group in the cholesterol polymer and a decrease in intensity of carboxyl groups in the soybean oil. This reveals that a mixture of the two polymers yields very little significant change (Figure 4.6 (a) and (b)).

The polymers used included chitosan, widely used because of its non-toxic but mucoadhesive properties. Studies show that chitosan and PEG do not react to form a new compound, but the two compounds pack onto one another (Prego et al., 2006; Aktas et al., 2005).

Thus, the question remains as to the mechanism of interaction between the polymers. FTIR analysis of soybean oil, cholesterol, and chitosan reveals few significant changes in the native forms of all the polymers. The mixing of the polymers revealed a disappearance of a band in the native chitosan, as compared to the mix. This band was at 1540 and $1558 cm^{-1}$ respectively. At these bands, in native chitosan, those bonds correspond to the presence of double bonds. Thus in the mixing of the polymers, these double bonds in chitosan are broken or the unsaturated carbonyl compound are reorganised. A cholesterol, soybean oil, and chitosan mixture elicits a high intensity band at 1054 cm^{-1}. This band is at a lower intensity in native chitosan. This means that a mixture of these polymers yields carbon-oxygen bonds at 1054 cm^{-1}, which corresponds to carbonoxygen stretching .This may correspond to stronger formation of intermolecular forces, mainly hydrogen bonding between the carbonyl oxygen bonds and hydroxyl groups found. Cholesterol contains carbonyl bonds and chitosan has a hydroxyl group. The FTIR spectra corroborates largely a packing motion with the forming of new bonds with oxygens derived from carboxylic acid groups.

The addition of PEG to the soybean oil, cholesterol, and chitosan mixture reveals an addition of an H-C-H bond, but with the loss of intensity of a signal of a double bond at 3200cm-1, mostly likely at a carboxyl end of chitosan.

Worth investigating is the drug interaction with the polymers involved. FTIR spectroscopy reveals that the functional groups detected at 3300-2800 are maintained when mixed with soybean oil and cholesterol only.

Interactions with cholesterol only and indomethacin reveal the additions of hydrogen bonds. These are very strong intermolecular bonds. Indomethacin alone shows symmetric and asymmetric carbon hydrogen stretches, and the intensity of their presence is diminished once mixed with cholesterol. Thus the structure of indomethacin is maintained (Figure 4.6(d)).

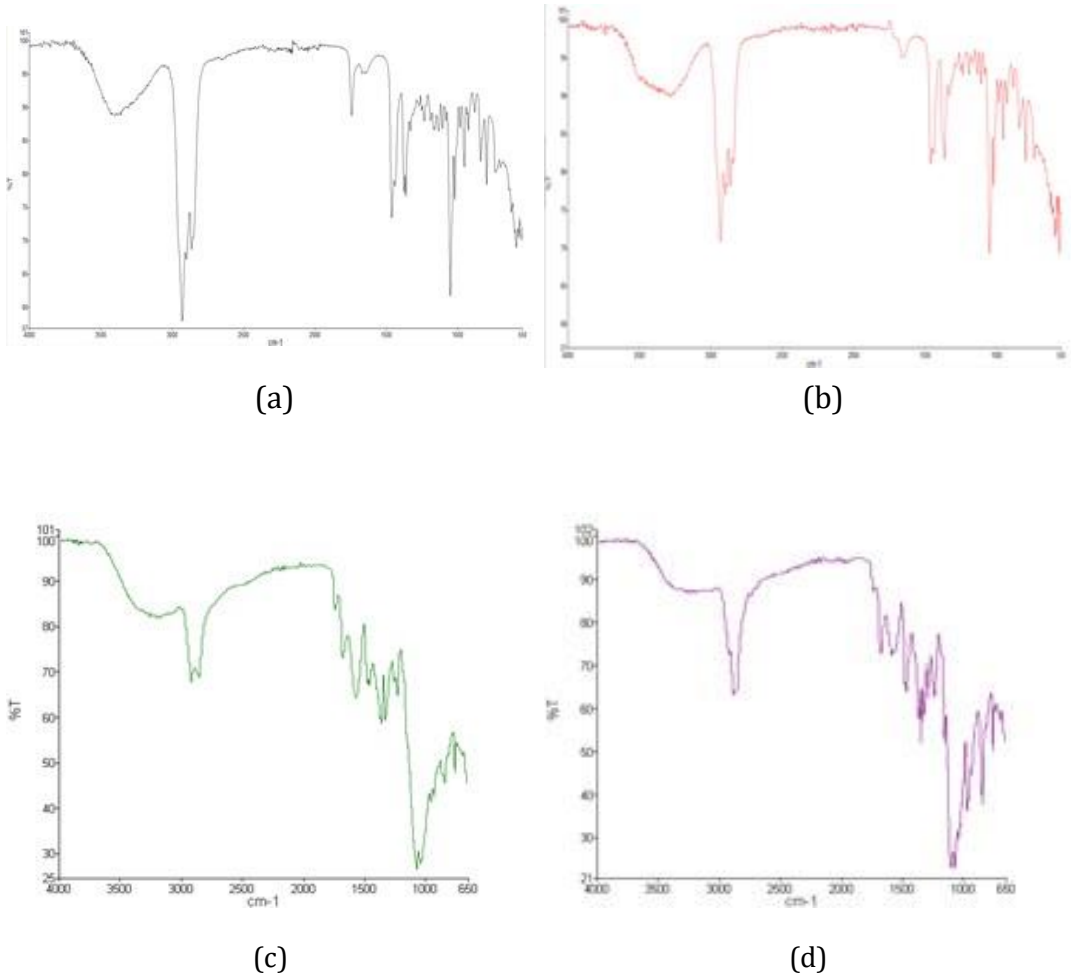


Figure 4.6: FTIR spectra illustrating the chemical behaviour of the optimised formulations (a) FTIR spectrum of drug-free nanoparticulate system with the first two layers present (innermost layer is soybean oil and cholesterol, and outermost is chitosan); (b) FTIR spectrum illustrating FTIR profile of placebo of the all layers of the nanoparticulate system (innermost layer is soybean oil and cholesterol, and chitosan and third layer polyethylene glycol 4000); (c) FTIR profile of the optimized formulation, with only the first 2 layers; (d) Diagram illustrating FTIR profile of the optimised drug entrapped, fully layered formulation

4.3.5. X-Ray diffraction analysis for determination of the degree of crystallinity of the optimised nanosystem

X-Ray crystallography has been a proven technique for analysing the possible crystalline nature of polymers. The crystalline and amorphous nature of polymers assist in ascertaining change in polymeric character in the polymers used and if there is a possibility that new bonds have been made. All these aspects will affect the rate of drug

release and stability. These aspects acted as predictors in determining whether the drug would be stably enclosed within the nanoparticulate system.

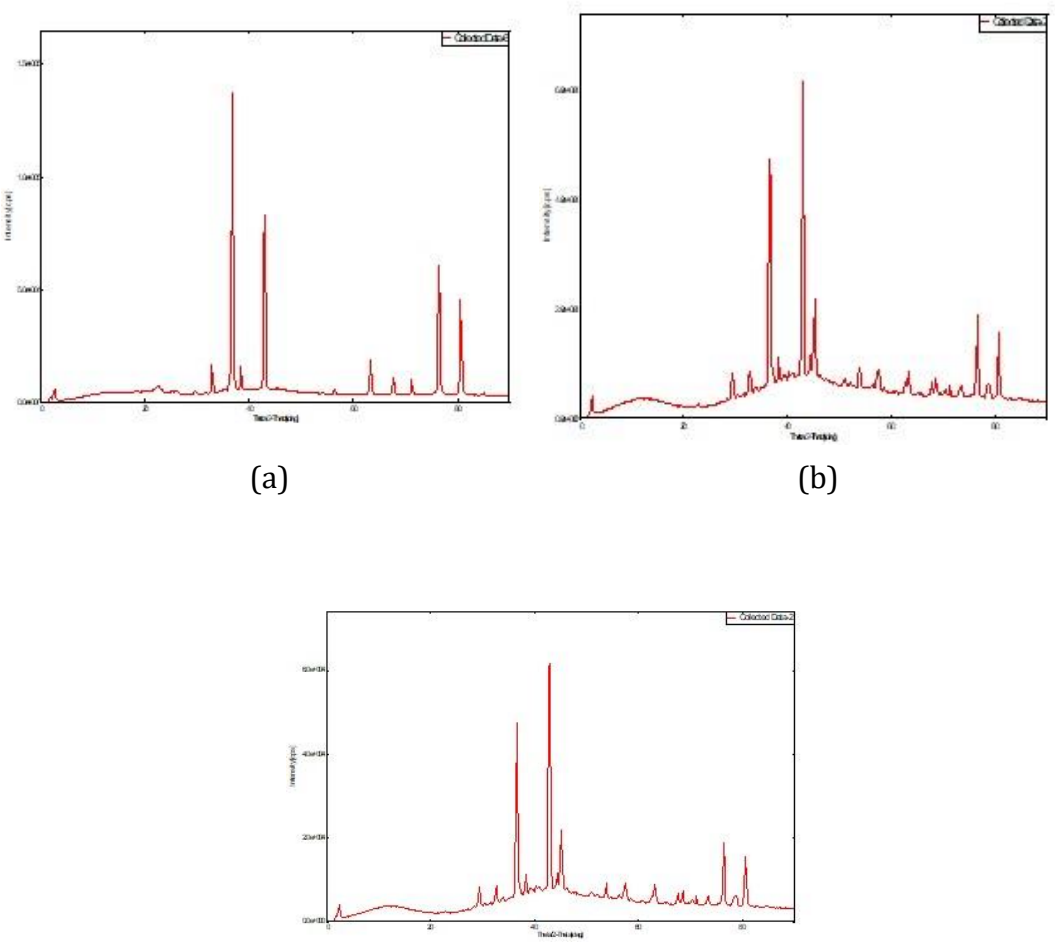
X-Ray crystallography is important in layer-by-layer drug delivery system as it sheds light on the possible interactions between the polymers involved, namely the cholesterol, soybean oil, chitosan, and polyethylene glycol. X-Ray crystallography demonstrated changes in the crystalline nature of the nanosystem.

The FTIR analysis revealed that any new bonds formed are likely to be from CHO bonds. The lack of presence of a completely new polymer corroborates a packing action in which the polymers lie closer to each other and in which the long and short polymer chains stack onto one another to form a stronger, and enclosed system. However, this may affect the crystalline nature of the drug delivery system.

X-Ray crystallography of the innermost later revealed a structure with high crystalline character. There is a small amorphous moiety. However, the larger portion of the graph elucidated a crystalline nature (Figure 4.7). There are 2 regions which reveal a high crystalline intensity. Much of the region is semi-crystalline. The addition of a layer of chitosan had significant effects on the crystalline nature of the layered system. An addition of chitosan revealed intense levels of peaks, which points to a more crystalline structure. The addition of the polyethylene glycol 4000 further enhances the crystal structure of the nanoparticulate system. The once amorphous system forms a semicrystalline structure.

X-Ray crystallography of the placebo layer-by-layer IONBs revealed the effect indomethacin has on the drug structure. A placebo structure of the IONBS had almost no amorphous region. Thus, the addition of drug affects the overall structure of the drug delivery system. The FTIR reveals that there is packing of molecules and possible hydrogen bonding. This may assist in building bonds to stabilise the drug within the inner structure. The breaking of the double bonds will likely expose the hydrogen ions and oxygen ions to participate in hydrogen bonding. These are very strong intermolecular forces and this may stabilise the IONBS structure. The innermost layer of the IONBs with drug added seems to be almost identical to the placebo IONBs. This means that the drug was unchanged when mixed with the polymers and is thus stable. Thus any new bonds were caused by the interaction between the soybean oil and cholesterol.

The structure of the placebo IONBs innermost layer was highly crystalline in structure. This structure revealed the interaction of the soybean oil and the cholesterol. The bulky nature of the double bonds would not promote a highly ordered crystal structure. The region of double bonds which are either C=C or O=O are electron rich and would be a region which would strongly repel other negatively charged ions and would strongly attract positively charged ions. The double bond environment also allowed for strong intermolecular forces, such as hydrogen bonding. Breaking of double bonds would be very challenging and require high energy.



(a) (b)

(c)

Figure 4.7: X-Ray crystallography patterns of the different formulations (a) The X-Ray crystallography profile of the optimised product (b) First 2 layers, soybean oil, and chitosan (c) First layer of the nanoparticulate system of the soybean oil

4.3.6. Rheological studies

A 1:1 ratio of Pluronic F68 and F127 was employed as the possible suspension. Rheological investigation showed an increase in yield stress and an increase in viscosity over time (Figure 4.8). This graph demonstrated a shear thickening flow of shape characterisation. An increase in viscosity may be advantageous because it may increase residence time on the ocular surface and allow for increased availability of the drug to the eye. The viscosity in the eye is approximately 3*MPas* and the tears exhibit a non-Newtonian rheological behaviour (Tseng et al., 1997). Measuring the rheological behaviour is important because changes in viscosity could irritate the eye and cause adverse effects. The shear rate during blinking also affects the bioavailability of the drug (Saettone et al., 2013). Pluronic F68 and F127 has also been used in other formulations and has been proven to increase residence time in the eye (Wei et al., 2002). A shear thickening character flow character will be able to overcome the high shear rate in the eye and be able to increase residence time in the rabbit eye and has been achieved in other research.

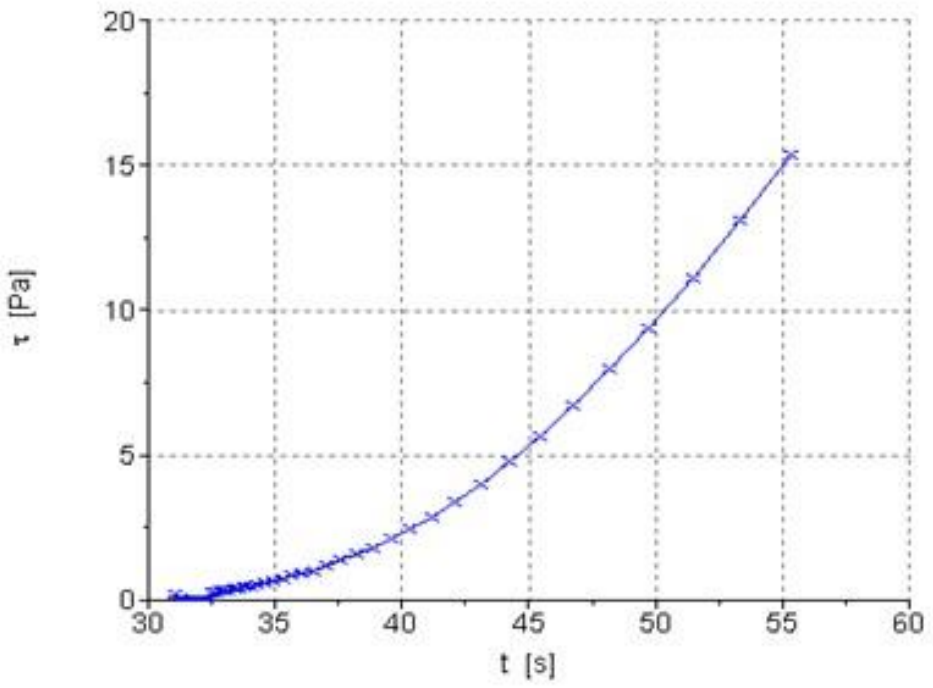


Figure 4.8: Rheological behaviour of nanobubble formulation

4.4. COMPARATIVE OVERVIEW

In comparing the studies and results in this studies, the novelty of this study is reinforced given that there is not a lot of body of work in nanobubbles, but more on nanoliposomes and nanoparticles. More recent studies in nanobubbles were used for intravitreal injections, Ultrasound was used to manage responsiveness and management of migration of the nanobubbles, and not for real-time viewing (Thakur et.al, 2019).

Other studies have prepared nanoparticles for ocular drug delivery (Balguri et al., 2016). These particles also made use Tween®80 and pluronic, the drug used was indomethacin. The particles were approx. 265nm in size and the highest polydispersity was 0.3. The drug entrapment efficiency was 81%. Chitosan chloride was use to modify the lipid surface. This study reinforces all the combinations and formulations of the study undertaken. This study also intended the particles for topical administration. PLGA nanoparticles were also produced for posterior ocular drug delivery for topical administration (Tahar et al.,2017). These particles were modified with chitosan and Tween®80. The modifications were studies to determine which modification enhances penetration. The mouse retina was the model to examine penetration. In this case, the use of Tween®80 was used to modify the nanoparticulate surface and enhance penetration into ocular tissues. The particle sizes varied depending on the modifications used. The sizes were 240.7nm modified with Tween®80, 332.7 with chitosan and 518.6nm with glycol chitosan (Tahar et al.,2017). Stability and heat signatures are also an important part of formulation analysis. Other nanoparticulate formulation for topical ocular drug delivery have been examined. Differential Scanning Calorimetry (DSC) of PLGA nanoparticles for topical formulation using the model drug hydrocortisone butyrate (Yang et al., 2016) showed that addition of polymer may affect the crystalline behaviour of the model drug. In our formulation, the structure of drug was not changed as per DSC analysis, and corroborated by X-Ray Crystallography analysis. The particles by Yang et al. are also densely packed as with ones viewed by this study using AFM. Other formulations, have made use of Tween®80 and pluronic-F68, along with Triamcinolone acetonide as a drug to produce nanoparticles for the posterior eye. The polymers used were glyceryl monostearate and Compritol® 888ATO, with the addition of gellan gum and sodium carboxy methyl cellulose in situ gel (Tatke et al., 2019). These nanoparticles

were topical administration to the posterior eye. In this study the size differing concentrations of polymer, drug and in situ gel produced nanoparticles with a size range of 187.5 nm to 400nm. The drug entrapment efficiency was above 90% in all formulations. In the DSC analysis, of the particles, no endothermic peak is observed for the drug, showing integration of the drug into the polymer formulation.

4.5. CONCLUDING REMARKS

The optimised formulation is stable at physiological temperatures. The optimised formulation does not have a viscosity profile that is adverse to the eye as poloxamers have been proven to be mucoadhesive. The final structure is more crystalline and loses amorphous traits with the addition of chitosan and polyethylene glycol. Indomethacin forms bonds with the non-polar cholesterol and oil, but its structure is not altered, thus it retains its therapeutic capability.

In other studies, a more amorphous structure is achieved and is evidenced by DSC studies and X-Ray Crystallography studies. A few comparisons reinforce the novelty of the study with regards to the use of nanobubbles specifically for real-time tracking. Comparative studies also show that the formulation requires further optimisation to improve on drug entrapment efficiency.

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CHAPTER 5
IN VIVO PERFORMANCE OF THE INTRAOCULAR NANOBUBBLE SYSTEM

5.1. INTRODUCTION

As discussed, creating an ocular formulation that ensures high compliance will contribute to the most effective treatment of uveitis and ensure that its treatment does not cause further complications. Rabbits are widely accepted to be the common model for ocular pharmaceutical research (Barar et al., 2009). The rabbit eye has similar dimensions and drug delivery transport model to the human eye. The rabbit eye is 0.25mm thick, while the human eye is 0.39 mm thick at its equator (Olsen et al., 1998). As compared to the human sclera, the rabbit sclera has similar permeability (Ambati et al.; 2000; Crysberg et al, 2002). The rabbit a vitreous volume of 1.5ml to 2.5ml (Killey et al., 1978), while the human vitreous has an approximate volume of 4ml.

In previous chapters, the optimised formulation of the nanoparticles was determined and its characteristics delineated using various methods. The action of the nanoparticles *in vivo* is a consideration in determining whether the nanobubbles formulation would be suitable for use in humans.

This investigation entailed observing and ascertaining whether the formulation irritates the eye, or has adverse side effects.

Another important aspect is determining whether the formulation can be tracked using ultrasound techniques.

5.2. MATERIALS AND METHODS

5.2.1. Studies in determining reaction of nanobubble formulation *In vivo*

The degree of anterior inflammation in both eyes was visually quantified by applying slit-lamp examinations for evaluating the symptoms of uveitis. Clinical distinctions for inflammation within eyes was performed using Hogan's classification method (Hogan et al., 1959) and quantified via a scoring system of symptoms (conjunctival hyperaemia, photophobia, pain, watering/ lacrimation), where Grade 0= absent, Grade 1 = mild, Grade

2 = moderate, Grade 3 = severe and Grade 4 = extremely severe. Rabbits were divided into three test groups for experimental study.

In vivo animal studies were conducted on 60 rabbits (ethics clearance number: 2013/44/04) at the Wits Central Animal Service in order to determine the ability of the nanobubbles to be tracked using ultrasound technology, per Figure 5.1. Sixty rabbits were equally divided into 3 groups and were sacrificed at specific time points. Prior to any sacrificing, the rabbits were placed under anaesthesia using ketamine and xylazine. Group 1 was administered the nanobubbles suspended in a mixture of 1ml Pluronic F68 and F127. Rabbits were administered with the nanoparticulate system in the cul de sac of the eye system. These rabbits were sacrificed at time points, 1 hour, 6 hours, 18 hours, 24 hours and 48 hours. Five rabbits were euthanised at each time point. Group 2 received a placebo of the nanoparticulate system suspended in the Pluronic mixture. These rabbits were sacrificed at time points of 1 hour and 48 hours. Group 3 involved injecting a 200µL saline solution of indomethacin. The administered eye was viewed using a Vevo®2100 ultrasound system. The eye was then enucleated and Vevo viewing was conducted again. The Vevo viewing was conducted from the anterior (the cornea) and from the posterior (near the optic nerve) of the eye.

Test group 1(n=25): These rabbits were administered the intraocular nanobubble system (IONBS) in the left eye, with the drug indomethacin incorporated into the drug delivery system. The movement of the IONBS was monitored using the *Vevo®2100* viewing system at 1, 6, 12, 24, 48 hours after administration of the IONBS. Sampling and euthanasia took place subsequent to monitoring at each time point.

Test group 2 (n=10): The rabbits received the intraocular drug delivery system with no drug (i.e. placebo) in the right eye. The movement of the placebo IONBS was monitored using the *Vevo®* at 1 and 48 hours after administration of the IONBS. Sampling and euthanasia took place subsequent to monitoring. The rabbits were sacrificed subsequent to viewing on the *Vevo®2100* system and intraocular pressure examination at the proposed time points.

Test group 3 (n=25): This third group received intraocular injection of a dilute solution of indomethacin. No viewing of nanoparticles was done in this group as only a dilute solution was used and it has no contrasting agent.

In order to verify that the nanobubbles could be tracked for theranostic purposes, animal studies were necessary. Tween®80 found in the innermost layer the formulation was considered to be a contrasting agent and could assist in the viewing process (Wang et al., 2010). However, in order to amplify the ability to view the particles, sulphur hexafluoride was incorporated into the nanoparticles to produce nanobubbles. In order to do this, a solution of the particles was sonicated in the presence of the gas.

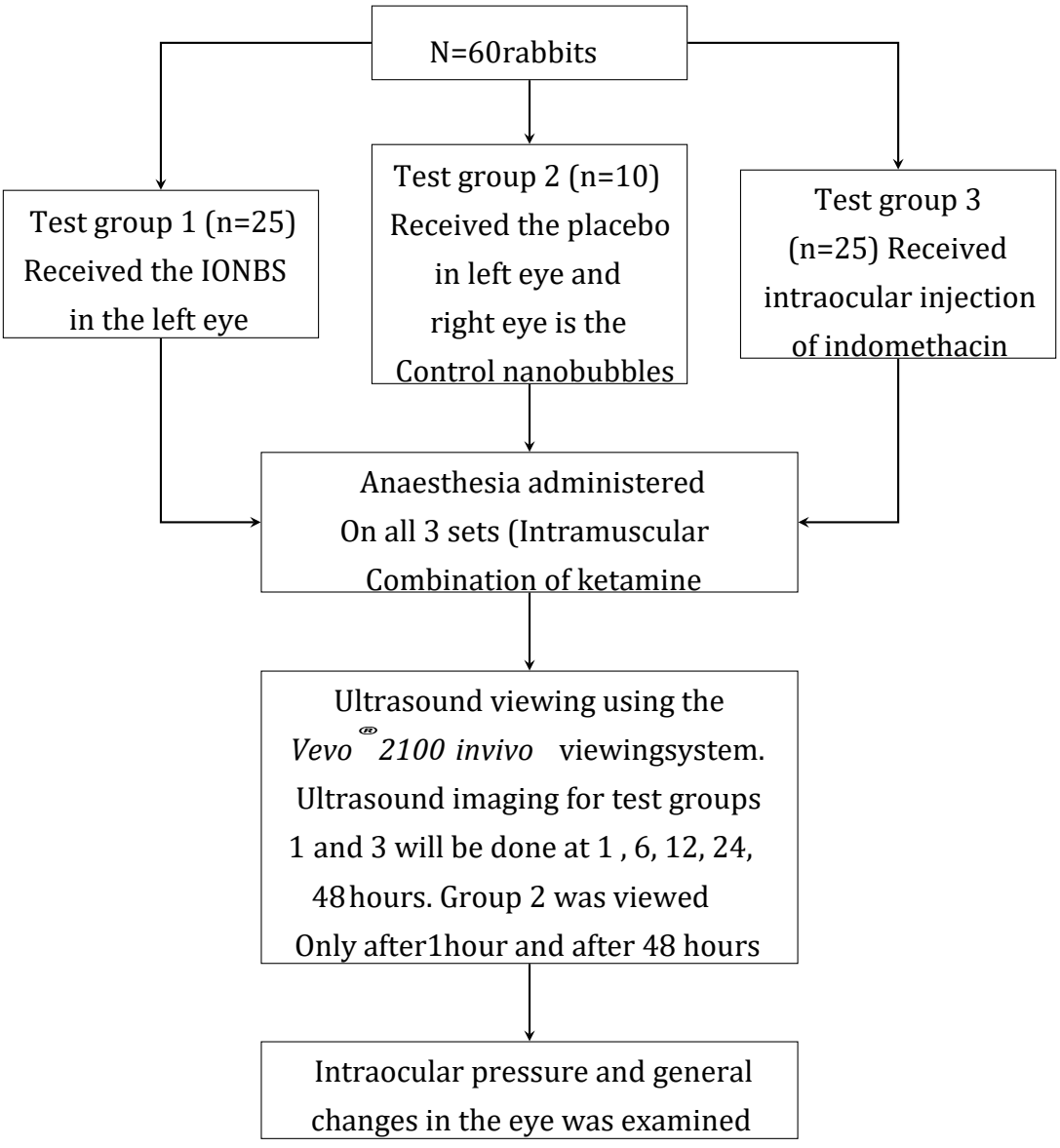


Figure 5.1: Flow diagram illustrating the process followed for *in vivo* rabbit studies

5.3. OBSERVATIONS, RESULTS & DISCUSSION

5.3.1. Ultrasound viewing of the nanobubbles *In vivo*

Ultrasound viewing elucidated the high probability that the theranostic possibilities are viable in the posterior segment of the eye. The images demonstrated an outline of the eye especially of the cornea. However, due to the use of the larger probe, the resolution was poorer, as the probe was large. Another image below shows an ultrasound image using a GE model ultrasound device with a small probe and the image is clear. In ensuring that nanobubbles were being viewed, a control in which there were no particles revealed a clear screen in which no interference was viewed. In this control, only the general outlines of the eye such as the cornea, were observed. What was evident was the fact that bubbles were still identifiable at 48 hours. The longer the bubble lifetime, the greater the time for therapeutic dose to be released. This includes the possibility that perhaps the suspension can be applied only once daily because bubble lifetime may aid longer drug release. This may be related to the half-life of the drug and the bubble lifetime. It was noted that the particles were easier to visualise and track once the eyes were enucleated as the probe could move easily. Worth noting is the fact that it was easier to view the nanoliposomes in earlier time points such as 1 hour and 6 hours. They were more challenging to spot in the later time points.

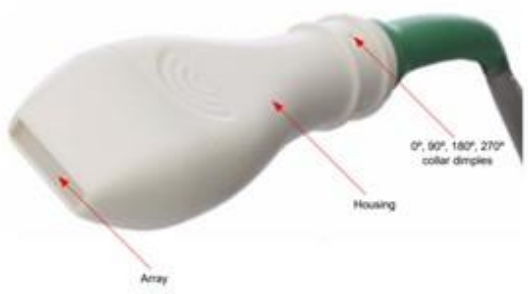


Figure 5.2: Probe used by the *Vevo®2100* in viewing the rabbit eyes. This probe allowed for the viewing of the rabbit eyes through ultrasound techniques

5.3.2. Histological analysis of the rabbit tissue post-mortem to determine side effects of the nanobubble formulation

Histological analysis confirmed previous research findings that intravitreal injections (group 3) caused notable trauma to the eye. This corroborates studies that show that intravitreal injections cause damage to the human eye. Histological analysis was performed by Idexx laboratories (Pretoria, South Africa). Inflammatory responses were detected in eyes after each time point. The limbus and conjunctiva were highly inflamed. There was a high concentration of lymphocytes and heterophils. Mild oedema was also present in some eyes, such as in the conjunctival area. In the later time points, heterophils and lymphocytes were also spotted in the optic nerve and vitreous humours. In the later hours, the vitreous humour was found to be highly eosinophilic. Eosinophils are inflammatory cells that are elicited when the immune system detects entrance of foreign bodies in the body (Hogan et al., 2008). Indomethacin was introduced intravitreally.

Group 3 was the use of an intravitreal needle to inject indomethacin. The conjunctiva is the most inflamed area, in all the samples. The vitreous humour was inflamed; however, the inflammation was not present at 48 hours. The retina and optic nerve had little inflammation, which is a good sign in terms of a drug delivery.

Group 2, receiving the placebo nanoparticulate system showed signs of mostly mild inflammation. The group 2 rabbit eyes, which are the placebo group, were also used as positive controls as the nanobubbles had no drug. Worth noting is that the eyes with no drug delivery system and no placebo, i.e. the positive control also contains inflammatory cells. High inflammation was spotted in the limbus and conjunctiva. In all the group 2 samples, the vitreous was filled with eosinophils. This may be due to the stress of the rabbits having to rehabilitate from their original habitats or the introduction of a foreign body such as the nanoparticulate system.

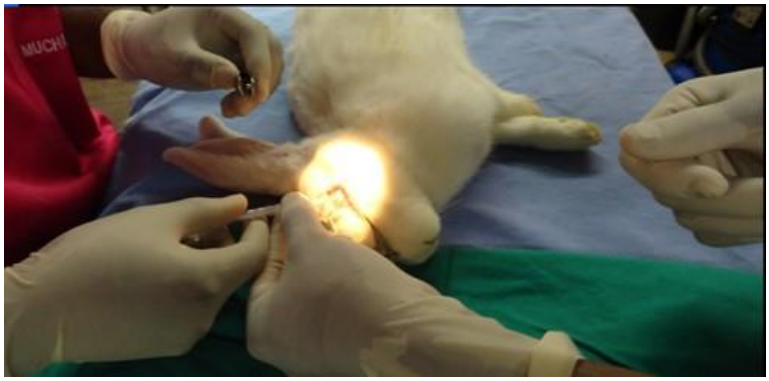
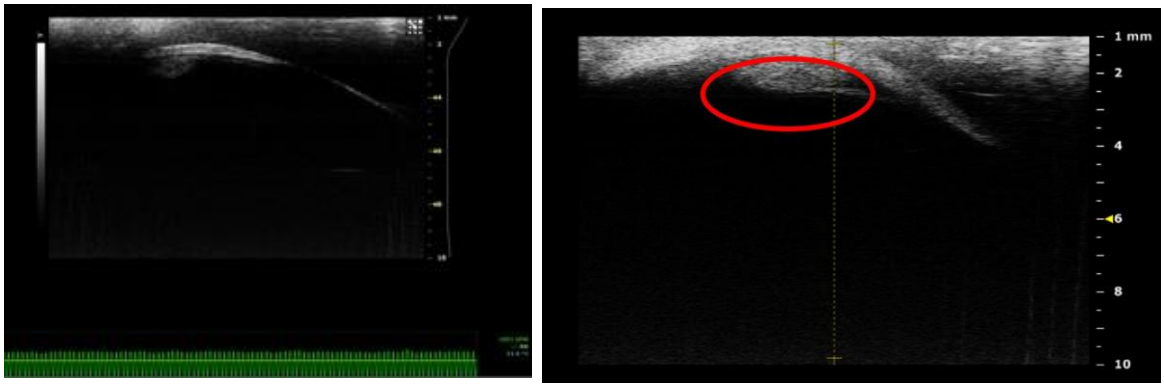
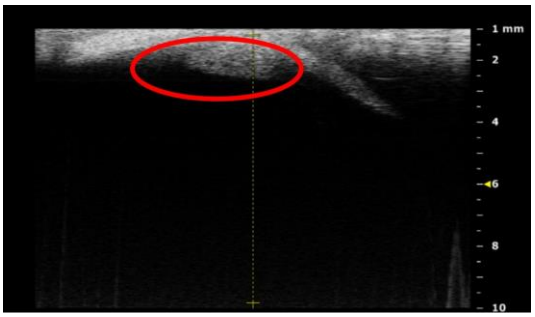


Figure 5.3: Handling of the rabbits as IONBS is inserted in the rabbit eye



(a) (b)



(c)

Figure 5.4: Diagram illustrating the rabbit eye viewed by means of ultrasound viewing using a *Vevo®2100* (a) Ultrasound image of the eye using a *Vevo®2100* of a rabbit eye with no nanoparticles. This would be the control; (b) Ultrasound image showing outline of cornea and movement of nanoparticles behind ocular surface; (c) Ultrasound image showing outline of cornea and movement of nanoparticles behind ocular surface. This is 1 hour post administration of the nanoparticles move.

In Group 1, the rabbits received the drug-loaded intraocular nanobubble system. Much of the organs of the eye seemed to react compatibly to the drug delivery system. Irritation was spotted in the conjunctiva and limbus. Other key tissues such as the anterior uvea, choroid and sclera showed little or no inflammation. The vitreous humour seemed to produce eosinophils and this could have been caused by the gas used to fill the nanobubbles.

Ocular irritation is an important part of assessing whether a formulation is safe to use in rabbit eyes. The study conducted measured irritation and determined low to moderate inflammation, A Draize test may be used to measure safety for the eyes (Platania et al, 2019). Ocular irritation can also be studied at the ex vivo model and studying the levels of heat shock protein. Other studies have tested gels on rabbit eyes for opthalmological use. One of the more recent studies have been conducted by Ranch et al. (2019). This study aimed to determine the optimised combination for the use of Carbopol, gellan gum and benzododecenium bromide. Ocular irritation was found to be minimal and thus the formulation was considered safe.

Recent studies are showing that ocular irritation is an important marker and this is tracked through a number of ways. Cellular response was tracked in this study, and ocular irritation was spotted, and thus the formulation would require further optimisation.

5.4. CONCLUSION

Topical ocular administration for the treatment of inflammation in the posterior segment of the eye is indeed possible as viewed by the *Vevo®2100* imaging system showing the movement of the IONBS through the eye. The use of contrast agents allows for monitoring of therapeutic efficiency in real time – thereby giving the nanoparticulate system theranostic capabilities. However, the probe used should be smaller and able to adequately ensure resolution is high. Further considerations and improvements should be around improving the entire system (including the gas and suspension) to ensure that there are much fewer inflammatory responses. The improvement should entail a smaller probe with higher resolution capability to navigate the ocular orbit. However, lack of necrosis during tissue analysis revealed an intraocular nanobubble system with much

potential as an alternative to the use of invasive techniques in treating diseases present in the posterior segment of the eye.

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CHAPTER 6
CONCLUSIONS AND RECOMMENDATIONS

6.1. INTRODUCTION

Nanotechnology has been hailed as an emerging technology and has had many applications in various other fields of science. As discussed earlier, treatment of posterior ocular disease and inflammation has seen growth and evolution, ranging from intraocular injections to implants. However, due to the associated risk, treatment administered topically would be the most optimal solution.

The review demonstrated the use of nanoparticulate systems as a viable manner to combat posterior uveitis. The anatomical barriers related to ocular drug delivery were discussed. The different drug delivery systems were discussed and the advantages and disadvantages of each system. The progression of uveitis and how it affects the different areas of the eye was discussed. The complications related to the use of intravitreal injections was also highlighted. Ideal polymers related to ocular drug delivery have been noted as Distearoylphosphatidyl choline (DSPC) and distearoylphosphatidyl-ethanolaminepoly (ethylene glycol) (DSPE-compounds). However, there is also an emphasis on ensuring that the polymers used do not elicit an immunogenic response.

This study has proven that more ubiquitous polymers not needing high levels of biological modification can still be of use in ocular drug delivery. This study revealed lower drug entrapment efficiencies than those observed in other studies. Thus the current formulation may require further optimisation. This may mean that using fewer layers, and still optimising for a small size may(approx. 220nm), may improve entrapment efficiency, which is a clear shortfall in the study conducted.

Preformulation studies were also conducted and they highlighted the importance of a contrasting agent, Tween®80. Ubiquitous polymers such a cholesterol and soybean oil were used. Eudragit® was also used as an option for preformulation studies. The studies also involved the use of chitosan and polyethlene glycol as additional layers to further protect the innermost layer, containing indomethacin. Pre-formulation studies also aided

in determining the factors of the optimal size, drug entrapment efficiency and drug release profile.

In the Box-Behnken design, the exact parameters for an optimised formulation were determined. Further studies characterised were the properties of the optimised formulation: thermogravimetric analysis, differential scanning calorimetry and Zetasizing studies and Fourier Transform Infra Red (FTIR). The factors affecting size and increased drug entrapment efficiency were also examined.

In Vivo studies were conducted on the optimised formulation and the ultrasound directed real-time viewing studies were completed.

Ultrasound imaging allows for real-time viewing and tracking of the nanobubbles. This has significant implications in ocular treatment as this eliminates invasive treatment of inflammation in the posterior segment of the eye. The ability to view the nanobubbles in real time allows for both therapeutic treatment and for diagnostic capabilities of the nanobubbles.

The use of intraocular injections, currently the gold standard, comes with many complications such as the formation of cataracts, at least 2 years after, and thus a further surgical intervention would be required.

6.2. CONCLUSIONS

6.2.1. Benefits of nanotechnology

Polymeric nanotechnology has shown benefits in various pharmaceutical applications. The use of polymers and the processes in which the particles are formulated allow for manipulation of the device. This means that one can control release of the drug under varying conditions such change as temperature and change in pH changes although the IONBS in this study were temperature independent and pH independent. Nanotechnology also allows more effective treatment because the use of nanotechnology has the ability to ensure high drug entrapment. This is because of the high surface to area ratio for drug entrapment, which will ensure that the drug may only need to be administered once or twice a day, also in the case of ocular drug delivery. Nanotechnology

is applicable in other environments too. Nanotechnology has been used in the mining industry to extract minute parts of precious minerals.

6.2.2. Challenges and solutions to ultrasound directed real-time viewing

Ultrasound has been used in many aspects of science and is a sound imaging and tracking method as it does not affect the cells exposed to it. Ultrasound in this study would for instance allow for viewing and examining for disorders such as retinal detachment. Ultrasound viewing has shown to be promising when viewing internal organs. Ultrasound has also been shown to be effective in viewing and measuring blood velocity in the posterior segment of the eye.

A possible improvement to the study could have been to use the surfactant as independent variable in the study. It might have been optimal, to produce the nanoliposomes, then introduce the gas, follow up with view using the ultrasound viewing system, in order to measure what factors contribute to the quality of the resolution, in order to optimise on real time viewing. Using ultrasound, the images had a poor resolution. The outline in the images shows the outline of the eye. However, the full eyeball is not visible due to the size of the probe. This hinders viewing of the system past the mid-point of the vitreous humour although histological studies show that the vitreous humour is exposed to the IONBS. Finding the best concentration for the contrast agent in relation to size would have strengthen resolution in viewing

6.3. RECOMMENDATIONS AND FUTURE OUTLOOK

The challenge associated with the study is the resolution of the images. This can be resolved using a smaller probe with higher resolution and further investigating on the use of better surfactants and appropriate concentrations. Further studies need to examine the safety of the gases used as part of the IONBS. According to the histology report, there is an immunogenic response to the use of the nanoparticles. The conjunctiva is inflamed and contains heterophilic cells. This takes place within the first hour.

However, after 24 hours, inflammation clears and the cornea, sclera, retina, and lens are normal – but the vitreous humour appears to be inflamed. In the animal studies where the IONBS was administered, the limbus is the most inflamed, while with the rabbits that

received the placebo, the conjunctiva was inflamed. For the group that received a saline solution, the uvea and vitreous humour were most inflamed.

The formulation of the IONBS has applications in other areas. The IONBS can also be modified with antibodies. The addition of antibodies can assist to improve specificity. For instance, antibodies to immune cells can be chemically attached to the surface of the nanoparticulate system. This can assist to treat inflammation and achieve high specificity. Genetic markers and sequences can be incorporated into the nanoparticulate system to anneal to specific sequences which may be found in cancer cells and thus assist with theranostic treatment in cancer.

These formulations may also be used in other settings outside of biomedical sciences. Nanoparticle systems can be used in water treatment by incorporating a treatment agent into the nanoparticles. The release of the treatment agent can be pH or temperature controlled.

Real-time ultrasound-assisted theranostic treatment of the diseases and/or inflammation in the posterior segment of the eye is possible through the use of echogenic and safe polymers, along with a contrasting agent able to improve resolution in viewing of an intraocular nanobubble system. Gases incorporated into the nanobubble system are able to improve contrast and resolution. However, it needs to be examined whether the incorporation of gases is safe for use in the human eye. In this study, nanobubbles were viewed in real time as they moved through the eye in order to treat uveitis. However, further exploration is necessary to determine whether the nanobubbles are able to reach the site of infection and have the potential to release a therapeutic dose. A possible area of examination would be enhancing the specificity of the IONBS through the use of antibodies to the inflamed cells. This study serves as an important milestone in improving compliance for treatment of uveitis as it will render current invasive procedures unnecessary.

Appendix 1:
Formulation, characterisation and design of an intraocular anti-inflammatory nanoparticulate drug delivery system

Kealeboga Mokolobate, Yahya E. Choonara, Lisa Du Toit, Viness Pillay

School of Therapeutic Sciences, Department of Pharmacy and Pharmacology,
University of the Witwatersrand, Medical School.

The posterior segment of the eye poses challenges in terms in terms of drug delivery. Procedures such as intraocular injections are invasive and may yield health hazards such as cataracts. This study aimed to design a layer-by-layer-originated lipo-polymeric intraocular nanoparticulate system, for administration in the cul-de-sac of the eye and penetration through the ocular barriers, for the treatment of uveitis. These nanoparticulate systems were produced by dissolving lipids (cholesterol and soybean oil), buffer pH 7.4 and drug in chloroform. This emulsion was sonicated, and the organic solvent was removed, followed by freeze-thawing. Following that, a layer of chitosan was added to the nanoparticles, allowed to deposit for 12 hours. Finally, a layer of polyethene glycol was added and allowed to deposit for 4 hours. Zetasizing was used to ensure stable particles within the nanometre range (200nm). Drug release studies (over 24 hours) were conducted on the formulations. Images highlighted nanoparticles with a thick shell due to the reinforced layering, effectively entrapping the drug. This study demonstrated how the variables involved (i.e. time of sonication, number of freeze-thaw cycles and amount of soybean oil), affect size and stability of the drug delivery system and enabled determination of an optimized formulation. This formulation (once incorporated into a suitable suspension) will circumvent the need for invasive procedures; and improve compliance regarding anti-inflammatory drug delivery to the posterior segment of the eye for uveitis treatment. Furthermore, the optimized product will be further modified to facilitate targeted drug release, using antibody conjugation.

Appendix 2:
Formulation, characterisation and design of an intraocular anti-inflammatory nanoparticulate drug delivery system

Kealeboga Mokolobate, Vines Pillay, Yahya Choonara, Lisa Du Toit

School of Therapeutic Sciences, Department of Pharmacy and Pharmacology,
University of the Witwatersrand, Medical School

Abstract

The posterior segment of the eye poses the most challenges in terms in terms of drug delivery. Procedures such as intraocular injections are invasive, are associated with low compliance, and may yield health hazards such as cataracts. This study conducted aimed to design a layer-by-layer-originated lipo-polymeric intraocular nanoparticulate system, administered in the cul-de-sac of the eye, for the treatment of uveitis. These nanoparticulate systems were produced by dissolving the lipids (cholesterol and soybean oil), buffer pH 7.4 and drug in chloroform. This emulsion was sonicated and the organic solvent was subsequently removed using a rotary evaporator. The emulsion was then freeze-thawed. Following that, a layer of chitosan was added to the nanoparticles, allowed to deposit for 12 hours, and finally, a layer of polyethene glycol was added and allowed to deposit for 4 hours. Zetasizing was used to ensure stable particles within the nanometre range (approx 200nm). Drug release studies (24hour) were conducted on the formulations. Images highlighted nanoparticles with a thick shell due to the reinforced layering, effectively entrapping the drug. This study illustrated how the variables (time of sonication, number of freeze-thaw cycles and amount of polymer (soybean oil) involved affect size and stability of the drug delivery system and enabled determination of an optimized formulation. This formulation (once incorporated into a suspension) will circumvent the need for invasive procedures and improve patient compliance and thus assist in bringing about a breakthrough in drug delivery and compliance regarding the posterior segment of the eye uveitis.

Appendix 3:

Ethics Clearance

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2013/44/04

APPLICANT: **Ms** K Mokolobate

SCHOOL: Medical School
DEPARTMENT: Pharmacy & Pharmacology
LOCATION: Faculty of Health Sciences

PROJECT TITLE: *Microimaging of an intraocular drug delivery system using New Zealand white rabbits*

Number and Species

60 New Zealand white rabbits

Approval was given for to the use of animals for the project described above at an AESC meeting held on 20 August 2013. This approval remains valid until 19 August 2015.

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and is subject to any additional conditions listed below:

1. **Observations should be done 3 times per day.**
2. **A score sheet should be used to assess health.**

Signed: _____ (Chairperson, AESC) Date: 10/9/2013

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____ (Registered Veterinarian) Date: 10/9/2013

cc: Supervisor: Prof V Pillay
Director: CAS

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