POLYMERIC TRANSFECTION AGENTS FOR DRUG DELIVERY TO TREAT RETINAL DAMAGE AND DISEASE

by

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ABSTRACT

Diseases and damage in the posterior segment tissues of the eye mainly the retina-choroid and the optic nerve such as glaucoma, age-related macular degeneration (AMD), diabetic retinopathy, and traumatic optic neuropathy (TON) together constitute the biggest incidence of irreversible blindness globally due to their neurodegenerative nature. Current clinical management approaches of such diseases involve frequent intravitreal injections which besides being uncomfortable, can also cause serious complications, and thus lead to low compliance and a huge cost burden on health systems. The aim of this thesis was to overcome this challenge using non-invasive and minimally invasive prolonged acting sustained-release ocular drug delivery technologies based on biodegradable and biocompatible polymers to achieve a therapeutic bioavailability in the posterior segment and possibly deliver neuroprotective drugs. This aim was approached using two different platforms – a topical eye-drop formulation containing penetration enhancing agents (PEAs) to increase the permeability of an anti-inflammatory glucocorticoid dexamethasone sodium phosphate (DSP) across the defensive barriers of eye, and a sustained-release intravitreal depot-forming system using microspheres to deliver endogenous neuroprotective antioxidants. The PEAs included a novel polyacetylene (pAc) polymer synthesised in-house and we compared it with two previously described cell penetrating peptide (CPP) based PEAs, TAT and penetratin, with respect to increasing transcorneal permeability of dexamethasone in an *ex-vivo* model of porcine cornea, and also tested them for their toxicity both *ex-vivo* by immunofluorescent staining of tight junctions in corneal epithelium and *in-vitro* cytotoxicity in a cell line of human retinal epithelium. A significant increase of up to 5-7 times in the transcorneal permeability of DSP in formulation with PEAs was observed after 60 minutes. It was also established that the

PEAs cause no damage to the barrier integrity of the defensive barrier *ex-vivo* and are also very well tolerated *in-vitro* in cell cultures. Second, the *in-vivo* efficacy of poly(lactic-co-glycolic acid) (PLGA) microspheres containing small-molecule endogenous antioxidants vitamin-E (VE) and melatonin (MEL), and dexamethasone (DEX) in different combinations, to provide neuroprotection and regeneration was tested in an optic nerve crush (ONC) model of degenerative TON in rats. Retinal ganglion cell (RGC) survival, and glial activation of astrocytes, Müller cells, and microglia in the retinas, and axonal regeneration in the optic nerves in immunostained tissue sections was measured 21 days post injury. It was observed that none of the formulations could provide neuroprotection to RGCs and instead exacerbated their degeneration and also increased the inflammatory response of the injury in the retina. However, VE containing formulations promoted axonal regeneration in the optic nerve, non-significantly, due to independent pathways involved in RGC survival and axonal regeneration. This was due to the inability of slow-release formulations to cope with the rapidly developing pathophysiology of the model characterised by exponential RGC loss very early in the injury. Overall, the topical drug delivery technology could potentially be used to achieve a significantly higher intraocular therapeutic bioavailability after topical eye drop administration, than currently afforded, whilst the intravitreal PLGA platform need to be tested as a pre-treatment in milder models of neurodegeneration for neuroprotective efficacy.

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PUBLICATIONS & PRESENTATIONS

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LIST OF ABBREVIATIONS

aFGF: Acidic fibroblast growth factor

Akt: Protein kinase B

AMD: Age-related macular degeneration

ARPE-19: Human retinal pigment epithelium cells

ARRIVE: Animal research: reporting of in-vivo experiments guidelines

ARVO: Association for research in vision and ophthalmology

BAB: Blood-aqueous barrier

BAC: Benzalkonium chloride

BBB: Blood-brain barrier

BCL-2: B-cell CLL/lymphoma 2

BDNF: Brain-derived neurotrophic factor

BM: Bruch’s membrane

BRB: Blood-retina barrier

BSA: Bovine serum albumin

CD: Cyclodextrins

CDS: Chromatography data system

CMC: Carboxymethylcellulose

CMCS: Carboxymethyl chitosan

CNS: Central nervous system

CNTF: Ciliary nerve trophic factor

CNV: Choroidal neovascularization

CPP: Cell penetrating peptide

CS: Chondroitin sulphate

DAPI: 4′,6-diamidino-2-phenylindole

DEX: Dexamethasone

DMO: Diabetic macular oedema

DPI: Days post injury

DR: Diabetic retinopathy

DSP: Dexamethasone sodium phosphate

EDTA: Ethylenediaminetetraacetic acid

EGFR: Epidermal growth factor receptor

ELISA: Enzyme-linked immunosorbent assay

Es: Endostatin

ET-1: Endothelin 1

EtE: Efficiency for traversing eye to retina

FA: Fluorescein angiography

FGF: Fibroblast growth factors

FITC: Fluorescein isothiocyanate

GAG: Glycosaminoglycan

GAP43: Growth-associated protein 43 neuromodulin

GCL: Ganglion cell layer

GDNF: Glial-derived neurotrophic factor

GFAP: Glial fibrillary acidic protein

GPC: Gel permeation chromatography

HCEC: Human corneal epithelial cells

HEPES: 4-(2-Hydroxyethyl)-1-piperazine ethanesulfonic acid

HIV: Human immunodeficiency virus

HPLC: High-performance liquid chromatography

HRP: Horseradish peroxidase

HS: Heparan sulphate

HUVEC: Human umbilical vein endothelial cells

IC_{50}: Half-maximal inhibitory concentration

ICH: International council for harmonisation of technical requirements for pharmaceuticals for human use

IGF: Insulin-like growth factor

IHC: Immunohistochemistry

IL: Interleukin

ILM: Inner limiting membrane

INL: Inner nuclear layer

IOP: Intra-ocular pressure

IP: Intellectual property

IPL: Inner plexiform layer

IR: Ischemia–reperfusion

IS: Inner segment

LC: Long chain

LD: Laser diffraction

L-DOPA: L-3,4-dihydroxyphenylalanine

LOD: Limit of detection

log P: Logarithm of the octanol/water partition coefficient

LOQ: Limit of quantification

MAPK/Erk1/2: Mitogen-activated protein kinases / extracellular signal-regulated kinases 1 and 2

MEL: Melatonin

M_n: Number average molecular weight

MS: Microspheres

MS-B: Blank microspheres

MS-MD: Microspheres loaded with melatonin and dexamethasone

MS-V: Microspheres with vitamin-E additive

MS-VMD: Microspheres with vitamin-E additive and loaded with melatonin and dexamethasone

MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide

M_w: Weight average molecular weight

NC: Nanocarriers

NC3Rs: National centre for the replacement, refinement and reduction of animals in research

NFL: Nerve fibre layer

NF-κB: Nuclear factor κB

NGF: Nerve growth factor

NHC: Human conjunctival epithelial cells

NLC: Nanostructured lipid carriers

NLD: Nasolacrimal duct

NMDAR: N-methyl-D-aspartate receptors

NP: Nanoparticles

NT: Neurotrophin

oBRB: Outer blood-retina barrier

OCT: Optical coherence tomography

OCT: Optimal cutting temperature compound

OIR: Oxygen-induced retinopathy

OLM: Outer limiting membrane

ON: Optic nerve

ONC: Optic nerve crush

ONL: Outer nuclear layer

OPL: Outer plexiform layer

OS: Outer segment

pAc: Polyacetylene (poly(O-propargyl-N-amino carbamate))

PACAP: Pituitary adenylate cyclase activating polypeptide

PACG: Primary angle-closure glaucoma

PAMAM: Poly(amidoamine)

pAntp: Antennapedia peptide

P_{app}: Apparent permeability coefficient

PBocAC: O-propargyl-N-(tert-butoxycarbonyl) amino carbamate

PBS: Phosphate-buffered saline

PCL: Poly(ε-caprolactone)

PDR: Proliferative diabetic retinopathy

PEA: Penetration enhancing agent

PEDF: Pigment epithelium-derived factor

PEG: Poly(ethylene glycol)

PFA: Paraformaldehyde

PK/PD: Pharmacokinetics/pharmacodynamics

PLGA: Poly(lactic-co-glycolic acid)

POAG: Primary open-angle glaucoma

POD: Peptide for ocular delivery

PPII: Polyproline type II

pRFP: Red fluorescent protein plasmid

PRISMA: Preferred Reporting Items for Systematic Reviews and Meta-Analyses

PTD: Protein transduction domain

PUFA: Polyunsaturated fatty acids

PVA: Poly(vinyl alcohol)

RBPMS: RNA-binding protein with multiple splicing

RCT: Randomised controlled trials

RGC: Retinal ganglion cell

RGD: Arginine-glycine-aspartic acid

RNV: Retinal neovascularization

RoB: Risk of bias

ROS: Reactive oxygen species

RPE: Retinal Pigment Epithelium

RT: Room temperature

SDS: Sodium dodecyl sulphate

SEM: Standard error of mean / Scanning electron microscopy

siRNA: Small interfering RNA

SLN: Solid lipid nanoparticles

SLS: Static light scattering

STAT3: Signal transducer and activator of transcription 3

TAT: Trans-activator of transcription

TEER: Transepithelial electrical resistance

TGF: Transforming growth factor

TLR2: Toll-like receptor 2

TNF-α: Tumour necrosis factor alpha

TON: Traumatic optic neuropathy

TrkB: Tropomyosin-related kinase receptor B

Trp: Tryptophan

TUDCA: Tauroursodeoxycholic acid

TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labelling

UDCA: Ursodeoxycholic acid

UK: United Kingdom

UV: Ultraviolet radiation

VE: Vitamin-E

VEGF: Vascular endothelial growth factor

VIP: Vasoactive intestinal peptide

WT: Wild type

ZO-1: Zonula occludens-1

CHAPTER 1

General Introduction

The posterior segment constitutes the back two-thirds of the eye and consists of vitreous humour, retina composed of neural retina and retinal vasculature including inner & outer blood-retinal barriers and choroid, sclera, and the optic nerve (Figure 1.1) (Cioffi, 2020).

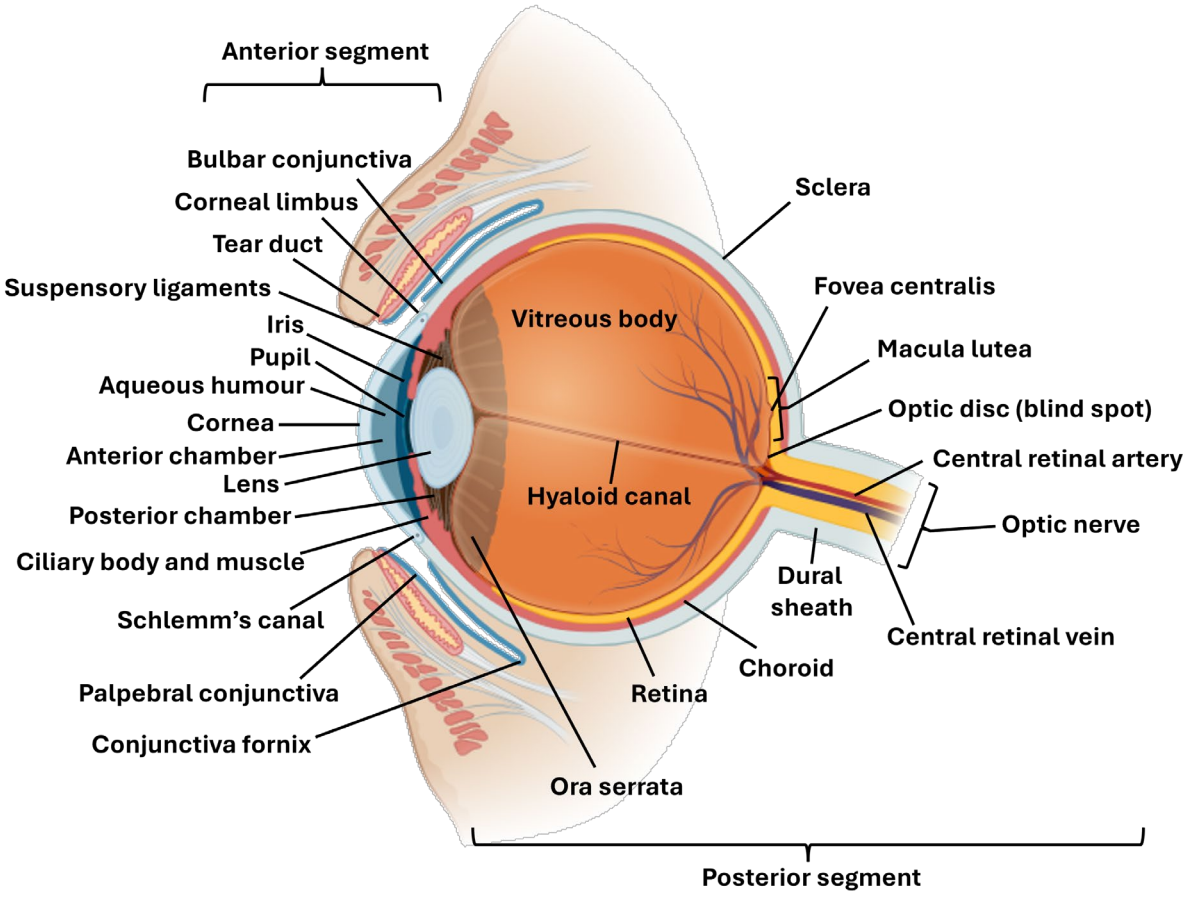

Figure 1.1:Sagittal section of a human eye illustrating the anatomical structures in the anterior as well as posterior segments. Created in BioRender and MS PowerPoint.

1.1. The Retina

The neurosensory component of the eye is called retina. It is an embryological part of central nervous system and almost 80% of sensory information in humans originates

in the retina (Hildebrand and Fielder, 2011). It has a unique cytoarchitecture and a sophisticated neurocircuitry (Figure 1.2). The choroid is a vascular layer that supplies the outer part of the retina, while the sclera is a tough outer layer that protects it (Hildebrand and Fielder, 2011; Cioffi, 2020).

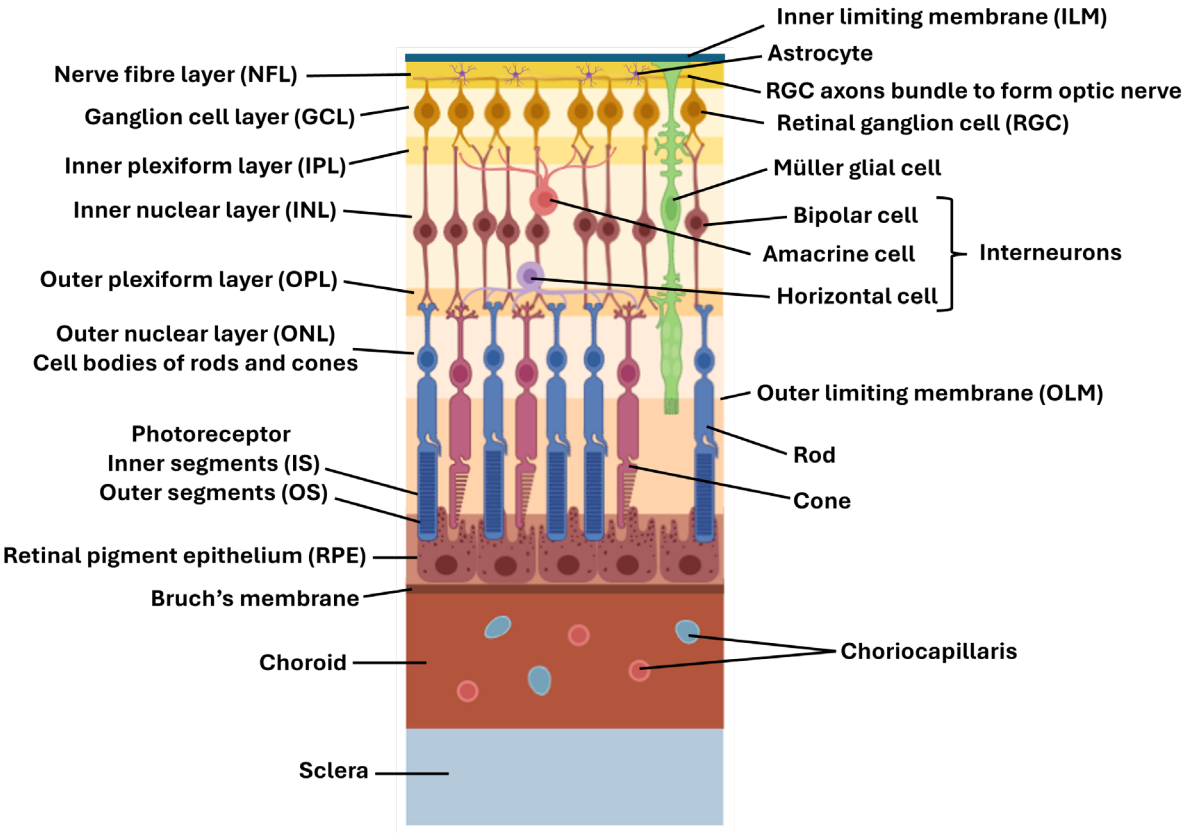

Figure 1.2: Illustration of the anatomy describing the cytoarchitecture, neurocircuitry, and histological layers of the human retina. Created in BioRender and MS PowerPoint.

1.1.1. Retinal Pigment Epithelium (RPE)

This is a very crucial cell layer in the visual system and a vital component to safeguard the integrity of the rod and cone outer segments. RPE serves a lot of complex roles in the retina, it absorbs the light focused on retinal surface from the lens, maintains the transepithelial transport of nutrients and ions between photoreceptors and the choriocapillaris, stores and converts vitamin A esters, forms an acid-

mucopolysaccharide complex that protects the outer segments of photoreceptors, and performs phagocytosis of photoreceptor outer segments (Strauss, 2005). There are around 3.5 million RPE cells in an adult human retina (Panda-Jonas, Jonas and Jakobczyk-Zmija, 1996). RPE cells form a single cuboidal epithelium layer in the central retina where they are most tightly packed and assume the shape of regular hexagonal tiles. The outer blood-retina barrier (oBRB), formed by tight junctions (zonulae occludentes) between neighbouring RPE cells, is a significant physiologic barrier to the free passage of molecules between the leaky choriocapillaris and the photoreceptors of the neuroretina. RPE is a pigmented layer due to the presence of melanin which renders it dark brown to black (Strauss, 2005; Hildebrand and Fielder, 2011; Cioffi, 2020).

1.1.2. Photoreceptors

Photoreceptors perform phototransduction in the retina which facilitates sensory perception of the visual system, by converting photon capture into a neural signal which is then propagated as an action potential through the optic nerve to the visual cortex and are thus called sensors of the visual pathway. The photoreceptor layer is the only part of the neural retina that is sensitive to light (Hildebrand and Fielder, 2011; Cioffi, 2020). There are two types of photoreceptors, rods and cones, with approximately 4 to 6 million cones and 77 to 120 million rods in an average healthy human retina. Rods being significantly more photosensitive than cones, can detect even singular photons. Therefore, rods take-over in low-light conditions and are exclusively accountable for scotopic vision. Whereas, cones operate under ambient and bright-light conditions due to significantly higher thresholds of photon saturation in cones and are therefore accountable for high visual acuity and temporal resolution, called photopic vision and

also facilitate colour perception (Molday and Moritz, 2015; Cioffi, 2020). Furthermore, there are three types of cone photoreceptors depending upon the photopigments they contain that absorb visible light at different wavelengths. Among them, red cones or long-wavelength responsive cones (63%) far outnumber green or medium-wavelength responsive cones (32%) and blue or short-wavelength responsive cones (5%) (Hildebrand and Fielder, 2011; Cioffi, 2020). The structure of a photoreceptor can be described as consisting of an outer segment that contains the photon-capturing pigment called photopigment, an inner segment that contains the cellular machinery which is necessary to maintain the high metabolism of photoreceptors, a nucleus, an inner fibre which is analogous to an axon, and the synapses. Photoreceptor terminal endings play a very crucial part in the physiology of transmission and early sensory processing of visual signals in the retina by interacting with surrounding photoreceptors and interneurons (Hildebrand and Fielder, 2011; Molday and Moritz, 2015; Cioffi, 2020).

1.1.3. Interneurons

The photoreceptor and the ganglion cell layers are connected to each other by interneurons in the inner nuclear layer (INL) of the retina. Bipolar, horizontal, amacrine, and interplexiform cells make up interneurons, and together they constitute complex neuroretinal circuitries in the outer (OPL) and inner (IPL) plexiform layers that interpret and compile signals from photoreceptors and transmit this information to the ganglion cell layer (GCL) (Hildebrand and Fielder, 2011; Cioffi, 2020).

1.1.4. Retinal Ganglion Cells (RGCs)

RGCs are situated in the innermost retinal layer and transmit visual signals from the retina to the brain. Signals from retinal bipolar and amacrine cells are received and

processed by RGCs, which are subsequently transformed into action potentials that travel through the optic nerve to the brain. As many as 100 rods and cones can provide information to one RGC. The ganglion perikarya are found in GCL, their dendrites contact with bipolar and amacrine cells in the IPL and their axons combine to form the optic nerve. There are about 20 different types of RGC in a human retina, however two types known as the midget and parasol cells are the best known and make up about 80% of RGC population. The human GCL contains, on an average, about 1.2 million RGCs (Hildebrand and Fielder, 2011; Wurtz, R.H., Kandel, 2012; Cioffi, 2020).

1.1.5. Glial Cells

Müller cells, astrocytes, microglia, and oligodendrocytes are the only four types of glial cells found in the retina; Müller cells being the primary glial cells of the retina which are present in all of retinal layers. The proximal and distal extensions of the Müller cells give rise to inner (ILM) and outer (OLM) limiting membranes respectively, thus providing structural support to the retina. Müller cells maintain the local environment in the retina to allow optimal functioning of the visual system (Hildebrand and Fielder, 2011; Cioffi, 2020).

1.1.6. Histological Layers of the Retina

Architecturally, the structure of cellular components in a retina can be differentiated into 10 distinct layers. At the outermost side is the RPE which forms the barrier to the choroid. Next to RPE towards the internal side lies the photoreceptor layer, consisting of the outer segment (OS) and inner segment (IS) layers of rods and cones photoreceptors (Masland, 1986; Hildebrand and Fielder, 2011; Hoon *et al.*, 2014; Cioffi, 2020). Next inwards is the OLM formed by the attachment of the distal extensions of Müller cells to each other and with the inner segments of the

photoreceptors via desmosomes or adherens junctions. The OLM acts as a semipermeable barrier surrounding IS & OS photoreceptors to the inner retinal layers. Moreover, it also imparts mechanical strength and provides support to the whole neural retina architecture. The outer nuclear layer (ONL) containing the cell bodies of photoreceptors is located further inwards beneath the OLM (Masland, 1986; Hildebrand and Fielder, 2011; Hoon *et al.*, 2014; Cioffi, 2020). The ONL is followed by OPL consisting of synaptic junctions between axonal ends of photoreceptor and dendritic processes of bipolar and horizontal cell interneurons. INL is next inwards which contains the cell bodies of bipolar, horizontal and amacrine cell interneurons, and Müller cells. The synaptic junctions between bipolar cells, amacrine cell interneurons and RGCs are located in the IPL. The GCL after IPL contains the RGC cell bodies followed by the ILM consisting of expanded terminations of proximal ends of Müller cells covered by a basement membrane. The innermost layer is ILM which provides a barrier for retina to the vitreous chamber, as well as providing mechanical strength and architectural support for the retina (Masland, 1986; Hildebrand and Fielder, 2011; Hoon *et al.*, 2014; Cioffi, 2020).

1.1.7. The Macula

The macula, at the posterior pole, is located in the central posterior portion of the retina and facilitates central high-resolution visual acuity due to the densest concentration of photoreceptors in this region which allows a person to see fine detail, read, and recognize faces. It is completely pigmented with carotenoids lutein and zeaxanthin and thus is yellowish in colour. It has a peculiar histological structure and is organized into four distinct concentric regions: perifovea, parafovea, foveal slope, and foveola (Hildebrand and Fielder, 2011; Cioffi, 2020).

1.2. Diseases of the ocular posterior segment

Diseases affecting the posterior segment ocular tissues, particularly the retina-choroid, are a leading cause of vision impairment worldwide. Around 2.2 billion people globally are believed to have some form of visual impairment, with illnesses of the ocular posterior segment, such as age-related macular degeneration (AMD) (10.4 million), glaucoma (6.9 million), and diabetic retinopathy (DR) (3 million) accounting for a considerable number of them (World report on vision, 2019).

1.2.1. Age-related macular degeneration (AMD)

Close to 8.7% cases of irreversible blindness around the world can be attributed to AMD, and estimates suggest a staggering 196 million people suffering with some form of AMD in 2020, rising to almost 288 million individuals by 2040 (World report on vision, 2019). AMD unsurprisingly also remains the single most commonly known cause of irreversible blindness in the elderly in first-world countries, particularly in those over 60 years old (Prokofyeva and Zrenner, 2012; Wong *et al.*, 2014; World report on vision, 2019). Some studies have suggested a higher prevalence of AMD in individuals of European descent than those of other races, as evidenced by the largest prevalence of AMD in Europe anywhere in the world (Prokofyeva and Zrenner, 2012; Wong *et al.*, 2014; Colijn *et al.*, 2017).

At the early onset of AMD, the central vision becomes blurred, and images appear as distorted, also known as metamorphopsia, which eventually leads to an obscured central field of vision over time and finally, complete loss of central vision (scotoma) at the advanced stages. The disease is clinically characterised by focal deposition of drusen, a yellow-coloured lesion of acellular, polymorphous debris between RPE and Bruch's membrane (BM) at the early stages in the macula and peripheral retina. A few

small and hard drusen are usually present in the eyes of people over 50 and is considered a normal process in ageing, and thus every ageing person with drusen cannot be considered to have AMD. However, excess drusen causes damage to the RPE and a chronic aberrant inflammatory response which leads to large areas of retinal atrophy or expression of angiogenic cytokines such as vascular endothelial growth factor (VEGF), or both and consequently results in choroidal neovascularization (CNV). Drusen consists of multiple substances including proteins, lipids, cholesterol, and cellular fragments of the RPE. Slight drusen appears in the early stages which is often asymptomatic and visual function is not affected much. As the disease progresses from early to intermediate form, there is a visible accumulation of drusen and change in the pigmentation of retina due to degenerating RPE cells. The final stage or late AMD can manifest in two distinct forms: neovascular AMD, also known as wet or exudative AMD, or geographic atrophy, also known as advanced dry or non-exudative AMD. In wet AMD, CNV is observed accompanied by increased vascular permeability and fragility. Newly formed abnormal blood vessels from choriocapillaris infiltrate into the retina through breaks in the BM and easily haemorrhage due to extreme fragility, thus causing fluid exudation and accumulation of blood in the retina along with either deposition of lipids, detachment of RPE from choroid, fibrotic scars, or a combination of these abnormalities. This may result in sudden profound vision loss within days to weeks. Whereas advanced dry AMD is characterized by gradual degeneration of RPE and photoreceptors and constriction of choroidal blood vessels. Dry-AMD would usually result in gradual, insidious loss of sight with central or peri-central visual scotomas over the course of months to years. More than 80% cases of severe vision loss or irreversible blindness caused by AMD can be attributed to the

neovascular form, though wet AMD represents only 15 to 20 percent of the total AMD cases (de Jong, Geerlings and den Hollander, 2019; James, 2024).

For the control and treatment of wet AMD, conventionally intraocular injections of drugs targeting vascular endothelial growth factor (anti-VEGFs) to treat CNV have been employed to very high success rates including an RNA oligonucleotide pegaptanib sodium and antibodies like ranibizumab, bevacizumab, and aflibercept. However, there is currently no treatment available for early, intermediate, and advanced dry forms of AMD, and no effective means to halt the disease progression (Eandi *et al.*, 2016).

1.2.2. Glaucoma

An estimated 76 million people suffer from glaucoma worldwide, with the numbers expected to reach as high as 111.8 million by 2040 (World report on vision, 2019). It is a significant public health concern as it leads to the second highest incidence of usually irreversible blindness in the world only after cataract, with approximately 10% affected people going blind due to glaucoma. Global prevalence of glaucoma is as high as 2.2% in people aged 40 to 80 years, and 2.51% in Europe in the same age group. In the UK as well it remains a critical disease affecting 2% of the population over 40 years old, and much higher at 10% in people older than 75 years, particularly in individuals of African-Caribbean descent (World report on vision, 2019; Allison, Patel and Alabi, 2020).

Glaucoma constitutes a group of optic neuropathies resulting in progressive degeneration of RGCs and eventually optic nerve, which causes a characteristic appearance of the optic disc, also called cupping, and vision loss. How the disease develops biologically, and the factors that contribute to its progression, is not clearly understood yet. The most dangerous aspect of glaucoma is that without screening, it

is often detected at severe stages as it does not exhibit any symptoms at early stages of disease progression. Glaucoma can be classified into two varieties: open-angle glaucoma and angle-closure glaucoma, and each one of them can be further sub-divided into primary or secondary disease. Though the damage to optic nerve may be attributable to an increase in intra-ocular pressure (IOP), there has been no clearly identified cause for it in primary or idiopathic glaucoma. Whereas in secondary glaucoma, it is mainly caused due to trauma, certain medications like corticosteroids, inflammation, tumour or underlying medical conditions. Open-angle glaucoma constitutes majority of the disease prevalence, however angle-closure glaucoma causes a disproportionate incidence of blindness. IOP is regulated by a balance between secretion of aqueous humour by the ciliary body and its drainage through two independent pathways – the trabecular meshwork and uveoscleral outflow. In open-angle glaucoma, trabecular meshwork develops resistance to aqueous outflow and causes an increased IOP. On the other hand, in angle-closure glaucoma, IOP is raised due to iris apposition obstructing the access to aqueous drainage pathways by forming an anatomically closed angle and thus the name.

A high IOP puts mechanical stress on the posterior segment ocular tissues, mainly lamina cribrosa and surrounding areas which results in axonal damage and disrupts the transport of essential trophic factors to RGCs from their brainstem target, leading to apoptosis. This may also cause neurodegeneration of other neurons in the retina and cells in the central visual pathway by altering their environment and increasing susceptibility to damage (Weinreb, Aung and Medeiros, 2014).

The only proven method to treat primary open-angle glaucoma (POAG) is IOP reduction by pressure lowering drugs like prostaglandin analogues, carbonic

anhydrase inhibitors, β-adrenergic blockers and α-adrenergic agonists applied topically, but most of them carry significant systemic side-effects. While in primary angle-closure glaucoma (PACG) and severe cases of POAG, surgical interventions are the only options available (Weinreb, Aung and Medeiros, 2014).

1.2.3. Diabetic Retinopathy (DR)

Among the working adults, diabetic eye disease including DR and diabetic macular oedema (DMO) contributes the highest causes of damage or loss of vision in the world (World report on vision, 2019). An estimated 146 million people suffer from DR globally, 6.4 million of them in Europe alone with a predicted increase to 8.6 million by 2050, having a high disease prevalence of 34.6% and 29.4% in global and European diabetes patients respectively (Yau *et al.*, 2012; World report on vision, 2019; Li *et al.*, 2020).

DR and DMO are commonly occurring microvascular complications in diabetics that have chronic hyperglycaemia, complexed with hyperlipidaemia and hypertension, which may cause sudden and crippling effect on visual acuity, gradually causing blindness. At the early stages, there are observable changes in the structure and cellular composition of the microvasculature. Damage to endothelial cells, which are essential for maintaining the blood-retinal barrier, leads to increased vascular permeability. Pericytes which are a very important cellular component in the control of retinal capillary perfusion, are also damaged in diabetes which results in alterations in retinal haemodynamics, causing abnormal autoregulation of blood flow in the retina and thus their apoptosis is a key characteristic of early-stage DR correlating with formation of microaneurysms. Capillary basement membrane thickens and there is an increased deposition of extracellular matrix components. An increased level of

leukostasis in the diabetic retina results in capillary nonperfusion, endothelial cell damage, and vascular leakage in the retinal microcirculation. Advanced DR results in abnormal growth of blood vessels in the retina secondary to ischemia called retinal neovascularization (RNV). Occluded capillaries in the retina cause ischemia, which in turn leads to a pathologic neovascularization mediated by angiogenic factors, such as VEGF resulting in proliferative diabetic retinopathy (PDR). The purpose of these abnormal blood vessels is to supply oxygenated blood to the hypoxic retina. DMO develops during DR progression and is characterized by thickening of the retina in and around macula where extracellular fluid accumulates as a result. It is preceded by breakdown of BRB due to leaking dilated hyperpermeable capillaries and microaneurysms. Only treatments available currently involve optimal glycaemic control to slow the progression of disease, but once the visual function is affected this option becomes very redundant. Laser photocoagulation therapy to reduce VEGF production and leakage, intravitreal anti-VEGF and steroid injections to reduce DMO and vitrectomy to treat intractable haemorrhage and retinal structural abnormalities caused by scarring are the only treatment options for severe disease (Ciulla, Amador and Zinman, 2003).

1.2.4. Traumatic optic neuropathy (TON)

TON is characterised by a partial or complete loss of visual function upon trauma to the optic nerve. This could be due to a direct injury like skull fractures or orbital fractures, or an indirect injury like concussion. Contusion or laceration of the optic nerve disrupts the axons and myelin sheath, fractures in the orbit or skull can cause compressive injury or complete transection. Secondary pathophysiology includes ischaemia, chronic inflammation, and axotomy leading to neuronal apoptosis and

degeneration of nerve fibres. TON can manifest clinically from mild vision disturbances to complete vision loss. Current clinical management strategies include controlling inflammation pharmacologically or through decompressing surgical intervention for structural issues (Steinsapir and Goldberg, 1994).

1.3. Topical drug delivery to the ocular posterior segment

Conventionally, the only successful route for administration of drugs to the posterior segment tissues has been intravitreal and periocular injections. But these are invasive methods that usually cause discomfort in patients leading to low compliance and limiting acceptance except for the most severe and sight-threatening disease, requiring specialist clinic settings and expert administration. Additionally, the associated complications of intraocular injections are aplenty including endophthalmitis, increased intraocular pressure, cataract, glaucoma and retinal detachment (Sampat and Garg, 2010; Sen *et al.*, 2014; Smith, Smith and Mohney, 2014). This makes topical drug delivery for the posterior segment a “holy-grail”. Topical ocular administration via corneal or conjunctival/scleral routes can attain therapeutic levels in the anterior and posterior segments. Either of the routes follow specific pathways of drug penetration and distribution into the posterior ocular tissues (Hughes *et al.*, 2005):

i. Diffusion of drug through the cornea, into the root of the iris, posterior chamber aqueous humour, and lens / lens zonules then into the vitreous and finally in the posterior segment tissues.

ii. Lateral transscleral diffusion at the pars plana of the ciliary body, through the pars plana and into the vitreous, without encountering the blood–retinal barrier.

iii. Lateral transscleral diffusion followed by penetration of Bruch's membrane and the RPE.

iv. Absorption into the systemic circulation either via conjunctival vasculature or through nasolacrimal duct and subsequently gaining systemic access to retinal vasculature.

Drugs partition to the corneal epithelium in the short contact span on the corneal surface where they distribute in the tear film, lipophilic drugs are retained in there and are slowly released to the corneal stroma and then into the anterior chamber. Upon application of eye drops, it takes 20-30 minutes for the drug to reach its peak concentration in the anterior chamber, however it is usually 2 orders of magnitude lower than the concentration instilled on the corneal surface, even for the most lipophilic drugs. From the aqueous humour, the drug has a wide access to the iris and ciliary body, where it may bind to melanin to form a reservoir for a slow and gradual release to the cellular microenvironment, thereby prolonging the drug activity. Drug distribution to the lens is way slower compared to distribution to the uvea because of the tightly packed protein rich structure of lens where drug partitioning happens slowly unlike porous uvea (Urtti, 2006).

Non-corneal drug absorption into the intraocular tissues also takes place by penetration across the conjunctiva and sclera which is the most significant route of transport for compounds with poor corneal permeability. Sclera is uniformly composed of a large amount of type I collagen, a few elastic fibres, and some fibroblasts. Proteoglycans make up most of the interfibrillar space in the form of an amorphous gel and are believed to be mainly responsible for the diffusional pathway for hydrophilic

drugs. Collagen fibres cross the eye as parallel bundles below the episclera and provide a free aqueous space in the surroundings that also facilitates a diffusion pathway for molecules, albeit with a lower resistance compared to the proteoglycan matrix. Drugs passing across the conjunctiva and sclera can then penetrate into the iris root and gradually to the aqueous humour in the posterior chamber. Some lipophilic drugs can also penetrate the RPE and may directly access the posterior segment tissues by lateral diffusion across the sclera after penetrating the Bruch's membrane and RPE (Hughes *et al.*, 2005).

Drug elimination from aqueous takes place by two main mechanisms: aqueous turnover through the trabecular meshwork and Schlemm's canal, and by venous blood flow in the anterior uvea. Elimination by the first mechanism takes place independent of the drug as a convective flow at about 3 µL/min. While the second mechanism works based on the drug's permeability across the endothelium of uveal blood vessels. That ensures that lipophilic drugs are cleared faster than hydrophilic drugs from the anterior chamber at a rate of about 20–30 µL/min and most of this clearance takes place through uveal blood flow (Urtti, 2006).

1.4. Barriers to topical drug delivery to the posterior segment

Topical drug delivery with eye drops for the treatment of posterior segment ocular diseases is hugely obstructed by the physiological and anatomical defensive barriers in the eye resulting in very low bioavailability, typically less than 5% (Hughes *et al.*, 2005; Urtti, 2006; Loftsson *et al.*, 2008).

1.4.1. Pre-corneal barriers

Upon topical application, lacrimal fluid flow quickly clears the applied drug from ocular surface. Despite a low tear turnover rate of only about 1 µL/min, excess drug

(increasing tear film volume) rapidly flows out to the nasolacrimal duct (NLD) in a matter of minutes. Systemic absorption rather than ocular absorption is another source of non-productive drug elimination. Systemic absorption can occur directly though conjunctival sac vasculature or after the fluid has passed into the nasal cavity through the NLD, which in turn significantly reduces drug concentration in the lacrimal fluid (Hughes *et al.*, 2005; Urtti, 2006; Loftsson *et al.*, 2008).

1.4.2. Epithelial barriers

Drug absorption into the eye from lacrimal fluid is obstructed by the corneal epithelium which is formed of 6 layers of mature epithelial cells (Figure 1.3) (Hughes *et al.*, 2005; Urtti, 2006; Loftsson *et al.*, 2008). These cells migrate from the corneal limbus towards the centre and apical surface of the cornea. Cells at the most apical surface form tight junctions that limit paracellular transport of drugs. This is the reason the permeability of hydrophilic drugs is typically an order of magnitude lower than that of lipophilic drugs. Still transcorneal transport remains the main route of drug entry into the aqueous humour from lacrimal fluid. Conjunctiva, while also having tight junctions of multi-layered epithelial cells, is a leakier epithelium and has a 20 times larger surface area compared to the cornea. It is lined with stratified columnar epithelium of two to seven layers of cells resting on a continuous basal lamina. Bulbar conjunctiva is also considerably permeable to hydrophilic and large molecules and hence can facilitate absorption of a variety of drugs. Major barriers to drug permeation across conjunctiva/sclera are the conjunctiva, sclera, and the RPE-choroid. Sclera is highly permeable to molecules of up to 70kDa. RPE also has tight junctions composed of a cuboidal monolayer of epithelial cells separating the outer surface of neural retina from the choroid. Drug transport across any epithelial barrier takes place by passive

(paracellular or transcellular) and active means (transcellular involving carrier-mediated membrane transporters) (Hughes *et al.*, 2005; Urtti, 2006; Loftsson *et al.*, 2008).

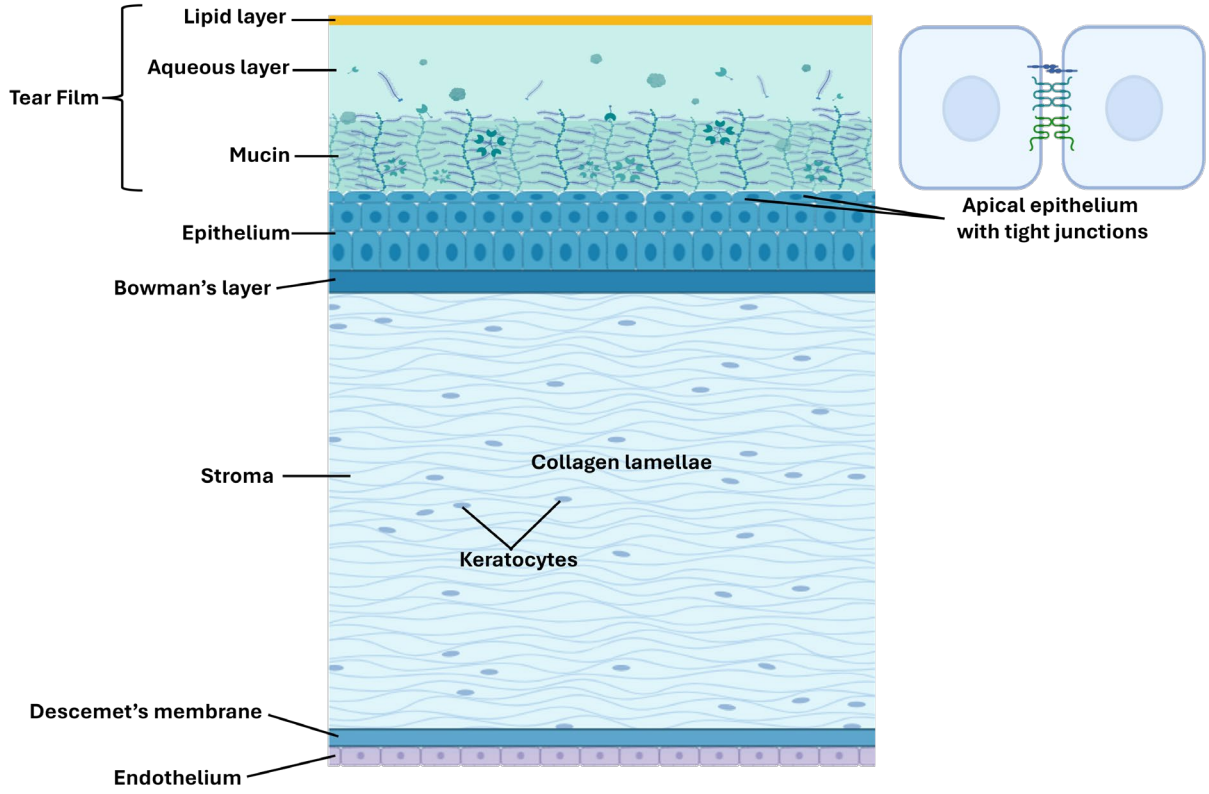

Figure 1.3: Sagittal cross-section of a human cornea illustrating the different anatomical layers and cellular arrangement, with the physiology of tear film and tight junctions between the cells in the apical layers that prevent paracellular transport of hydrophilic molecules.

1.4.3. Blood-ocular barriers

Blood-ocular barriers protect the eye from xenobiotics in the blood stream. There are two blood-ocular barriers in the eye: the blood aqueous barrier in the anterior segment and the blood-retina barrier (BRB) in the posterior segment. The blood-aqueous barrier consists of endothelial cells in the uvea and prevents escape of plasma albumin, and hydrophilic drug molecules from plasma to the aqueous humour. In the posterior segment, the blood-retinal barrier is constituted by endothelial tight junctions in the

walls of retinal blood vessels and RPE tight junctions against the choriocapillaris. The choriocapillaris has extensive blood flow and leaky walls unlike the retinal blood vessels and thus exhibits ample permeability to drugs in the choroidal extravascular space but their retinal distribution is inhibited by the RPE and retinal endothelia (Hughes *et al.*, 2005; Urtti, 2006; Loftsson *et al.*, 2008).

1.4.4. Other constraints

The lens blocks access to the vitreous from posterior chamber of the anterior segment to the posterior segment. Clearances from aqueous through trabecular meshwork and Schlemm's canal, and the uveo-scleral outflow also prevent longer half-lives of drugs in the anterior chamber. Aqueous reverse flow from posterior chamber to anterior chamber of the anterior segment also limits transport to the posterior segment. Further the iridolenticular diaphragm obstructs the posterior movement after corneal absorption. A reverse gradient of drug concentration is developed extending towards the aqueous humour from the vitreous as soon as the concentration of drug falls in the aqueous upon diffusion into the vitreous, which also leads to extremely low concentrations of drug in the vitreous and retina. Another limiting factor is the anterior blood flow in the choroid and fenestration of choriocapillaris which leads to fast scleral clearance (Hughes *et al.*, 2005; Urtti, 2006; Loftsson *et al.*, 2008).

1.5. Penetration Enhancing Agents (PEAs) in posterior segment delivery

PEAs present a very promising approach in topical drug delivery towards increasing the drug bioavailability in the fundus oculi by facilitating increased penetration across bio-barriers along with cellular internalization in the tissues and longer retention in the posterior segment. PEAs can be chemical or biological agents that induce an enhanced permeability of the defensive ocular barriers and membranes of cells and

tissues to pharmaceutical molecules by altering their properties through known and unknown mechanisms. There are three main mechanisms of PEA action, that are known so far (Kaur and Smitha, 2002; Moiseev *et al.*, 2019; Thareja *et al.*, 2021)

i. Alteration of tear film stability and the mucous layer on the ocular surface.

ii. Modification of membrane components like lipid bilayers of the associated epithelial cells by surface active PEAs for transcellular transport.

iii. Loosening up the epithelial tight junctions for paracellular transport.

PEAs may use either of these mechanisms or a combination of them, depending on the chemical and biological nature of both the PEAs and the barrier. The paracellular pathway is comprised of the intercellular space with intercellular junctions. At the most apical zone of contact the intercellular junctions comprise tight junctions. Adherens junctions lie adjacent to the tight junctions and below these two junctions, desmosomes and gap junctions are present. An ideal PEA should be non-toxic and non-irritating, should work at low concentrations, act fast and have a reversible effect (Kaur and Smitha, 2002; Moiseev *et al.*, 2019; Thareja *et al.*, 2021). The transmembrane protein cadherin, which is responsible for Ca^{2+} dependent cell-cell adhesion, forms the adherens junctions. Cells are also bound to one another via desmosomes. Gap junctions consist of connexons and facilitate communication between cells by means of porous channels. Apart from tight junctions that act as the primary restriction to the entry of molecules through paracellular transport, none of the other structures provide any barrier to drug penetration (Kaur and Smitha, 2002; Moiseev *et al.*, 2019; Thareja *et al.*, 2021).

Tight junctions are bandlike structures where plasma membranes of adjacent cells come in close apposition completely encircling the superficial epithelial cells. A variety of factors modulate the permeability of tight junctions, such as degree of maturation of epithelium, response to physiological requirement, changes in environmental conditions including osmolarity and ionic strength, and presence of drugs, vitamins, and hormones (Kaur and Smitha, 2002).

There can be multiple classes of PEAs based on the mechanism of their penetration across ocular barriers. A few of them include cyclodextrins, calcium chelators (EDTA), surfactants (BAC), bile acids and bile salts, fatty acids, azones, mucoadhesive polymers (chitosan, hyaluronic acid), cell-penetrating peptides like the trans-activator of transcription (TAT), Penetratin, etc. They can be formulated as additives or excipients, as drug delivery carriers, or as conjugates with drug delivery carriers including nano/microparticles, liposomes, nano/microemulsions, etc. However, most of the PEAs are effective only for increasing the corneal penetration and bioavailability of molecules into the anterior segment tissues and only a very few of them actually ever increase the uptake into the posterior segment as well (Kaur and Smitha, 2002; Moiseev *et al.*, 2019; Thareja *et al.*, 2021).

1.6. Cell-penetrating peptides (CPPs)

CPPs are short chain peptides, usually comprising 30 amino acid residues or less, exhibiting a distinct property to internalize across cellular membranes with negligible cytotoxicity, via an energy dependent or independent pathway or a combination of both, and without any need for chiral interactions with surface receptors (Bechara and Sagan, 2013). Most commonly studied CPPs are amphipathic cationic peptides, however a few anionic or hydrophobic CPPs have also been reported (Bechara and

Sagan, 2013; Thareja *et al.*, 2021). CPPs can facilitate the transport of a variety of covalently or non-covalently linked molecules of all sizes into living cells. Based on their origin, CPPs can be classified into three distinct categories: peptides derived from proteins, chimeric peptides constituting two natural sequences fused together, and synthetic CPPs that are sequences designed rationally based on structure-activity studies. A lot of research is being done on the use of CPPs in drug delivery, e.g., topical transdermal delivery, nasal delivery to pulmonary system, and delivery to the central nervous system across the blood-brain barrier. Their mechanisms of penetration are still debated. TAT and Penetratin are two CPPs that have been extensively researched for drug delivery. TAT, is a protein transduction domain (PTD) derived from the HIV-1 Tat protein, a trans-activator of transcription, and has a general sequence of H – YGR KKR RQR RR – OH, whereas Penetratin is a similarly derived PTD from a nonviral antennapedia homeodomain protein in *Drosophila melanogaster*, with a sequence of H – RQI KIW FQN RRM KWK KGG – OH (Bechara and Sagan, 2013; Thareja *et al.*, 2021).

1.6.1. Protein transduction technology

The process of membrane transduction takes place in three steps. Initially ionic interactions take place between the basic groups of the CPP-PTD side-chains and negative charges of the plasma membrane. There is also evidence suggesting cell-surface proteoglycans like heparan sulphate (HS) and glycosaminoglycan (GAG) can act as receptors for CPP binding to the plasma membrane. In the next step, these electrostatic interactions could result in penetration across the membrane either through a novel, currently unknown mechanism(s), or internalization by endocytosis or a combination of both. Different headgroups in the peptide backbone are necessary

for an efficient cellular entry. Finally, the biological function of the internalized PTD cargo is expressed, however its potency might depend upon the route of uptake (Kabouridis, 2003).

1.6.2. Structural requirements for uptake of CPPs

The electrostatic interactions of cationic CPPs with the negatively charged phospholipids and proteoglycans on the surface of plasma membrane is generally acknowledged to be the initial step before internalization, though it might not be always required for cellular uptake. The insertion and membrane binding property of the amphipathic CPPs may lead to endocytosis or direct translocation. These electrostatic or hydrophobic interactions are significantly controlled by the number of the positive charge and charge density, the hydrogen bonds, the size, and the secondary structure of the CPPs. The positive charge on the CPPs plays a very important role in enhancing their uptake. Arginine residues are more effective due to the presence of guanidine headgroups in the arginine side-chains as it forms bidentate hydrogen bonds with negatively charged phosphate, sulphate, and carboxylate groups on the surface of plasma membrane, resulting in counteranion scavenging that helps attenuate guanidium polarity by producing a polar ion-pair complex capable of diffusing into the membrane (Bechara and Sagan, 2013).

Another important factor is the hydrophobicity of the peptide. Hydrophobic residues in the sequence of a CPP play an important part in their interaction with the cell membrane bilayer and thus enhance the peptide translocation. However, deeper insertion in the lipid bilayer due to high hydrophobicity of CPPs might also be a constraint leading to low internalization as the CPPs risk getting stuck in the plasma membrane. Aromatic functional groups in the hydrophobic residues and the non-

covalent interactions of aromatic π-electron density are crucial for biological functions. Aromatic residues including tyrosine and tryptophan are predominantly present at the membrane surface in the membrane proteins and carry favourable free energies of insertion into the lipid bilayer interface. Tryptophan residues in particular are very effective in enhancing the uptake of certain CPPs due to their interactions with lipid bilayers of the plasma membrane. Trp residues in basic CPPs increase their affinity for chondroitin (CS) and heparan sulphate (HS), the sulphated sugars present on the membrane surface, and form stable aggregates, enhancing uptake (Bechara and Sagan, 2013).

Secondary structure of the peptide also impacts the membrane perturbation and subsequent internalization. CPPs are usually unstructured in aqueous solutions and adopt different shapes when they interact with membrane lipids like an α-helix, a β-strand, β-turn or β-sheet conformation. Structural flexibility is very important as polymorphism to interact with different lipid environments and malleability of CPPs determine their membrane interactions and route of internalization. Depending on the structure adopted by the peptide, it might favour one mode of internalization over another (Bechara and Sagan, 2013).

1.6.3. Internalization mechanisms of CPPs

Most of the CPPs can internalize across cell membranes through receptor-mediated or independent routes, via endocytosis or direct translocation, but mostly a combination of multiple routes at the same time as most of these methods are connected and occur simultaneously, and the down-regulation of one pathway might lead to up-regulation of the other. Endocytosis is a naturally occurring process in all the cells triggered either by electrostatic interactions of CPPs with cell surface

proteoglycans or by direct interaction with the plasma membrane. It takes place in two steps: endocytic entry followed by endosomal escape. Escape is crucial to prevent the lysosomal degradation of cargo and to enable it to reach its extra-endosomal target and execute its biological function. Often times CPPs bound to GAGs might enter after the recycling of GAGs that are internalized constantly, or endocytosis can be triggered more effectively by CPPs through GAG clustering, activation of intracellular signals and actin remodelling. All different routes of endocytosis have been observed in cellular uptake of CPPs including macropinocytosis, clathrin and caveolae mediated endocytosis. Direct translocation of CPPs requires destabilization of the membrane in an energy and temperature independent manner. Initially it was believed to be the primary mechanism of CPP internalization but was later refuted as an artifact of fixation (Richard *et al.*, 2003; Duchardt *et al.*, 2007; Vivès, Schmidt and Pèlegrin, 2008). There have been multiple hypotheses proposed to explain the direct translocation of CPPs across the lipid bilayer of plasma membrane including inverted micelle formation, adaptive translocation, pore formation, electroporation like permeabilization and microdomain boundary entry. The exact mechanism of cellular entry of CPPs still remains inconclusive and a topic of debate (Richard *et al.*, 2003; Duchardt *et al.*, 2007; Vivès, Schmidt and Pèlegrin, 2008; Bechara and Sagan, 2013; Thareja *et al.*, 2021).

1.7. Polymers in ocular drug delivery

Biodegradable polymers in ocular formulations facilitate increased retention time, higher bioavailability, and controlled release via mucoadhesion to the corneal and conjunctival epithelia in eye (Lynch *et al.*, 2019). Their ability to biodegrade after administration ensures that they are broken down by the body into non-toxic by-products. Meanwhile it also helps in maintaining a sustained drug release profile, as

the drug releases while the polymer breaks down. There can also be stimuli-responsive polymers that react or release an active ingredient when a change in conditions like temperature, pH, or pressure, etc. is affected, and are called smart polymers that have been used as in-situ gelling systems. These formulations undergo a change in viscosity as a response to change in the physiological environment and can remain at the site of application for longer times, thereby allowing time for the drug to permeate across the cornea (Lynch *et al.*, 2019). Other bio-responsive polymers can undergo a variety of changes in response to a stimulus, including changes in permeability, shape, or phase separation, which allows the drug in the formulation to be released upon requirement. Usually, a combination of different polymers is used in a single formulation to overcome any disadvantages of a certain polymer (Lynch *et al.*, 2019). Many polymers can interact with each other and form polyelectrolyte complexes, which are formed upon interaction of a positively charged polymer with a negatively charged one to form a cross-linked system. These polyelectrolyte complexes are used to make nanoparticles to be employed in drug delivery. Different polymers used in ocular drug delivery include natural polymers like chitosan, hyaluronic acid, sodium alginate, carboxymethylcellulose sodium (CMC), etc. and synthetic polymers like Poly(lactic-co-glycolic acid) (PLGA), Poly(ε-caprolactone) (PCL), Poly(ethylene glycol) (PEG), etc. (Lynch *et al.*, 2019).

1.8. Neuroprotection in treatment of retinal diseases

Neuroprotective strategies promote survival of the neurons by preserving their structure and function, with a potential to protect against or slow down the progression of vision loss and retinal cell death in case of retinal damage and disease (Ahmed *et al.*, 2011, 2019; Morgan-Warren *et al.*, 2016; Pardue and Allen, 2018). Successful

neuroprotective therapies have the ability to provide months or years of useful vision to patients, allowing them to continue to be gainfully employed while maintaining their independence and have a reasonable quality of life. Neuroprotective strategies are broadly targeted towards the survival pathways of photoreceptors and RGCs and the mechanisms of their degeneration and death in disease and can be used in multiple retinal diseases. These approaches may target a specific pathway or multiple pathways but mostly have pleiotropic effects that result in enhanced survival of retinal neurons and preserved visual function. Experimental neuroprotective therapeutics involve synthetic bile acids ursodeoxycholic acid (UDCA) and its taurine conjugated derivative tauroursodeoxycholic acid (TUDCA) (Daruich *et al.*, 2019), steroidal hormones progesterone and oestrogen (Nuzzi *et al.*, 2018), dopamine based agents L-DOPA (l-3,4-dihydroxyphenylalanine) or dopamine agonists (Aung *et al.*, 2014), neurotrophic factors including neurotrophins like nerve growth factor (NGF) (Garcia, Hollborn and Bringmann, 2017), brain-derived growth factor (BDNF) (Kimura *et al.*, 2016), neurotrophin 3 (NT-3), and neurotrophin 4 (NT-4) (Peinado-Ramón *et al.*, 1996), glial-derived neurotrophic factors (GDNF) like neurturin, artemin, and persephin (Hauck *et al.*, 2006) and others like pigment epithelium-derived factor (PEDF) (Vigneswara *et al.*, 2013; Vigneswara and Ahmed, 2019), fibroblast growth factors (FGF) (Di Pierdomenico *et al.*, 2018), gp130-binding growth factors (such as ciliary nerve trophic factor (CNTF)) (Leibinger *et al.*, 2009), insulin-like growth factors (IGF) (Arroba *et al.*, 2011), and transforming growth factors (TGF) (Braunger *et al.*, 2013), and siRNA mediated knockdown of pro-apoptotic factors (Ahmed *et al.*, 2011; Vigneswara *et al.*, 2014). However, these treatments often need to be started at the

earliest stages of pathology when they can be most effective in halting progression (Ahmed *et al.*, 2011, 2019; Morgan-Warren *et al.*, 2016; Pardue and Allen, 2018).

1.9. Aims and Hypotheses

1.9.1. Aims

This thesis aimed to achieve a therapeutic bioavailability of anti-inflammatory and/or neuroprotective drugs in the posterior segment of the eye for the treatment of diseases related to the retina and optic nerve such as glaucoma, traumatic optic neuropathy (TON), age-related macular degeneration (AMD), diabetic retinopathy, etc. via non-invasive or minimally invasive sustained-release drug delivery technologies based on biodegradable and biocompatible polymers. This is an unmet clinical need as the current management modalities involve frequent intravitreal injections and even then, the degenerative loss is not recovered fully. The first aim was to identify most suitable penetration enhancing agents for a topical formulation to deliver a glucocorticoid dexamethasone sodium phosphate (DSP) to the posterior segment and synthesise a library of PEAs. Secondly, it was aimed to evaluate the ability of synthesised PEAs to transport therapeutically useful titres of DSP through biological defensive barriers *ex-vivo* and also assess their safety in maintaining the barrier integrity *ex-vivo* and *in-vitro* cytotoxicity and tolerance. Final aim was to deliver and investigate the *in-vivo* therapeutic efficacy of sustained-release intravitreal depots of biodegradable 50:50 PLGA microspheres co-encapsulated with anti-oxidants α-tocopherol (vitamin E) and melatonin, and an anti-inflammatory corticosteroid, dexamethasone, in an optic nerve crush (ONC) model of TON in rats.

1.9.2. Hypotheses

The first hypothesis postulated that the PEAs are able to transport a therapeutic titre of DSP across the cornea and could potentially lead to increased bioavailability in the posterior segment than is currently possible. The second hypothesis postulated that intravitreally injected PLGA microspheres containing vitamin-E, melatonin, and dexamethasone would provide neuroprotection in the retina by protecting RGC apoptosis and promote axonal regeneration in the optic nerves *in-vivo* 21 days after ONC.

1.10. Experimental approach

To achieve the aims and test the hypotheses in this thesis, in chapter – 2, a systematic literature review of the available experimental literature on PEAs that can efficiently enter the posterior segment of the eye after topical administration was conducted with the aim of identifying the most effective PEA for non-invasive delivery of drugs to the posterior segment of the eye. This was done by literature search from three electronic databases, establishing an inclusion and exclusion criteria, measuring risk of publication bias, and data extraction all based on the guidelines outlined by Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA).

In chapter – 3, we investigated the ability of penetratin, TAT and pAc in improving bioavailability of DSP across complex defensive ocular barriers in a medium-throughput and rapid-assay *ex-vivo* model of permeability using porcine eye tissue, developed in our group. pAc was synthesised using a patented protocol. We first tested the permeabilities of pAc compared with that of fluorescently labelled CPPs, across porcine cornea and sclera. DSP was then formulated with pAc and non-labelled CPPs for topical application and assayed for permeability. DSP concentration in the

receptor chamber was quantified using high-performance liquid chromatography (HPLC) to ascertain the enhancement in its permeability with PEAs. This was followed by safety assessment of barrier integrity of corneal epithelium using transepithelial electrical resistance (TEER) measurements and immunofluorescent staining of excised cornea with epithelial tight junction markers ZO-1 and occludin, and finally *in-vitro* cytotoxicity assessment of PEAs in human ARPE-19 cell line using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay.

In chapter – 4, we investigated the *in-vivo* therapeutic efficacy of intravitreal depots of biodegradable 50:50 PLGA microspheres co-encapsulated with anti-oxidants α-tocopherol (vitamin E) and melatonin, and an anti-inflammatory corticosteroid, dexamethasone, in a rat model with optic nerve crush (ONC) injury mimicking the pathophysiology of neurodegeneration in TON and glaucoma. The encapsulated microspheres were prepared using an oil-in-water emulsion solvent extraction/evaporation technique and injected intravitreally in ONC rats allowing a sustained release of active substances for 21 days. Postmortem immunohistochemical analyses in the eyes and optic nerves were carried out to determine $RBPMS^+$ RGC survival and $GAP43^+$ axonal regeneration, $GFAP^+$ Müller cell gliosis and $OX\text{-}42^+$ microglial activation for neuroinflammation, and $CD\text{-}68^+$ macrophage migration for immune response.

1.11. Collaborative work credits

The work included in chapters 3 and 4 is outcome of inter-university and inter-ORBITAL consortium collaborations and secondments. The polyacetylene (pAc) polymeric PEA used in chapter – 3 (section 2.1 – Materials and Methods) was synthesised and characterised by Dr Thomas Leigh in the research group of co-supervisor Dr Francisco

Fernandez-Trillo at the School of Chemistry, University of Birmingham, UK and was used as provided for all the work done in the chapter. Similarly, the drug loaded PLGA microspheres were fabricated and characterised (section 2.1 – Materials and Methods, section 3.1 – Results) by Dr Marco Brugnera in the research group of Prof Rocio Herrero-Vanrell at the Faculty of Pharmacy, Complutense University of Madrid, Spain and were brought to University of Birmingham as part of an academic secondment within the EU-project ORBITAL. Other than the abovementioned, Abhinav Thareja conceived and conducted the experiments and analysed the corresponding data.

1.12. References

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CHAPTER 2

Penetration Enhancers for Topical Drug Delivery to the Ocular Posterior Segment — A Systematic Review

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Abstract

There is an unmet clinical need for eye drop formulations to efficiently treat the diseases of the posterior ocular segment by non-invasive topical administration. Here, we systematically reviewed the literature on ocular penetration enhancers and their ability to transfer drugs to the posterior segment of the eye in experimental studies. Our aim was to assess which penetration enhancer is the most efficient at delivering drugs to the posterior segment of the eye, when topically applied. We conducted a comprehensive search in three electronic databases (Ovid Embase, Ovid MEDLINE, and PubMed) to identify all the relevant manuscripts reported on ocular penetration enhancers based on the PRISMA guidelines. We identified 6540 records from our primary database search and filtered them per our inclusion/exclusion criteria to select a final list of 14 articles for qualitative synthesis. Of these, 11 studies used cell penetrating peptides (CPPs), 2 used chitosan, and 1 used benzalkonium chloride (BAC) as the penetration enhancer. Cationic and amphipathic CPPs, transactivator of transcription (TAT), and penetratin can be inferred to be the best among all the identified penetration enhancers for drug delivery to the fundus oculi via topical eye drop instillation. Further high-quality experimental studies are required to ascertain their quantitative efficacy.

2.1. Introduction

Eye diseases originating in the posterior segment ocular tissues, mainly in the retina-choroid, are a major cause of visual impairment across the globe. It is estimated that worldwide, around 2.2 billion people have some sort of visual impairment and out of these, people affected by diseases of the ocular posterior segment including age-related macular degeneration (AMD) (10.4 million) and diabetic eye disease (3 million) constitute a large proportion (World report on vision, 2019). Specifically, in Europe, AMD and diabetic eye disease lead to a critically high incidence of blindness in older populations and together constitute approximately 35% of the causes of blindness, thus putting a huge burden on public health expenditure (Prokofyeva and Zrenner, 2012). AMD is the reason for 8.7% blindness globally with projected estimates at 196 million people having this disease in 2020 and expanding to 288 million by 2040 and is also the single and most widely recognized cause of elderly blindness in developed nations, especially in individuals over 60 years of age (Prokofyeva and Zrenner, 2012; Wong *et al.*, 2014; World report on vision, 2019). Studies also provide strong evidence that AMD is more prevalent in people of European ancestry as compared to other ethnicities, with Europe having the highest prevalence of AMD anywhere in the world (Prokofyeva and Zrenner, 2012; Wong *et al.*, 2014; Colijn *et al.*, 2017). In the working adult population of the world and particularly in Europe however, diabetic eye diseases comprising Diabetic Retinopathy (DR) and Diabetic Macular Oedema (DMO) is the most common reason for vision impairment and blindness, with a prevalence of approximately 34.6% among global diabetic population and 29.4% in diabetic Europeans, and an estimated increase from current 6.4 million affected people in Europe to 8.6 million in 2050 (Yau *et al.*, 2012; Li *et al.*, 2020).

Topical delivery of drug formulations through the eye surface, mainly via eye-drops, to treat diseases of the posterior segment is significantly inhibited by the various physiological and anatomical defensive ocular barriers including clearance by lacrimal fluid and drainage mechanisms, conjunctival systemic absorption, cornea, and different tissues in the anterior segment. On the other hand, systemic administration of drugs is hindered by the blood-ocular barriers comprising blood-aqueous barrier (BAB) and blood-retina barrier (BRB), resulting in negligible ocular bioavailability of less than 5% (Urtti, 2006; Loftsson *et al.*, 2008). The only feasible conventional routes of drug administration to the posterior segment are intravitreal and peri-ocular injections that are highly invasive, cause huge patient discomfort, thus low compliance and require specialist clinicians, besides leading to serious complications including endophthalmitis, increased intra-ocular pressure, cataract, glaucoma and even retinal detachment in rare cases (Sampat and Garg, 2010; Sen *et al.*, 2014; Smith, Smith and Mohney, 2014). Another option is sustained-release drug-eluting ocular implants, but that too requires surgical intervention, suffers from common post-operative complications, and needs to be replaced after a time.

Among various emerging technologies to optimize topical ocular drug delivery congruent with an increased drug bioavailability towards the posterior segment tissues, use of penetration enhancers (PEAs) could be the most promising and viable means to achieve drug formulations that exhibit much higher uptake, cellular internalization, and longer retention into the posterior segment via simple eye-drop instillation. As the name suggests, ocular PEAs are chemical or biological agents capable of modifying the various defensive barriers and membranes of cells and tissues in the eye, through known and unknown mechanisms, to affect an enhanced passage across them.

Multiple types of PEAs have been used in pharmaceutical formulations and reported in literature, such as cyclodextrins (CD), calcium chelators (EDTA), surfactants (BAC), bile acids & bile salts, fatty acids, azones, mucoadhesive polymers (chitosan, HA), and cell-penetrating peptides (CPP) (Kaur and Smitha, 2002; Zambito and Di Colo, 2010; Moiseev *et al.*, 2019). PEAs can be used as additives or excipients; as drug carriers conjugated with the drug; or as conjugates with drug carriers like micro/nanoparticles, liposomes, micro/nano-emulsions, etc. The majority of these PEAs barring a few, however, are effective only for transcorneal permeation to enhance bioavailability in the anterior ocular segment and have negligible or no effect on uptake in the tissues of the ocular posterior segment.

There are a few literature reviews on different reported PEAs for topical ocular drug delivery, but none specifically on the PEAs that can access the ocular posterior segment (Kaur and Smitha, 2002; Moiseev *et al.*, 2019). The present report systematically reviewed the available experimental literature on PEAs that can efficiently enter the posterior segment of the eye after topical administration with the aim of identifying the most effective PEA for non-invasive delivery of drugs to the posterior segment of the eye. This will help in developing non-invasive, novel pharmaceutical formulations with enhanced drug bioavailability in the ocular posterior segment to treat the complex eye diseases like AMD and diabetic eye disease through easy to apply, non-invasive and cost-effective mode of eye-drop instillation.

2.2. Methods

2.2.1. Review Process

This systematic review was conducted based on the guidelines outlined by Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) (Liberati *et al.*,

2009; Moher *et al.*, 2009). The reporting parameters were modified in context of animal studies. All of the review steps were duly performed by two authors (A.T. and Z.A.) independently and any conflicts or disagreements were resolved after discussion to reach a consensus.

2.2.2. Literature Search

Published reports on PEAs were identified by running exhaustive searches in three electronic databases: Ovid Embase, Ovid MEDLINE, and PubMed from the time of inception of the databases to 30th December 2020. We formulated a common search string for all the databases containing the terms ((penetration enhancers) AND (retina OR retinal ganglion cells OR retinal pigment epithelium)). The search was restricted to English language reports only. Duplicates were removed and manuscripts were initially shortlisted by referencing the titles and abstracts. Final selection was done by reviewing full texts against the inclusion and exclusion criteria by the two authors (A.T. and Z.A.). Additional publications were identified upon reviewing the references of shortlisted articles.

2.2.3. Inclusion and Exclusion Criteria

To be eligible for review, the screened articles had to fulfil certain criteria: (a) only manuscripts of completed experimental studies published in a journal were included; conference abstracts and in-process or non-indexed citations were not included; (b) only studies that investigated topical route of administration of PEAs were included; (c) only animal studies were included; clinical or human trials or randomised controlled trials (RCTs) were not; (d) rat, mouse and rabbit models only for live animal testing; (e) most importantly, only those studies were included where PEAs were able to reach the tissues of the ocular posterior segment mainly retina-choroid *in-vivo*, and studies must

demonstrate and compare qualitative and/or quantitative *in-vivo* biodistribution and efficacy in the posterior segment of eyes with and without PEAs, using standard analytical techniques. Eligibility assessment of studies was performed in an unblinded standardized manner.

2.2.4. Risk of Publication Bias

Risk of bias (RoB) was evaluated using a modified version (Fabian-Jessing *et al.*, 2018) of SYRCLE's risk of bias tool for animal studies (Hooijmans *et al.*, 2014), which itself is adapted from the Cochrane Collaboration's tool for assessing risk of bias in RCTs (Higgins *et al.*, 2011). Briefly, each article was assessed on 13 different risk parameters and the responses were recorded as: (a) Yes, if the parameter was positively reported in the article with sufficient information on its methodology; (b) Yes*, if the parameter was reported positively but with insufficient information or without a methodology; (c) No, if the parameter was negatively reported or not reported at all. In all the final shortlisted studies, we surveyed the various critical aspects of bias that play a vital role in animal experiments, to determine the methodological quality of those studies.

2.2.5. Data Extraction

Data was collected and summarized in a pre-designed electronic data extraction form with multiple sections to address all the key aspects of the study including author(s), journal and year of publication, type of PEA used, *in-vivo* animal models, *in-vitro* or *ex-vivo* tests, cell viability and toxicity analyses, and efficacy tests and biodistribution in the posterior segment tissues *in-vivo.* Since efficacy of drug penetration to the posterior chamber was mostly analysed by immunohistochemistry, meta-analysis was not possible and hence we qualitatively reviewed the reports to answer our review aims.

2.3. Results

6540 records were identified from our primary literature search on the databases and after filtering duplicates, 5559 unique original published articles were identified. These were then subjected to a preliminary screening of titles and abstracts, and a list of 28 articles were found to be relevant for review. The whole focus was on identifying PEAs that could efficiently access or facilitate penetration of cargo to the fundus oculi by topical eye-drop instillation, and to the best of our knowledge we have obtained all-inclusive search results that correspond to our eligibility criteria. Full texts of these 28 shortlisted manuscripts were then examined; any relevant references were also analysed and a final selection of 14 eligible studies was made for qualitative synthesis in our review after application of inclusion and exclusion criteria. The literature identification strategy is demonstrated by means of PRISMA flow diagram in (Figure 2.1). Of the 14 selected studies, 11 or around 78.6% were exclusively dealing with CPPs as PEAs for delivery to the ocular posterior segment and the rest (3) were based on other compounds. 13 studies (92.9%) employed rat or mouse models for *in-vivo* efficacy and biodistribution tests and 1 study used rabbits. Since none of the studies quantitatively analysed penetration efficiency to the posterior segment, a meta-analysis of the data was not possible and hence what follows is a qualitative analysis of the results presented for the different PEAs. The characteristics of the study are defined in (Table 2.1).

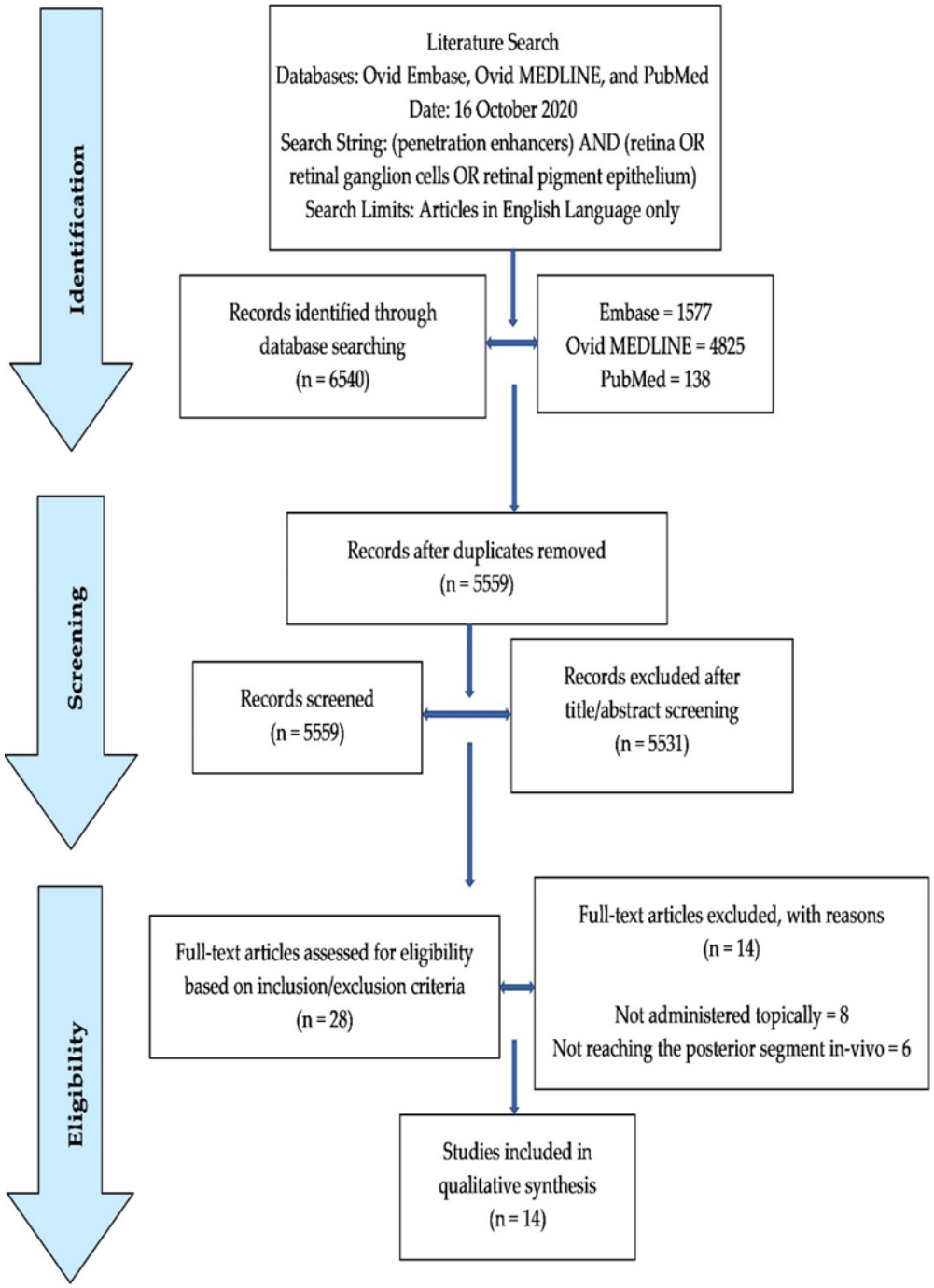

Figure 2.1: Prisma flow diagram demonstrating the literature search strategy

Table 2.1: Study characteristics

Publication	Penetration Enhancers (PEAs)	Animal model	*In-vivo* Efficacy and Biodistribution
Johnson *et al.*, 2008	CPP: Peptide for Ocular Delivery (POD) – GGG(ARKKAAKA)$_4$	C57BL/6J mice	Microscopy – POD conjugates transduced sclera/choroid and dura of the optic nerve 45 min post application and was eliminated within 24 h; no staining in non-POD groups
Wang *et al.*, 2010	TAT – CPP	Sprague-Dawley rats	Immunohistochemistry – TAT conjugates readily detected in retina 30 min post administration, mainly in RGCs in the GCL, peaked around 30 min to 1 h, detectable till up-to 8 h; no detection in non-TAT retinas
Liu *et al.*, 2014	Penetratin – CPP	Sprague-Dawley rats	Fluorescence Microscopy – Penetratin reached both anterior and posterior segments of the eyeballs 10 min after application, fluorescence observed in GCL, IPL, OPL, INL, ONL, RPE and the photoreceptor segments, peaked at 30 min and persisted till 6 h; no fluorescence in untreated eyes
Zhang *et al.*, 2015	TAT – CPP	Kunming mice and C57BL/6 mice	Immunohistochemistry – Groups treated with TAT conjugated drug had high drug distribution in the retina after eye-drop administration; unconjugated drug groups showed no concentration
Mahaling & Katti, 2016	Benzalkonium chloride (BAC)	Pigmented mice	Fluorescence Microscopy - The results indicate that the presence of BAC lead to varying degrees of increase in bioavailability of different nanoparticles to retina as compared to non-coated nanoparticles
Li *et al.*, 2016	TAT – CPP	C57BL/6 mice	Quantitative analysis (ELISA) - Concentration of drug in the retina groups treated with TAT conjugated drug was approximately 14 times

			higher than in those with non-conjugated drug, concentration peaked around 1 h, and the half-life was around 11 h
Liu *et al.*, 2016	Penetratin – CPP	Sprague-Dawley rats	Fluorescence Microscopy – Penetratin conjugated complexes reached posterior segment in 10 min post application, fluorescence in photoreceptors segments, RPE, GCL, IPL, OPL, choroid, INL and ONL; no fluorescence in eyes treated with unconjugated complexes
Chu *et al.*, 2017	TAT – CPP	Brown Norway rats	Confocal Microscopy – Eyes treated with TAT modified complexes exhibited 11 times higher fluorescence in the retina-choroid than those treated with non-modified complexes.
de Cogan *et al.*, 2017	CPP: (5[6]-carboxyfluorescein-RRRRRR-COOH)	Adult Sprague-Dawley rats and Wild-type (WT) C57BL/6J mice	Quantitative analysis (ELISA) – Concentration of drug detected in groups treated with CPP conjugated drug is 10-11 times higher as compared to the groups with non-conjugated drug, drug levels peaked at 40 min post administration and was eliminated by 24 h
Jiang *et al.*, 2017	Penetratin derivatives - CPP	Mice	Fluorescence Microscopy – Fluorescence from retinas treated with hydrophobic Penetratin derivatives > wild-type Penetratin > hydrophilic derivatives; peptides reached the posterior segment within 10 min post instillation and fluorescence peaked around 1 h
Atlasz *et al.*, 2019	TAT – CPP	Rats	Fluorescence Microscopy - TAT bound complexes reached the retina with the efficiency about three-fold that of non-conjugated drugs
Li *et al.*, 2019	Chitosan	C57BL/6 mice	Optical Coherence Tomography – Eyes treated with chitosan conjugates emitted higher levels of fluorescence from the posterior

			segment that those eyes treated without chitosan; fluorescence started at 10 min, peaked at 6 h, and disappeared 12 h post application
Yang *et al.*, 2019	Penetratin – CPP	ICR mice	Fluorescence Microscopy – Retinas treated with Penetratin modified complexes demonstrated much higher fluorescence intensity (about 3 times) as compared to the retinas with non-modified complexes; detected in retinas 1 h post administration, in RPE within 2 h, peaked at 8 h and still detectable until 12 h
Wang *et al.*, 2020	Carboxymethyl chitosan (CMCS)	New Zealand albino rabbits	Quantitative analysis (HPLC) – Drug concentration in retina-choroid treated with CMCS conjugates was 2.5 times higher than in those treated with non-conjugates; drug concentration peaked at 1 h still detectable up-to 3 h post application

2.3.1 Cell Penetrating Peptides (CPP)

CPPs can be defined as short chain peptides, often consisting of 30 or less amino acid residues, that exhibit a well-defined property of internalization across cellular membranes with negligible cytotoxicity, through an energy dependent and/or independent mechanism, without the need of chiral interactions with surface receptors. The most commonly studied CPPs are of cationic nature, however a number of anionic or hydrophobic peptides have also been reported. CPPs are able to transport a multitude of covalently or non-covalently linked cargoes of different molecular weights into living cells (Bechara and Sagan, 2013). Different CPPs have been reported for drug delivery across the skin topically, nasal delivery for pulmonary diseases, and even for crossing the blood-brain barrier (BBB), though the mechanism of their cellular

internalization is still debated and is a contentious topic (Vivès, Schmidt and Pèlegrin, 2008; Bechara and Sagan, 2013). Lately, there has been a rigorous use of CPPs for ocular drug delivery, mostly to the anterior segment tissues of eye and here we report the use of CPPs as penetration enhancers for drug delivery to the ocular posterior segment. Of the total 11 CPP candidates in our review, 5 reports are on TAT, 4 on Penetratin and 2 on other peptides.

2.3.1.1. TAT

TAT or transactivator of transcription is a protein transduction domain (PTD) with general sequence GRKKRRQRRRPQ of the HIV Tat protein and is one of the most investigated CPP for drug delivery.

Wang *et al.* (2010) first demonstrated its potential to deliver a therapeutic growth factor across the posterior segment tissues by eye-drop instillation (Wang *et al.*, 2010). They investigated the penetration ability of TAT conjugated human acidic fibroblast growth factor (aFGF) in the retinas and its therapeutic efficiency *in-vivo* in a retinal ischemia–reperfusion (IR) injury model in 18 weeks old male Sprague-Dawley rats. A primary immunohistochemical analysis was made to assess tissue penetration of TAT-aFGF compared with non-conjugated aFGF, where TAT-aFGF was readily detected at 30 min post administration in the retinas whereas non-modified aFGF was not detected. TAT-aFGF was localized mainly in the retinal ganglion cells (RGC) in the ganglion cell layer (GCL), its concentration peaked around 30 min to 1 h, and gradually declined thereafter, but could still be detected up-to 8 h post administration. These results were reinforced with the histological examination of retinal injury from IR after application of TAT-aFGF and aFGF when retinas from TAT-aFGF treated eyes showed significantly less severe pathological changes, lesser damage to tissue morphology, and lower

depletion in RGCs than the untreated groups, whereas aFGF only treated groups were no different from untreated eyes. Furthermore, TUNEL staining of diseased retinas affirmed a lower instance of RGC apoptosis induced by IR upon treatment with TAT-aFGF. Apoptotic RGCs in the GCL of TAT-aFGF treated retinas were significantly less in numbers than in the groups treated with aFGF only. In addition, TAT-aFGF treated groups showed accelerated recovery of retinal function compared to those treated with aFGF as measured by electroretinography. The authors have suggested a non-corneal route of transport to the posterior segment as TAT-aFGF could not be detected in the deeper layers of cornea like stroma and endothelium and were only found in the superficial corneal epithelium cells, meaning it was not fully able to penetrate and pass through the cornea. The mechanism of cellular uptake of TAT is expected to be cell specific because of selective uptake by RGCs.

Zhang *et al.* (2015) demonstrated delivery of endostatin (Es), a specific inhibitor of endothelial cell proliferation and angiogenesis, with TAT via eye-drop instillation, tested their ocular penetration system in Kunming mice, and evaluated their inhibitory effects in a choroidal neovascularization (CNV) model in 5-week-old male C57BL/6 mice by immunohistochemistry analyses (Zhang *et al.*, 2015). Retinal distribution as observed by immunohistochemistry (IHC) revealed a good distribution profile of TAT-Es in the retina after eye-drop application whereas retinas treated with unconjugated Es showed no detection. The results were supported by the inhibitory effects of TAT-Es on CNV areas observed in choroidal flatmounts. There was a significant reduction in CNV areas in mice treated with TAT-Es eye drops (1378.4 ± 154.3 μm^2) as compared with negative controls (2623.6 ± 240.9 μm^2), while Es only eye drops showed no such protective effects and were similar to negative controls (2514.7 ± 260.7 μm^2). They

also observed that TAT-Es eye drops showed almost similar inhibitory effects as with positive control group receiving intravitreal injections of Avastin for 2 weeks (863.5 ± 50.1 μm^2) and intravitreally injected TAT-Es (961.2 ± 86.9 μm^2), which means eye-drop treatment was as efficient as intravitreal injections and could easily replace it. There have been multiple mechanisms suggested in the study for cellular internalization including energy independent direct translocation for a small portion, but majorly endocytosis dominated by macropinocytosis and relatively smaller instances of clathrin and caveolae-mediated endocytosis. It was speculated that since TAT bears a high net positive charge and could easily interact with negatively charged proteoglycans on the cell membrane like heparan sulphate that work as receptors for positively charged molecules, it may initiate a receptor mediated uptake via macropinocytosis.

Li *et al.* (2016) used TAT combined with a tripeptide of arginine-glycine-aspartic acid (RGD) as a novel fusion protein for cell-specific Es therapy for anti-angiogenesis effects and inhibition of retinal neovascularization (RNV) in DR in an oxygen-induced retinopathy (OIR) model in C57BL/6 mice via eye drops (Li *et al.*, 2016). ELISA detected 14-fold higher concentrations (9.42 ± 0.77 ng/mg) of TAT-Es-RGD in the retina at 1 h after topical application compared to that detected with unconjugated Es (0.68 ± 0.10 ng/mg). The concentration of TAT-Es-RGD was slightly higher than TAT-Es (8.43 ± 0.85 ng/mg) owing to the endothelial cell targeting ability imparted by RGD that binds preferentially to the $\alpha_v\beta_3$ integrin expressed on angiogenic endothelial cells. Concentrations of TAT-Es and TAT-Es-RGD peaked at 1.00 ± 0.00 h and 1.125 ± 0.35 h, respectively, and their half-lives were 9.64 ± 1.31 h and 11.03 ± 1.95 h, respectively. Further, fluorescence microscopy analysis of retinal flat mounts of OIR to check the inhibitory effect of TAT-Es-RGD on retinal neovascularization manifested a significant

reduction in avascular areas and vessel tufts in the groups treated with TAT-Es and TAT-Es-RGD via eye drops compared with those of the negative control group. While the Es and Es-RGD treated groups were no different from the negative control group. Breaking of endothelial cells from the inner limiting membrane (ILM) of the retina was also quantified as an evaluation index for hyperoxia-induced neovascularization. A histological analysis of the ILM of retinas proved there was a significant decrease in the number of endothelial cell nuclei, that broke through the ILM in areas of neovascularization in the TAT-Es and TAT-Es-RGD groups as compared with negative controls, whereas Es and Es-RGD groups had no significant difference when compared with negative controls. In addition to these results, levels of VEGF that induce angiogenesis in OIR mice were detected using IHC. The levels of VEGF in TAT-Es and TAT-Es-RGD groups were much lower than that of the negative control groups, whereas in the Es and Es-RGD groups the levels were similar to that of the negative control group. Eye drop delivery of TAT-Es-RGD reduced VEGF expression by a similar amount as intravitreal injection, signifying how eye-drops can easily replace invasive treatments.

Chu *et al.* (2017) reported topical ocular delivery of PEG-PLGA nanoparticles (NP) modified with TAT-RGD dual peptides for penetration enhancement and targeting in laser induced CNV models in male Brown Norway rats (Chu *et al.*, 2017). Choroid-sclera flatmounts of CNV region were examined using confocal microscopy to determine the extent of penetration and distribution of NPs. Fluorescent intensity in the CNV region was maximum in the groups treated with RGD and TAT dual-modified NPs and was almost 11 times higher than in the groups treated with non-modified NPs which showed negligible emission after eye drop administration in the CNV area.

Fluorescence intensity in different treatment groups was measured in the order of RGD-TAT-NP > TAT-NP > RGD-NP > NP. TAT modified NPs were able to penetrate the ocular barriers and internalize into retina-choroid, however the internalization was scattered and not focused in the CNV sites due to the absence of selectivity in TAT. RGD and TAT dual-modified NPs exhibited maximum localization and accumulation surrounding and particularly within the CNV region. This is attributed to the complementary targeting property of RGD peptide by binding with integrin $\alpha_v\beta_3$.

Atlasz *et al.* (2019) studied TAT bound delivery of vasoactive intestinal peptide (VIP), a retinoprotective peptide, and pituitary adenylate cyclase activating polypeptide (PACAP), a potent anti-inflammatory peptide, in male rats via topical eye-drop administration (Atlasz *et al.*, 2019). Retinal penetration was evaluated by fluorescence microscopy. Retinas of mice treated with eye drops of PACAP-TAT and VIP-TAT showed much higher fluorescence density per unit area than in retinas treated with PACAP/VIP, indicating that PACAP-TAT/VIP-TAT reached the retina more efficiently than PACAP/VIP. The Efficiency for Traversing Eye to Retina (EtE) was also calculated. EtE for PACAP-TAT/VIP-TAT (3.66 ± 0.67%, 3.05 ± 0.58%) was about three times higher than that for PACAP/VIP (1.23 ± 0.56%, 0.97 ± 0.47%).

2.3.1.2. Penetratin

Penetratin is another commonly used CPP derived from a nonviral antennapedia homeodomain protein in *Drosophila melanogaster*, with a general sequence of RQIKIWFQNRRMKWKKK and molecular weight 2.733 kDa.

Liu *et al.* (2014) first reported use of Penetratin as a penetration enhancer for drug delivery to the fundus oculi via eye-drop instillation (Liu *et al.*, 2014). Penetration efficiency and biodistribution of Penetratin was evaluated in 17-week-old male

Sprague−Dawley rats using fluorescence microscopy. Within 10 min of administration, Penetratin was able to internalize in both anterior and posterior segments of the eye. In the posterior segments it was distributed widely in almost all layers of retina-choroid with maximum uptake by the photoreceptor segments and retinal pigment epithelium (RPE) in the subretinal space displaying intense fluorescence in rods and cones, a moderate and uniform distribution in the GCL, inner plexiform layers (IPL), outer plexiform layer (OPL), and mild uptake into the inner nuclear layer (INL) and outer nuclear layer (ONL). Accumulation peaked at 30 min as evidenced by fluorescence intensity and was detectable for as long as 6 h after application suggesting lasting retention in these tissues. An in-vitro cytotoxicity assessment of Penetratin by MTT assay was performed on human conjunctival epithelial cells (NHC) and was found to be least toxic among a group of CPPs, with a high IC_{50} value of approximately 2.5 mM, even higher than that of TAT (2 mM). However, even at a concentration as high as 30 mM of Penetratin, the cell viability was still close to 100%. Both trans-corneal and conjunctival-scleral transport pathways contribute to Penetratin getting into the posterior segment and its penetration ability can be attributed to the high positive charge, its amphipathicity and polyproline type II (PPII) helix structure as observed by circular dichroism.

Liu *et al.* (2016) continued their explorations with Penetratin and this time around demonstrated retinal gene delivery of red fluorescent protein plasmid (pRFP) by PAMAM dendrimers conjugated with Penetratin, in 17 week old male Sprague−Dawley rats via topical instillation (Liu *et al.*, 2016). Their penetration and distribution were evaluated by fluorescence microscopy. Penetratin-PAMAM-pRFP complexes internalized into the posterior segment in merely 10 min, accumulation peaked from 1

to 2 h and persisted until 8 h post administration. Maximum fluorescence intensity was observed in photoreceptors and RPE, followed by GCL, IPL, OPL and the choroid, and faintish fluorescence in the INL and ONL. In contrast, eyes treated with nude pRFP had no detectable fluorescence in the retina. Furthermore, *in-vivo* gene expression was also measured and revealed that nude plasmids could not transfect retinas, while Penetratin-PAMAM-pRFP complexes displayed strong transfections in photoreceptors, IPL and OPL, which was in-line with the results of distribution.

Jiang *et al.* (2017) compared the *in-vivo* penetration ability of different derivatives of Penetratin with that of wild-type Penetratin via topical eye-drop administration in male mice (Jiang *et al.*, 2017). Pharmacokinetics and biodistribution of hydrophobic and hydrophilic derivatives in comparison with wild-type Penetratin were estimated by fluorescence microscopy. Initially all the peptides were able to internalize in both anterior and posterior segments within 10 min as evident by a bright fluorescence and their concentrations peaked around 1-h post eye-drop instillation. However, the retinas treated with the hydrophobic derivatives emitted overwhelming fluorescence, revealing that the hydrophobic derivatives internalized more efficiently than did the wild-type Penetratin. Whereas the retinas treated with the hydrophilic derivatives showed weaker fluorescence compared with wild-type Penetratin implying an impaired penetration ability. To back the results, it has been suggested that higher hydrophobicity induces more helix content in the peptide structure as seen in circular dichroism studies, which in turn helps in stronger affinity interactions with the biological membranes thus facilitating higher permeability. Toxicity analyses performed *in-vitro* on human corneal and conjunctival epithelial cells via MTT assay revealed no cytotoxicity, and *ex-vivo* in excised rabbit corneas and sclera by measuring the

hydration values also showed good biocompatibility and no leakage during translocation.

Yang *et al.* (2019) studied Penetratin and RGD peptide modified nanocarriers (NCs) of PEG-PAMAM dendrimers for enhanced penetration and complementary targeting to the ocular posterior segment in male ICR mice via topical application (Yang *et al.*, 2019). Ocular permeability and posterior segment distribution as estimated by fluorescence microscopy exhibited that although all the NCs were able to penetrate the posterior segment, the mouse retinas treated with NCs conjugated with Penetratin or RGD-Penetratin showed almost 3 times stronger fluorescence intensity than the retinas treated with non-conjugated nanocarriers, at their peak concentrations at 8 h post application. Penetratin-RGD-NCs initially entered the retinas within 1 h, penetrated the RPE within 2 h, and then gradually internalized to the inner retinal layers, and the accumulation reached a maximum at 8 h post administration. In addition, these retinas kept emitting measurable fluorescence for up to 12 h and were still detectable 24 h after instillation, indicating that the Penetratin-RGD modified NCs resided in the retinas persistently which was not the case with retinas treated with non-modified NCs as their fluorescence died out within 12 h. MTT viability assays to establish *in-vitro* cytotoxicity of the peptide-NC complexes in human conjunctival epithelial cells (NHC), human corneal epithelial cells (HCEC), and human umbilical vein endothelial cells (HUVEC) revealed that these complexes had no detectable cytotoxicity, and the percentage cell survival was 90%–110% after incubation with 0.2–20 μM NCs. PEG conjugation significantly attenuated the cytotoxicity of PAMAM dendrimers because of reduced amino groups on the surface and surface passivation, thus preventing contact between cells and primary amines.

2.3.1.3. Other CPPs

Johnson *et al.* (2008) reported a novel CPP called peptide for ocular delivery (POD) with a general sequence GGG(ARKKAAKA)$_4$ and molecular weight 3.5 kDa that could efficiently reach and deliver cargo to the fundus oculi *in-vivo* in C57BL/6J mice by topical eye-drop instillation (Johnson, Cashman and Kumar-Singh, 2008). Pharmacokinetics and ocular distribution in the posterior segment were determined by microscopic analysis which revealed that in the eyes treated with POD-drug complexes the sclera/choroid and dura of the optic nerve were transduced by these complexes in 45 min after application and it was eliminated within 24 h, whereas no such staining was observed in the groups with non-conjugated drug. They have also suggested that cellular uptake of POD is temperature dependant and does not happen by endocytosis as no reduction in peptide uptake was observed *in-vitro* in the presence of endocytosis inhibitors. Instead, a direct entry mechanism without plasma membrane fixation is supported as also cell-surface proteoglycans inhibited the uptake of POD *in-vitro.*

de Cogan *et al.* (2017) tested delivery of anti-VEGF drugs using another novel poly-arginine based CPP: (5[6]-carboxyfluorescein-RRRRRR-COOH) for their penetration and pharmacokinetics in male Sprague- Dawley rats, and inhibitory effects in a CNV model in Wild-type (WT) C57BL/6J mice, via eye-drop application (de Cogan *et al.*, 2017). Penetration and biodistribution was evaluated by ELISA. Mice treated with CPP-drug conjugates showed a peak drug concentration about 10-11 times higher in contrast to the groups treated with non-conjugated anti-VEGF drug. The levels of drug accumulation in CPP-drug conjugates in the retina peaked at 40 min after topical administration representing an upwards of 0.2% of the applied pay load, followed by elimination in 24 h. To estimate the inhibitory effects of CPP-drug conjugates on CNV

lesions, fluorescein angiography (FA), infrared imaging and IHC of disease biomarkers (collagen IV and isolectin B4) in RPE/choroid flatmounts showed that CPP-drug complexes caused considerable reduction to two-thirds extent in the area size of scar and neovascularization, whereas CNVs treated with non-complexed drugs had no apparent differences from the untreated or negative control groups. Also, the inhibitory effects of eye-drop instilled CPP-drug conjugates were similar to those achieved by intravitreal injection of drugs or CPP-drug conjugates, implying that non-invasive topical administration may be a preferred mode for treating posterior segment diseases. No cytotoxicity was detected for CPPs in *in-vitro* MTT assays in rat retinal cultures, human ARPE-19 cells, and human corneal fibroblasts.

2.3.2. Chitosan

Chitosan is the second most abundant natural polysaccharide derived from deacetylation of chitin and has a sugar backbone of β-1,4-linked glucosamine (Felt, Buri and Gurny, 1998). It is a polycationic biopolymer and a structural component of the exoskeletons of crustaceans and insects but also found in some fungi. It bears many favourable properties, like non-cytotoxicity, biocompatibility, and biodegradability and has been used in pharmaceutical and biomedical fields for decades, playing a tremendous role in advancement of drug delivery systems (Felt, Buri and Gurny, 1998). Chitosan and its derivatives have been long used as penetration enhancers for transdermal, oral, nasal, buccal and vaginal drug delivery (Caramella *et al.*, 2010). In ocular drug delivery it finds wide use as an excipient as penetration enhancer owing to its highly mucoadhesive nature that allows prolonging drug residence in the precorneal area and thus resulting in higher bioavailability.

Li *et al.* (2019) used chitosan as a penetration enhancer on liposomes for ocular delivery of triamcinolone acetonide via eye-drop instillation to the posterior segment of the eye in C57BL/6 mice (Li *et al.*, 2019). Optical Coherence Tomography (OCT) to evaluate penetration and distribution in the eyes showed that eyes treated with chitosan coated liposomes emitted relatively higher levels of fluorescence from the posterior segment of the eye when compared with non-coated liposome treated eyes, though it was not a very significant difference. The fluorescence emission in the posterior segment of the eye started at 10 min and peaked at 6 h after topical administration. Fluorescence from eyes treated with chitosan coated liposomes lasted longer (12 h) than that of non-chitosan groups (10 h) indicating that apart from slightly higher penetration, chitosan also enabled longer retention in the posterior segment.

Wang *et al.* (2020) demonstrated the penetration enhancing ability of carboxymethyl chitosan (CMCS) coating over nanocomposites to deliver dexamethasone (DEX) to the posterior segment of eye in New Zealand albino rabbits through topical eye-drops (Wang *et al.*, 2020). *In-vivo* activity and distribution of the CMCS coated nanocomposites was estimated by measuring the DEX concentrations in retina-choroid tissue homogenates using HPLC. Eyes treated with CMCS conjugated nanocomposites showed the highest DEX concentration in retina-choroid as compared to the groups treated with commercially available DEX eye drops and non-CMCS nanocarriers. In the commercial eye drop groups, DEX was not detectable in the tissues at any time point and its concentration was below quantification limits. Even with the non-CMCS nanocarriers, DEX was cleared from the choroid-retina within 1 h post application suggesting impaired retention of the nanocomposites. At 30 min however, DEX tissue concentration in CMCS-nanocomposite groups was 2.5 times

higher than that in the non-CMCS nanocomposite groups, which peaked at 1 h and was still detectable up-to 3 h post administration, implying greater penetration and longer residence time in retina-choroid. The *in-vivo* transport pathway for internalization of CMCS was visualized using fluorescence imaging of rabbit eyes after eye-drop instillation which measured the fluorescence intensity of all ocular tissues to track the CMCS coated nanocomposites revealed an overwhelmingly stronger fluorescence in the conjunctival-scleral pathway than in the corneal pathway, indicating that CMCS is transported to the posterior segment via conjunctival-scleral route. The primary mechanism of CMCS uptake in the human conjunctival epithelial cells is via energy-dependent and clathrin-mediated endocytosis as evidenced *in-vitro* by about 54% reduction in cellular uptake when inhibitors of clathrin-mediated endocytosis were used.

2.3.3. Benzalkonium chloride (BAC)

BAC is an age-old antimicrobial preservative widely used in ophthalmic formulations and may act as penetration enhancer, generally to the anterior segment tissues. It facilitates penetration by breaking down the physiological and anatomical diffusion barriers for solute and solvent molecules located in the outer layer of the epithelium (Kusano *et al.*, 2010).

Mahaling & Katti (2016) used BAC for enhanced penetration of nanoparticles into ocular tissues via topical eye-drop administration in pigmented mice (Mahaling and Katti, 2016). Bioactivity and distribution of BAC coated nanoparticles was determined by fluorescence microscopy. Although the effect of BAC on ocular penetration of nanoparticles and localisation in the retina showed promising results, the enhancement effect was not very pronounced. However, there was some vaguely reported significant

increase in bioavailability of BAC coated nanoparticles in the retina-choroid-sclera, as compared to non-coated nanoparticles, which also varied depending on the surface properties of nanoparticles.

2.3.4. Risk of Bias (RoB)

RoB analysis carried out on the methodological quality of the 14 included studies is depicted as a stacked bar-chart in (Figure 2.2). In the included studies, there is a high risk of bias since none of the studies reported treatment concealment, random housing of animals, blinding of interventions nor assessed outcomes randomly. More than >55% of studies did not include information on random sequence generations for animals, specified the correct timing of randomisation nor provided sample size calculations. Only 70% of the studies described baseline characteristics, 85% specified primary outcomes but 100% were deemed free from contamination (i.e., when controls groups inadvertently receive the treatment or are exposed to the treatment). The RoB analysis of the 14 articles demonstrates that some of the animal studies possess a high degree of bias that may negate the findings of the studies.

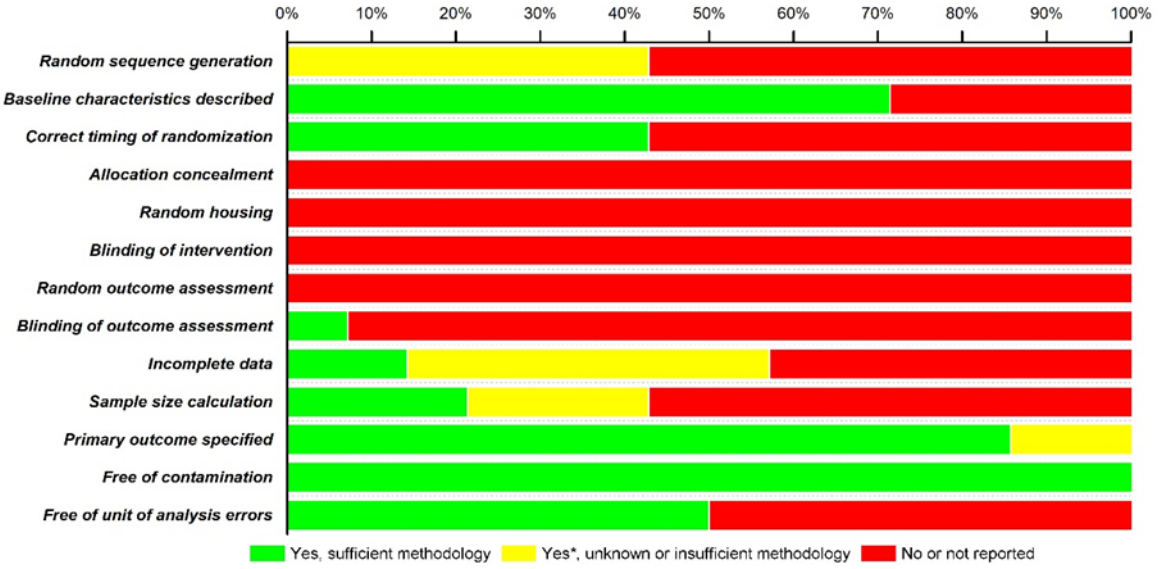

Figure 2.2: Risk of Bias (RoB) analysis.

2.4. Discussion

We systematically reviewed all the available literature on experimental PEAs for topical delivery to the posterior segment of the eye. From the primary search result of 6540 articles, we filtered the relevant publications to a final 14 in conformity with our inclusion criteria and qualitatively analysed them since a meta-analysis was not possible due to lack of a common standardized quantitative measurement of penetration efficacy in the selected studies. The search results included all the different ocular penetration enhancers that have been reported, however most of them were only effective to the anterior segment of the eye. Since decades, PEAs have been used in topical transdermal drug delivery to the skin and in intestinal mucosa by oral administration, and it is only recently that they are being employed for ocular delivery. Topical eye-drop formulations did not evolve functionally for diseases of the fundus oculi, but now there seems to be a renewed vigour in their development specially when PEAs can

overcome the biggest hinderance of low uptake and bioavailability in the posterior segment tissues.

This systematic review reveals that CPPs are the most favourable penetration enhancers for drug delivery to the fundus oculi with high cellular internalization and broad biodistribution in retina-choroid. CPPs have demonstrated their ability to increase the *in-vivo* uptake of various cargoes including protein drugs (Wang *et al.*, 2010; Zhang *et al.*, 2015; Li *et al.*, 2016; de Cogan *et al.*, 2017), nanoparticles (Chu *et al.*, 2017), genes and nucleic acids (Liu *et al.*, 2016), dendrimers (Liu *et al.*, 2016; Yang *et al.*, 2019), peptides (Atlasz *et al.*, 2019), etc. across the ocular bio-barriers and into the posterior segment via eye-drop instillation and in some cases affected a similar uptake and biodistribution as with intravitreal or intraocular injections thus providing a safe and effective alternative to invasive treatment procedures (Zhang *et al.*, 2015; Li *et al.*, 2016; de Cogan *et al.*, 2017). Pharmacokinetic distributions of all the CPPs were very impressive as well as they were detected in almost every layer of retina-choroid like RGCs – GCL, photoreceptor segments, RPE, rods & cones, IPL, OPL, ILM, INL and ONL. Though their penetration mechanisms and transport pathway in the eye need more detailed investigations, this review does show how PEAs may follow all the different internalization mechanisms from passive direct translocation across cellular membranes (Johnson, Cashman and Kumar-Singh, 2008; Zhang *et al.*, 2015) to active endocytosis including proteoglycans mediated macropinocytosis and clathrin or caveolae-mediated endocytosis (Zhang *et al.*, 2015) which is consistent with their general cellular uptake (Richard *et al.*, 2003; Duchardt *et al.*, 2007) and can access the ocular posterior segment from both transcorneal as well as conjunctival-scleral pathways (Liu *et al.*, 2014). CPP studies also encompassed almost all the different

animal models of major posterior segment pathophysiology including ischemia–reperfusion (IR) injury (Wang *et al.*, 2010), choroidal neovascularization (CNV) (Zhang *et al.*, 2015; Chu *et al.*, 2017; de Cogan *et al.*, 2017), retinal neovascularization (RNV) and OIR (Li *et al.*, 2016). Interestingly the CPPs could also be modified with RGD tripeptide for cell-specific and targeted uptake owing to its binding affinity to the $\alpha_v\beta_3$ integrin expressed on angiogenic endothelial cells (Li *et al.*, 2016; Chu *et al.*, 2017; Yang *et al.*, 2019). Also worth noting is their negligible or no cytotoxicity as investigated *in-vitro* using cell viability assays in different cell lines including human corneal (HCECs) and conjunctival epithelial cells (NHCs) (Liu *et al.*, 2014; Jiang *et al.*, 2017; Yang *et al.*, 2019), rat retinal cultures, human ARPE-19 cells, and human corneal fibroblasts (de Cogan *et al.*, 2017).

BAC is a well-known antimicrobial preservative that has been used in ophthalmic eye-drop formulations for a long time. Here we see it does work on penetration into the ocular posterior segment but only to a very limited and negligible extent and thus at best can be used for treatment of anterior segment diseases also by virtue of its internationalization mechanism as it works by breaking down the tight junctions of surface epithelia on cornea (Mahaling and Katti, 2016). Chitosan is reported to enhance the pre-corneal retention time of drugs due to its mucoadhesive nature thus effectively preventing elimination by tear flow. Chitosan was used as a PEA for posterior segment uptake without any encouraging efficacy but could enhance the residence of liposomes in fundus oculi to some extent (Li *et al.*, 2019), so we draw the inference that it could be used in combination with CPPs as to increase the residence time of eye-drop at the corneal surface resulting in higher uptake and will also increase therapeutic efficacy of the drug by increased retention in the target tissues.

Carboxymethyl chitosan (CMCS) on the other hand showed very promising results with a 2.5 times enhancement in the cargo uptake into the retina-choroid though their major transport was through conjunctival-scleral pathway (Wang *et al.*, 2020). As opposed to chitosan, CMCS is anionic in nature and bears a net negative charge but still retains mucoadhesive properties of chitosan residues and enables an increased pre-corneal and posterior segment residence time, and its uptake is defined as majorly by clathrin-mediated endocytosis.

The risk of bias (RoB) analysis on the methodologies of selected studies showed a relatively high tendency of bias. Though >70% of the studies reported baseline characteristics of animals, a poor randomization of allocation to experimental and control groups and absolutely no concealment of this gives rise to high selection bias. None of the studies report random housing of animals and blinding of investigators and animal caregivers at the intervention stage, thus resulting in extremely high performance bias. Random housing of animals before and after intervention is important because housing conditions like lighting, humidity, temperature, etc can influence outcomes of the study and behaviour of animals and thus should be comparable in experimental groups (Hooijmans *et al.*, 2014). While assessing the outcome, none of the studies report selecting animals at random for analysis and >90% of the studies did not ensure blinding of the investigator who made the assessment, which results in very high detection bias. A moderate attrition bias is observed in more studies addressing the incomplete outcome data, however with insufficient information on the methodology used. A good >85% of studies specified primary outcomes for each intervention, however >57% did not calculate a sample size and the few that did calculate had insufficient information on methodology in terms of reporting bias. On the

brighter side, there is absolutely no risk of contamination in any of the studies, i.e., neither did the experimental groups receive any additional treatments besides the primary intervention nor were the controls exposed to inadvertent interventions. A 50:50 bias due to unit of analysis errors is reported where one eye of the same animal was used as experimental and control. The high risk of bias can be avoided by adhering to a set of standardised techniques in animal experiments based on the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments) (du Sert, Ahluwalia, *et al.*, 2020; du Sert, Hurst, *et al.*, 2020).

2.4.1. Limitations

The leading limitation of this article was that a meta-analysis was not possible due to the lack of quantitative data in the included studies. Another limitation is that almost all of the included studies used immunohistochemistry to assess penetration of drugs to the posterior segment, which is subjective and is prone to inconsistencies due to antibody characteristics such as relative affinity, specificity and sensitivity to detect the target antigen. The use of poorly characterised antibodies has given rise to the inability of researchers to replicate published data, especially in the analysis of low abundance proteins such as G protein-coupled receptors, steroid hormones, ion channels and transporters (Schonbrunn, 2014). A quantitative method such as ELISA or HPLC would be preferrable when working out posterior segment PEA delivery efficiency. Therefore, future studies should take into account these limitations when designing studies to explore the efficacy of PEAs to deliver their payload to the posterior segment of the eye.

2.5. Conclusion

This systematic review suggests that cationic and amphipathic CPPs like TAT and Penetratin are the most promising PEA candidates in the present domain for facilitating an increased passage of cargo across the defensive barriers of the eye and into the fundus oculi via topical eye-drop administration. These CPPs not only exhibit greater permeability at ocular barriers but also an efficient biodistribution and cellular uptake in the tissues of the ocular posterior segment. A cell-specific and targeted uptake in diseased sites can be achieved in combination with RGD tripeptide. More *in-vivo* studies are required however, to determine their quantitative efficacy in terms of the proportional bioavailability in the posterior segments of the eye with that of topically applied payload. Most of the *in-vivo* uptake into the retina-choroid takes place via the conjunctival-scleral pathway. Bioavailability in these tissues can be further enhanced if CPPs can be used in combination with mucoadhesive polymers to increase pre-corneal residence time and thus effectively allowing for an augmented transcorneal internalization. Further investigations should be carried out on their *in-vivo* penetration mechanisms and toxicity and also whether the penetration causes any irreversible alterations in barrier properties of the defensive barriers which can cause more harm than good.

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CHAPTER – 3

Improving corneal permeability of dexamethasone using penetration enhancing agents: first step towards achieving topical drug delivery to the retina

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Abstract

With an ever-increasing burden of vision loss caused by diseases of the posterior ocular segment, there is an unmet clinical need for non-invasive treatment strategies. Topical drug application using eye drops suffers from low to negligible bioavailability to the posterior segment as a result of static and dynamic defensive ocular barriers to penetration, while invasive delivery systems are expensive to administer and suffer potentially severe complications. As the cornea is the main anatomical barrier to uptake of topically applied drugs from the ocular surface, we present an approach to increase corneal permeability of a corticosteroid, dexamethasone sodium-phosphate (DSP), using a novel penetration enhancing agent (PEA). We synthesised a novel polyacetylene (pAc) polymer and compared its activity to two previously described cell penetrating peptide (CPP) based PEAs, TAT and penetratin, with respect to increasing transcorneal permeability of DSP in a rapid *ex-vivo* porcine corneal assay over 60 minutes. The transcorneal apparent permeability coefficients (P_{app}) for diffusion of pAc, and fluorescein isothiocyanate (FITC) conjugated TAT and penetratin were up to 5 times higher ($p < 0.001$), when compared to controls. When pAc was used in formulation with DSP, an almost 5-fold significant increase was observed in P_{app} of DSP across the cornea ($p = 0.0130$), a significant 6-fold increase with TAT ($p = 0.0377$), and almost 7-fold mean increase with penetratin ($p = 0.9540$). Furthermore, we investigated whether the PEAs caused any irreversible damage to the barrier integrity of the corneal epithelium by measuring transepithelial electrical resistance (TEER) and immunostaining of tight junction proteins using zonula occludens-1 (ZO-1) and occludin antibodies. There was no damage or structural toxicity, and the barrier integrity was preserved after PEA application. Finally, an *in-vitro* cytotoxicity

assessment of all PEAs in human retinal pigment epithelium cells (ARPE-19) demonstrated that all PEAs were very well-tolerated, with IC_{50} values of 64.79 mM for pAc and 1335.45 μM and 87.26 μM for TAT and penetratin, respectively. Our results suggest that this drug delivery technology could potentially be used to achieve a significantly higher intraocular therapeutic bioavailability after topical eye drop administration, than currently afforded.

3.1. Introduction

Despite being the safest and most convenient method of ocular drug delivery, topical administration suffers from limited bioavailability of drug to internal structures beyond the ocular surface due to the presence of physiological and anatomical defensive barriers in the eye. Overall, less than 5% of the applied amount of a drug reaches even the anterior segment tissues after eye drop administration on the ocular surface (Hughes *et al.*, 2005; Urtti, 2006; Loftsson *et al.*, 2008). Inflammation plays a significant role in the pathogenesis of multifactorial diseases in the posterior segment of the eye. Recent studies have demonstrated the association between inflammatory and angiogenic cascades and how either could be a cause or consequence of the other in the pathophysiology of age-related macular degeneration (AMD) (Telander, 2011) and diabetic macular oedema (Romero-Aroca *et al.*, 2016). New long term clinical management strategies include intraocular injections or implants of steroids, sometimes in combination with anti-vascular endothelial growth factor (anti-VEGF) drugs (Abadia *et al.*, 2016). These invasive treatments are painful to administer, cause fear and anxiety, and are financially burdensome because of the requirement for expert administration, which can lead to non-adherence and inferior visual outcomes in patients (Ehlken *et al.*, 2020). Additionally, there is a well-documented risk of post injection complications including endophthalmitis (Sigford *et al.*, 2015), increased intraocular pressure & glaucoma (Good *et al.*, 2011), cataract (Thompson, 2006), and retinal tear & detachment (Karabag *et al.*, 2015). These issues make topical drug delivery for the posterior segment a “holy-grail”. Several nanoparticulate carrier-based approaches are currently being investigated extensively to achieve an effective bioavailability of drugs at the posterior segment via topical instillation (Wang *et al.*, 2018) including lipid based solid lipid nanoparticles (SLNs) and nanostructured lipid

carriers (NLCs) (Balguri, Adelli and Majumdar, 2016), polymeric nanoparticles (Tahara *et al.*, 2017), liposomes (Lai *et al.*, 2019), etc. However, majority of them follow the scleral route instead of corneal uptake.

Penetration enhancing agents (PEAs) can facilitate increased uptake of otherwise poorly absorbed drugs into cells and across impermeable tight epithelium membranes like the cornea and conjunctiva (Kaur and Smitha, 2002; Moiseev *et al.*, 2019; Thareja *et al.*, 2021). As opposed to nanocarrier drug delivery systems, PEAs are more versatile in their use because they do not require chemical surface processing for stability, do not need a consistent 3D shape, size or size distribution and hence are easier to scale-up, cost-effective, and do not carry the same risk of adverse health effects and hazardous environmental exposure as an obstacle in the regulatory pathway to their development (Desai, 2012). PEAs are often also used as surface coating on nanoparticles (Mahaling and Katti, 2016; Yang *et al.*, 2019). Synthetic helical polymers are increasingly being used in drug delivery. Polyacetylene (pAc) is one such class of helical polymers that can modulate their structural conformations in response to external stimuli and their helicity can be exploited for biomembrane interactions and enhanced cargo transport across ocular barriers (Leigh and Fernandez-Trillo, 2020). We have developed a novel pAc polymer for this purpose (Fernandez Trillo and Blanch, 2023). Cell penetrating peptides (CPPs) are short chain peptides consisting of 5-30 amino acid residues with an intrinsic ability to translocate across cell membranes through endocytic or non-endocytic mechanisms or a combination of both, which is not seen in other peptides, mainly attributable to the presence of positive charges and amphipathicity, although some anionic and hydrophobic CPPs have also been reported (Milletti, 2012; Bechara and Sagan, 2013).

In a recent systematic review, we identified penetratin and TAT, as two CPPs that were the most efficient PEAs for posterior segment drug delivery (Thareja *et al.*, 2021). TAT is a cationic CPP isolated from the Tat protein of the HIV-1 virus, whereas penetratin is a partially amphipathic CPP corresponding to the third helix of Antennapedia homeodomain protein of *Drosophila melanogaster* (Vivès, Schmidt and Pèlegrin, 2008; Milletti, 2012).

Dexamethasone sodium phosphate (DSP) is a polar, water soluble pro-drug of dexamethasone, a corticosteroid commonly used as gold standard treatment against oedematous vascular leakage in macular oedema secondary to diabetic retinopathy (Dugel, Bandello and Loewenstein, 2015) or retinal vein occlusion (Haller *et al.*, 2010), and to treat inflammation in uveitis (Saincher and Gottlieb, 2020), and sometimes as an adjunct therapy with anti-VEGF biologicals in neovascular AMD (Vakalis *et al.*, 2015), either as intraocular injections or through sustained release intraocular implants, primarily due to its anti-inflammatory properties complemented by angiostatic and anti-permeability effects (Ciulla *et al.*, 2004; Gaballa *et al.*, 2021). DSP, being hydrophilic, exhibits far inferior corneal permeability compared to dexamethasone (Civiale *et al.*, 2004).

In this chapter, we investigated the ability of penetratin, TAT and pAc in improving bioavailability of DSP across complex defensive ocular barriers in a medium-throughput and rapid-assay *ex-vivo* model of permeability using porcine eye tissue, developed in our group (Begum *et al.*, 2020). pAc was synthesised using a patented protocol (Fernandez Trillo and Blanch, 2023). We first tested the permeabilities of pAc compared with that of fluorescently labelled CPPs, across porcine cornea and sclera. DSP was then formulated with pAc and non-labelled CPPs for topical application and

assayed for permeability. DSP concentration in the receptor chamber was quantified using high-performance liquid chromatography (HPLC) to ascertain the enhancement in its permeability with PEAs. This was followed by safety assessment of barrier integrity of corneal epithelium using transepithelial electrical resistance (TEER) measurements and immunofluorescent staining of excised cornea with epithelial tight junction markers ZO-1 and occludin, and finally *in-vitro* cytotoxicity assessment of PEAs in human ARPE-19 cell line using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. This work adds a new horizon to a safe, simple, and affordable topical ocular delivery modality of corticosteroids by modulating the transcorneal route using PEAs without causing any irreversible damage to defensive barriers of the eye and potentially achieve therapeutic doses at the posterior segment tissues.

3.2. Materials and Methods

3.2.1. PEAs

A poly(*O*-propargyl-*N*-amino carbamate) (pAc) was prepared following a proprietary, patented protocol (Fernandez Trillo and Blanch, 2023), with all the chemicals and solvents (reagent grade) purchased from Sigma-Aldrich (Poole, Dorset, UK), Fisher Scientific (Leicester, UK), VWR (Peterborough, UK), or ThermoFisher (Ashford, UK). Briefly, an acetylene monomer *O*-propargyl-*N*-(*tert*-butoxycarbonyl) amino carbamate (PBocAC) was synthesised using propargyl alcohol, carbonyldiimidazole, and *tert*-butyl carbazate in ethyl acetate. PBocAC was then polymerised upon reaction with a rhodium catalyst [Rh(NBD)B(Ph)$_4$] in tetrahydrofuran to give the protected polymer compound poly(*O*-propargyl-*N*-(*tert*-butoxycarbonyl) amino carbamate) (p(PBocAC)). p(PBocAC) was finally dissolved in trifluoroacetic acid to obtain deprotected reactive

pAc polymer with the structure as shown in (Figure 3.1), which was later freeze-dried to a pale-yellow powder. The protected polymer (p(PBocAC)) was characterised by gel permeation chromatography (GPC) for the following parameters: weight average molecular weight (M_w) = 41158 ± 130 g/mol, number average molecular weight (M_n) = 24615 ± 734 g/mol, and polydispersity index (Đ) = 1.67 ± 0.04.

Freeze-dried CPPs (TAT and penetratin) used in this study were all commercially purchased – from AnaSpec – Kaneka Eurogentec (Seraing, Belgium) and their sequences and properties are listed in (Table 3.1).

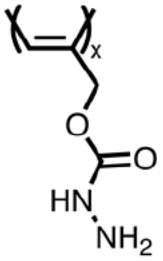

Figure 3.1: Formula structure of poly(O-propargyl-N-amino carbamate) illustrating polyacetylene backbone with x number of repeating units.

Peptide Name	Amino acid sequence (N terminal → C terminal)	Catalogue No.	Molecular weight (Da)
FITC-LC-TAT (47-57)	FITC - -LC-YG RKK RRQ RRR – NH_2	AS-27043	2063.0
FITC-LC-Antennapedia peptide/Penetratin	FITC - -LC-RQ IKI WFQ NRR MKW KK – NH_2	AS-24176	2748.9
TAT (47-57)	H – YGR KKR RQR RR – OH	AS-60023	1560.4
Penetratin	H – RQI KIW FQN RRM KWK KGG – OH	AS-64885	2362.5

Table 3.1: List of CPPs used in the study with their amino acid residue sequences. To study the permeability of CPPs, fluorescein isothiocyanate (FITC) labelled peptides were used, conjugated at N terminal with a long chain (LC) to prevent degradation of FITC.

3.2.2. *Ex-Vivo* Permeability Measurement of PEAs and DSP Formulations in Cornea and Sclera

3.2.2.1. *Ex-vivo* Porcine Eye model

An *ex-vivo* model of permeability using porcine cornea and sclera was used to rapidly assess transcorneal and trans-scleral permeability, which has been established and validated by our group (Begum *et al.*, 2020) (Figure 3.2). Fresh non-scalded porcine eyes (Medical Meat Supplies, Rochdale, UK), surplus tissue from the food industry, were obtained from a local abattoir within two hours of slaughter and transported on ice in an air-tight container. The eyes were then physically examined, and those with an opaque/cloudy cornea or a visible corneal injury/scar were discarded, using only clear transparent corneas. The attached muscles and optic nerve were removed, and the globes rinsed with multiple changes of phosphate buffered saline (PBS; Cat no. BR0014G, ThermoFisher, Hampshire, UK). A small incision was made at the corneal limbus using a scalpel blade and the cornea was carefully excised with a scleral rim using curved mayo scissors. The corneal button was rinsed multiple times with PBS and placed in a petri dish containing PBS, with the corneal epithelium facing down. The remaining globe without the cornea was then cleared out of all other tissues including the lens, vitreous, retina, and choroid to obtain only the sclera, which was then rinsed thoroughly in multiple changes of PBS. 5 mm discs of cornea and sclera were excised using biopsy punches (Stiefel, GSK, Waterford, Ireland) – 3 to 4 sections from each tissue.

Corneal discs were taken from superior, inferior, nasal, and temporal cornea and each disc included both central and peripheral cornea. Each 5 mm tissue disc was then placed in a CellCrown 96 well microplate insert (Cat no. Z682004, Sigma-Aldrich, Poole, UK) with the corneal epithelium or outer sclera facing up, thus simulating eye drop instillation. The tissue disc was placed at the base of the outer insert covering the aperture and the inner insert was then placed on top of the tissue and gently pressed in to create a watertight seal without deforming the tissue. Combined inserts containing tissue discs were then placed in a black wall, clear flat-bottom 96 well microplate (Cat no. 301002, Porvair, Norfolk, UK) containing 80 µL PBS in each well. The formulation to be tested was applied into the cavity in the inner insert that acted as the donor chamber, and the concentration after diffusion through the cornea or sclera was detected in PBS in the microplate well that acted as receptor chamber.

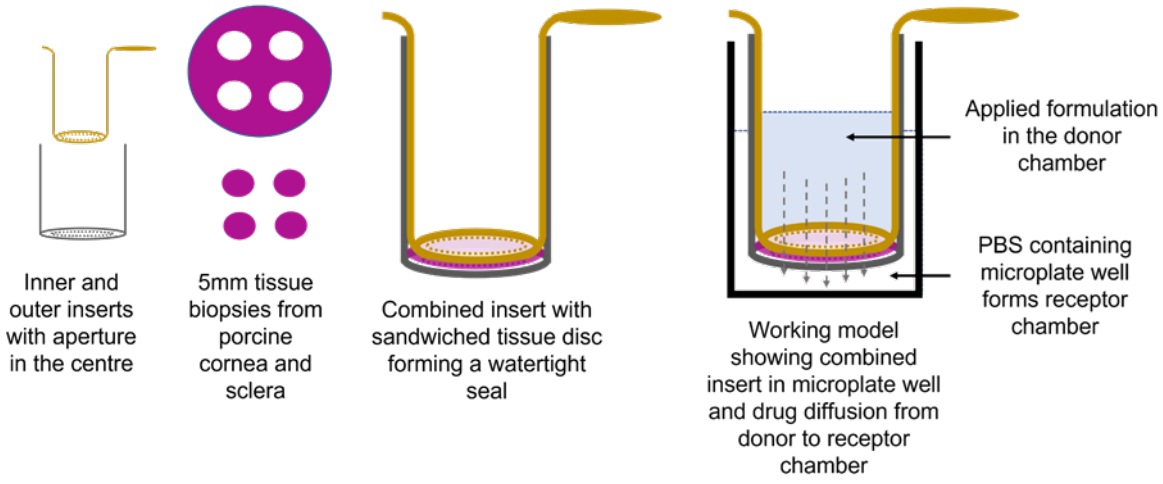

Figure 3.2: Illustration of the ex-vivo model of permeability using porcine cornea and sclera. The inner and outer insert apertures allow diffusion of applied formulation across the membrane of cornea or sclera discs taken using a biopsy punch which were then sandwiched between the two inserts forming a watertight seal. This combined insert was then placed in a 96-well microplate well containing PBS. The formulation was applied in the donor chamber in the inner insert and its permeability assayed by measuring the concentration in the PBS in microplate well, acting as the receptor chamber.

3.2.2.2. Permeability Measurements

The PEAs were first assayed for their permeabilities at 37 °C across porcine cornea (n = 12/PEA) and sclera (n = 9/PEA). Fluorescein sodium (Cat no. 46960, Sigma-Aldrich) was used as a positive control. The 3-4 biopsy discs of cornea and sclera were randomised across all PEA groups. 20 µL each of 2.21 mg/mL (19.36 mM) polyacetylene (pAc) suspension in PBS, and 100 µM PBS solutions of N-terminal FITC labelled CPPs FITC-TAT and FITC-penetratin, and fluorescein sodium, were applied in the donor chamber and their concentrations were measured in the PBS in microplate wells after 60 minutes. The inserts with formulations were carefully removed from the microplates and concentration analysed in a BMG Clairostar Plus microplate reader (BMG Labtech GmbH, Ortenberg, Germany) operated using in-built Read Control acquisition software and data collected in MARS data analysis software. pAc was detected by fluorescence (λ_{ex}: 410 nm, λ_{em}: 481 nm), FITC-TAT and FITC-penetratin were detected by measuring absorbance at 496 nm and fluorescein at 488 nm. The unknown concentrations were calculated by linear regression of standard calibration curves.

Further, formulations of dexamethasone 21-phosphate disodium salt (DSP 98%; Cat no. J64083, ThermoFisher, Oxford, UK) with PEAs were assayed for their permeability at RT across cornea (n = 12/formulation) and sclera (n = 9/formulation). The formulations were prepared in PBS as follows: 2.21 mg/mL pAc + 10 mg/mL DSP stirred overnight, 100 µM of CPPs TAT or penetratin + 10 mg/mL DSP mixed and vortexed just before use, and 10 mg/mL DSP only controls. The tissue sections were randomised as earlier and 20 µL of each formulation was applied in the donor chamber. The permeability of DSP with and without the PEAs was calculated by measuring its

concentration in the receptor chamber after 60 minutes. The inserts were removed carefully and the solution in microplate wells was collected in micro-inserts in vials for high-performance liquid chromatography (HPLC) analysis.

3.2.2.3. HPLC for DSP Quantification

DSP transcorneal and transscleral penetration was quantified using Agilent 1260 Infinity II HPLC equipment (Agilent, Santa Clara, CA, USA) fitted with a G7129A vial autosampler, G7111B quaternary pump and G7115A diode array detector, operated and data collected by OpenLab chromatography data system (CDS) software (Agilent), using a previously published and validated method (Milojevic *et al.*, 2002). The mobile phase consisted of a 65:35, v/v mixture of deionised water + acetonitrile (HPLC Plus, ≥ 99.9%; Cat no. 34998, Sigma-Aldrich) adjusted to pH 2.5 with phosphoric acid (ACS reagent, ≥ 85%; Cat no. 30417-M, Sigma-Aldrich) and degassed by vacuum filtration before running at an isocratic elution flow rate of 1 mL/min. The analytes were separated at 25°C in an Agilent ZORBAX Eclipse Plus C18 4.6 X 250 mm reverse phase porous silica column with 5 µm particle size and 95 Å pore size (Cat no. 959990-902, USA) fitted with a 4.6 X 12.5 mm guard column (Cat no. 820950-936, Agilent). 10 µL samples were injected in each run and DSP was detected at 242 nm UV absorbance after 3.8 minutes retention time. Standard calibrations for DSP were performed in triplicate in a concentration range of 0.2 – 400 µg/mL. The method was validated according to ICH Q2(R2) guidelines for validation of analytical procedures (European Medicines Agency, 2024), for linearity (r = 0.99986), precision and accuracy. The limits of detection (LOD) and quantification (LOQ) was calculated at 0.02736 µg/mL and 0.08291 µg/mL respectively. Unknown concentrations of DSP were calculated from the linear regression of standard curves.

3.2.2.4. Apparent Permeability Coefficient

The penetration of PEAs and DSP was expressed as a normalised apparent permeability coefficient (P_{app}, cm/s) calculated using the equation: $P_{app} = \frac{Q}{t}\left(\frac{1}{A.Q_0.60}\right)$ (Civiale *et al.*, 2004; Liu *et al.*, 2014; Begum *et al.*, 2020), where Q is the mass of translocated compound detected in PBS in the receptor chamber at t = 60 minutes, Q_0 is the initial mass of compound applied in the donor chamber and A is the area of tissue available for diffusion.

3.2.3. Transepithelial Electrical Resistance Measurement of Corneal Epithelium

Trans-epithelial electrical resistance (TEER) measurements were taken on the corneal discs in the *ex-vivo* permeability assays, to determine the PEA toxicity on the corneal epithelial barrier integrity using a previously described method (Juretić *et al.*, 2018; Begum *et al.*, 2020). TEER was measured on cornea discs treated with pAc, FITC-TAT and FITC-penetratin (n = 3/group) using Millicell ERS-2 (Electrical Resistance System) volt-ohm meter (Cat no. MERS00002; Sigma-Aldrich) at RT by placing the corneal discs in a Millicell 96-well transport analysis plate (Cat no. PSHT004S5; Sigma-Aldrich) wells containing PBS and measuring the resistance values on the epithelial surface by immersing the tip of a specially designed silver/silver chloride (Ag/AgCl) electrode for 96 well plates (Cat no. MERSSTX00; Sigma-Aldrich). The resistance values were recorded from the monitor in ohms (Ω) initially at 0 minutes before application of compounds and finally after removal of the compounds at 60 minutes. PBS only wells were measured for background resistance, which was subtracted from the measured values and TEER was calculated by multiplying the recorded resistance values with the cross-sectional area of 5 mm corneal discs and expressed in $\Omega.cm^2$. PBS treated corneal discs (n = 3) were used as controls for comparison.

3.2.4. Immunostaining of Epithelial Tight Junctions

To prepare corneal discs for cryosectioning prior to immunohistochemistry, corneal discs were recovered after permeability assays and fixed in 4% paraformaldehyde (BioReagent, ≥ 36.0%; Cat no. 47608, Sigma-Aldrich) in PBS overnight at 4°C followed by cryoprotection in increasing concentration of sucrose (≥ 99.5%; Cat no. S9378, Sigma-Aldrich, Switzerland) solutions – 10%, 20% and 30% for 8-14 hours each. The tissues were then embedded in Peel-A-Way embedding moulds (Cat no. 12677736, Epredia, Fisher Scientific, USA) with optimal cutting temperature compound (OCT; Cat no. 12678646, Fisher Scientific) and frozen on dry ice to be stored at -80°C. Frozen blocks were sectioned at 15 µm thickness using a cryostat (Brights Instruments, Huntingdon, UK) at -20°C, and the sections were collected on SuperFrost Plus adhesion glass microscope slides (Cat no. 10149870, Fisher Scientific, Ashford, UK). The sections were allowed to settle overnight at RT before storing at -20°C until use.

Corneal discs were immunostained for epithelial tight junction markers to detect structural damage to tight junction integrity. Frozen sections were thawed at RT for 30 minutes and washed two times for 5 minutes in PBS, followed by permeabilisation in 0.1% triton X-100 (Cat no. T8787, Sigma-Aldrich, USA) in PBS for 10 min. After further two washes for 5 minutes in PBS at RT, sections were blocked in 0.5% bovine serum albumin (BSA; Cat no. A3311, Sigma-Aldrich) + 0.1% triton X-100 in PBS for 30 min at RT to prevent any non-specific binding. The tissue sections treated with pAc, FITC-TAT, FITC-penetratin, fluorescein sodium and PBS controls (n = 3/group) were then incubated with primary antibodies: ZO-1 (1:100; Rabbit Polyclonal IgG, Cat no. 40-2200, ThermoFisher) and occludin (1:100; Rabbit Polyclonal IgG, Cat no. 71-1500, ThermoFisher) separately in PBS containing 0.5% BSA + 0.05% tween-20 (Cat no.

663684B, VWR) and left overnight at 4°C in a humidified chamber. The next day, sections were washed three times for 5 minutes in PBS and incubated with fluorescently labelled secondary antibody: Alexa Fluor Plus 594 (1:400; Donkey anti-Rabbit Polyclonal IgG; Cat no. A32754, ThermoFisher) in PBS containing 0.5% BSA + 0.05% tween-20 for 1 hour at RT. After further washes, three times for 5 minutes in PBS, coverslips were mounted with Vectashield Plus antifade mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) (Cat no. H2000, Vector Laboratories, Peterborough, UK). Sections were imaged on an inverted confocal laser scanning microscope with Airyscan (LSM880; Carl Zeiss Ltd, Cambridge, UK) and two to three images were obtained using 20X objective (200X magnification) for each section with red, green and DAPI filters by an experimenter masked to the treatment conditions.

3.2.5. MTT Assay for *In-vitro* Cytotoxicity of PEAs

In-vitro cytotoxicity of all the PEAs was determined using MTT assay-based Cell Proliferation Kit I (Cat no. 11465007001, Sigma-Aldrich). The assays were performed according to manufacturer's instructions on human retinal pigment epithelium, ARPE-19, cell line (ATCC-CRL-2302; ATCC, Manassas, VA, USA). The cells were cultured between passages 5 to 7 in a 75 cm^2 tissue culture flask (Cat no. 430641, Corning) with 1:1 mixture of Dulbecco's modified eagle medium and Ham's nutrient mixture F-12 basal media (DMEM/F-12; Cat no. 31330038, ThermoFisher) containing 4-(2-Hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) buffer, 2.5 mM L-glutamine and phenol red, and supplemented with 10% heat-inactivated foetal bovine serum (FBS; Cat no. 10500064, ThermoFisher) and 1% penicillin/streptomycin antibiotic mixture (5,000 U/mL; Cat no. 15070063, ThermoFisher). The cells were grown in an incubator at 37°C in 5% CO_2 and after reaching 80% confluency, cells were detached

using a mixture of 0.05% (w/v) trypsin and 0.53 mM ethylene-diamine-tetra acetic acid (Trypsin-EDTA, without phenol red; Cat no. 15400054, ThermoFisher) in sterile DPBS (Dulbecco's; Cat no. 14190094, ThermoFisher). Cells were counted and seeded at a concentration of 10^4 cells/well in 100 µL supplemented cell culture medium into tissue culture grade 96-wells flat bottom microplates (Cat no. 3596, Corning, Durham, NC, USA) and allowed to settle overnight in an incubator at 37°C and 5% CO_2.

The next day, media was carefully removed, and 100 µL each of the three PEAs at a range of different concentrations were added, and media only wells were used as controls. All treatments were prepared in sterile cell culture medium, and each experiment had at least 8 technical replicates for every concentration of each PEA. pAc was UV sterilised and CPPs were provided as sterile freeze-dried powders from the manufacturer. Cells were exposed to the PEAs for 24 hours at 37°C and 5% CO_2. The PEAs were removed, and fresh culture medium was added to allow for a recovery for 48 hours at 37°C and 5% CO_2. At this point, the media was removed and 100 µL of 0.5 mg/mL solution of MTT in sterile culture medium was added to the wells and incubated for 4 hours in the dark at 37°C, 5% CO_2. Finally, the purple formazan crystals were solubilised with the addition of 100 µL solubilisation buffer containing 10% sodium dodecyl sulphate (SDS) in 0.01 M hydrochloric acid (HCl). The plates were allowed to stand overnight at 37°C, 5% CO_2 for complete solubilisation of formazan crystals and the resulting purple solution was analysed by measuring spectrophotometric absorbance of the formazan product in the samples at 570 nm in a microplate reader (BMG Clairostar, Ortenberg, Germany). The experiment was repeated three times (n = 3), and half maximal inhibitory concentration (IC_{50}) values were calculated for each PEA from the non-linear sigmoidal fits of the dose-response curves.

3.2.6. Statistical Analysis

The data were analysed in OriginPro, Version 2021b SR1 (OriginLab Corporation, Northampton, MA, USA). All values were presented as mean ± standard error of mean (SEM). P_{app} data was tested for normality using Shapiro-Wilk test and accordingly parametric or non-parametric tests were used for further analysis. For a parametrically distributed data, one-way ANOVA with post-hoc Bonferroni's multi comparisons test was used, while Kruskal-Wallis ANOVA with post-hoc Dunn's pairwise comparisons test was used for non-parametrically distributed data. A p value < 0.05 was set as the threshold for statistical significance.

3.3. Results

3.3.1. All Three PEAs Increased Corneal Permeability of DSP *Ex-vivo*

pAc, FITC-TAT, and FITC-penetratin all showed significantly higher permeabilities compared to fluorescein sodium controls across both cornea and sclera in the permeability assays (Table 3.2 and Figure 3.3(a)). Among the three PEAs, FITC-penetratin had the highest corneal permeability, up to 5 times higher ($p < 0.0001$) than the corneal P_{app} of fluorescein after 60 minutes. All PEAs showed up to 2-fold higher ($p < 0.05$) permeabilities across the sclera compared to fluorescein controls. However, all the PEAs showed similar permeabilities amongst them across the sclera. Though pAc and fluorescein's scleral P_{app} values were almost double as compared to their corneal P_{app} values, CPPs showed slightly lower scleral permeabilities compared to their corneal permeabilities.

Table 3.2: P_{app} (Mean ± SEM) values for all PEAs and fluorescein from permeability assays, and ratios of means as a comparative analysis of permeability of PEAs to that

of fluorescein across cornea (n = 12/group) and sclera (n = 9/group). The statistical significance of the results is demonstrated by p values.

Compound	Cornea (n = 12/group)			Sclera (n = 9/group)		
	P_{app} (Mean ± SEM) X 10^{-4} cm/s	Mean ratio to Fluorescein Control	p value (compared with fluorescein)	P_{app} (Mean ± SEM) X 10^{-4} cm/s	Mean ratio to Fluorescein Control	p value (compared with fluorescein)
pAc (2.21 mg/mL; 19.36 mM)	1.09 ± 0.18	2.69	<0.001	1.90 ± 0.44	2.07	0.0195
FITC-TAT (100 µM)	1.72 ± 0.13	4.24	<0.0001	1.68 ± 0.28	1.84	0.01726
FITC-penetratin (100 µM)	2.01 ± 0.06	4.94	<0.0001	1.84 ± 0.26	2.00	0.0035
Fluorescein (100 µM)	0.41 ± 0.04			0.92 ± 0.07		

All three PEAs – pAc, TAT, and penetratin, significantly increased the corneal permeability of DSP in formulations in permeability assays (Table 3.3 and Figure 3.3(b)). TAT exhibited the most statistically significant enhancement of DSP corneal uptake after 60 minutes at 6-fold (p = 0.0377), pAc increased the DSP corneal permeability by 5-fold (p = 0.0130), whereas penetratin showed the highest increase in mean value of DSP corneal permeabilities by up to 7-fold higher (p = 0.9540). However, there was no enhancement in scleral permeability of DSP with any of the PEAs. DSP's scleral P_{app} with pAc and penetratin at 60 minutes was much lower – almost half compared to the scleral P_{app} of DSP by itself, and its scleral P_{app} with TAT remained similar to its scleral P_{app} by itself. The scleral permeability of DSP itself was upwards of 10 times higher than its permeability in the cornea. With pAc and penetratin,

DSP's scleral permeability was almost similar compared to the permeability in cornea, whereas its scleral permeability with TAT was almost double than the corneal permeability.

Table 3.3: Calculated P_{app} values (Mean ± SEM) of DSP alone and in formulations with different PEAs showing the comparative uptake across cornea (n = 12/group) and sclera (n = 9/group) with all PEAs, as observed by the ratios of means of DSP P_{app} with PEAs to the DSP P_{app} by itself, and their statistical significance (p values) in enhancing DSP uptake.

Formulation	Cornea (n = 12/group)			Sclera (n = 9/group)		
	P_{app} (Mean ± SEM) X 10^{-5} cm/s	Mean ratio to DSP only	p value (compared with DSP)	P_{app} (Mean ± SEM) X 10^{-5} cm/s	Mean ratio to DSP only	p value (compared with DSP)
DSP (10 mg/mL)	0.20 ± 0.04			2.26 ± 0.92		
pAc (2.21 mg/mL) + DSP	1.01 ± 0.33	4.93	0.0130	1.15 ± 0.75	0.51	0.1451
TAT (100 µM) + DSP	1.26 ± 0.68	6.18	0.0377	2.21 ± 1.74	0.98	0.0703
Penetratin (100 µM) + DSP	1.41 ± 0.74	6.88	0.9540	1.46 ± 0.77	0.65	0.2697

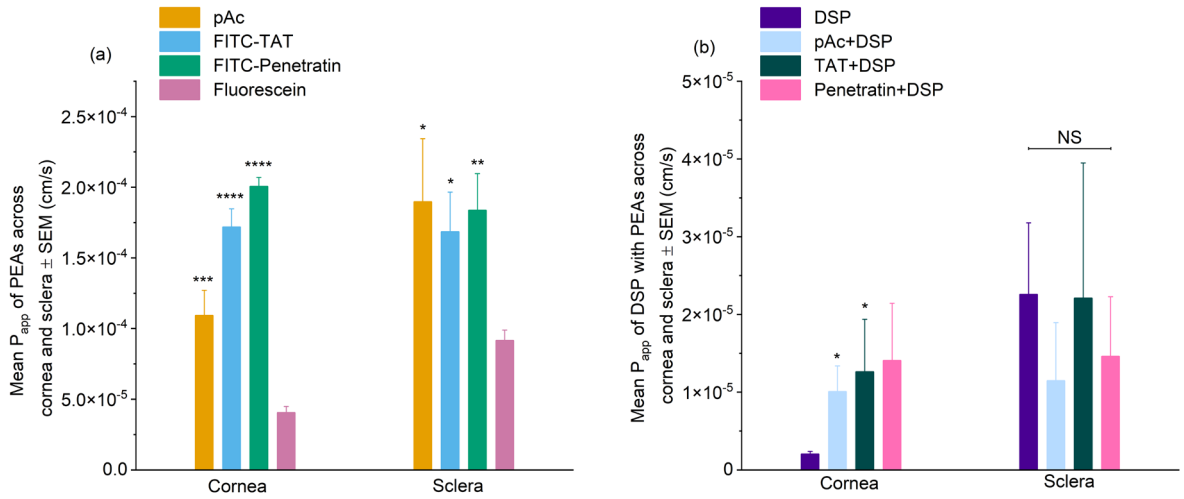

Figure 3.3: Mean ± SEM P_{app} plots of PEAs (a) and DSP in formulation with PEAs (b) in cornea (n = 12/group) and sclera (n = 9/group) from permeability assays at 60 minutes. (a) All three PEAs show higher permeabilities across both cornea and sclera compared with fluorescein controls. In cornea and sclera respectively: pAc ≈ 2.7X (***p < 0.001) and ≈ 2.1X (*p = 0.0195); FITC-TAT ≈ 4.2X (****p < 0.0001) and ≈ 1.8X (*p = 0.0173); FITC-penetratin ≈ 4.9X (****p < 0.0001) and ≈ 2X (**p = 0.0035). (b) All PEAs increase corneal uptake of DSP: pAc ≈ 4.9X (*p = 0.0130); TAT ≈ 6.2X (*p = 0.0377); Penetratin ≈ 6.9X (p = 0.9540). However, there is no enhancement observed in the uptake of DSP across sclera with any of the PEAs. NS = not significant.

3.3.2. PEAs Cause No Irreversible Damage to the Barrier Integrity of Corneal Epithelium or Epithelial Tight Junctions

TEER measurements on the corneal discs treated with PEAs and PBS controls showed no significant differences in TEER over 60 minutes as depicted in Figure 3.4. In fact, control discs exhibited the maximum drop in TEER values at 5.90 ± 2.91 % from 181.62 ± 22.52 Ω.cm^2 at 0 minutes to 172.1 ± 25.82 Ω.cm^2 at 60 minutes, compared to drop in pAc tissues at 2.33 ± 0.58 % from 75.07 ± 4.48 Ω.cm^2 at 0 minutes to 73.38 ± 4.8 Ω.cm^2 at 60 minutes, in FITC-TAT at 2.66 ± 0.36 % from 79.32 ± 8.63 Ω.cm^2 at 0 minutes to 77.24 ± 8.56 Ω.cm^2 at 60 minutes, and that of FITC-penetratin at 4.61 ± 1.59 % from 93.16 ± 4.29 Ω.cm^2 at 0 minutes to 88.74 ± 2.55 Ω.cm^2 at 60 minutes. Immunostaining in the corneal sections with ZO-1 and occludin showed

characteristic staining of tight junctions in the apical cell layers of corneal epithelium with all PEAs, and identical to PBS treated controls, as shown in different panels in Figure 3.4. There was no breakdown of surface cellular layers at any point confirming that the integrity of the corneal barrier is preserved.

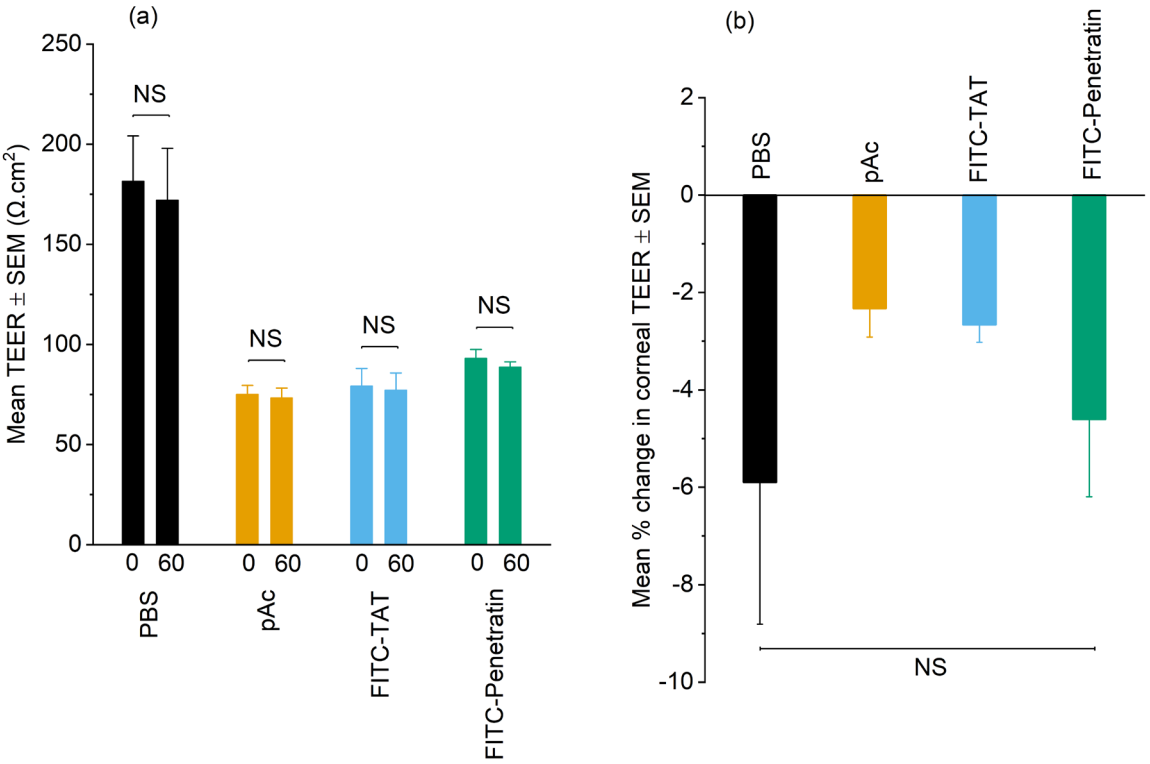

Figure 3.4: Plots representing (a) TEER values and (b) percentage change in TEER (mean ± SEM; n = 3/PEA) over 60 minutes of application of PEAs on corneal surfaces in assays. The drop in TEER values for all PEAs is lower than that of PBS controls. NS = not significant.

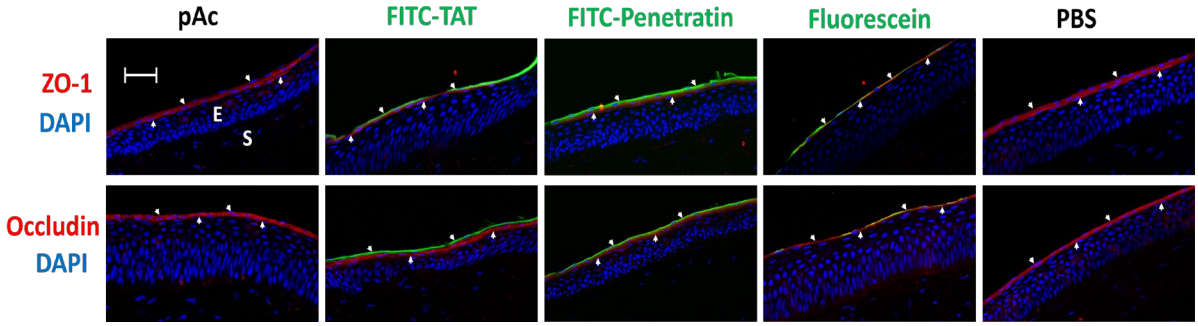

Figure 3.5: Confocal micrographs of cross sections of corneas at 200X magnification stained with ZO-1 and occludin after permeability assays showing corneal epithelium (E) and stroma (S). Tight junctions between the superficial stratified squamous epithelial cells with flat nuclei are stained in red for ZO-1 and occludin in the 2-3 apical layers of corneal epithelium as shown by white arrows. No staining was observed in either wing or basal cell layers in the epithelium. FITC conjugated CPPs and fluorescein also show a green-fluorescent layer on the top indicating surface retention of PEAs, however no such fluorescence was seen in deeper layers within epithelium or further down in stroma and endothelium. DAPI stained nuclei in blue; scale bar 50 µm; n = 3 sections per PEA.

3.3.3. PEAs exhibit Safe Levels of *In-vitro* Tolerance in ARPE-19 Cells at Clinically Relevant Concentrations

All the three PEAs were tolerated well *in-vitro* in ARPE-19 cell toxicity assays, as evidenced in the dose response curves in Figure 3.6. IC_{50} values as calculated from these curves for pAc was 64.79 mM, for CPPs TAT and penetratin it was 1335.45 µM and 87.26 µM respectively, which was much higher than we used *ex-vivo* in all cases except penetratin.

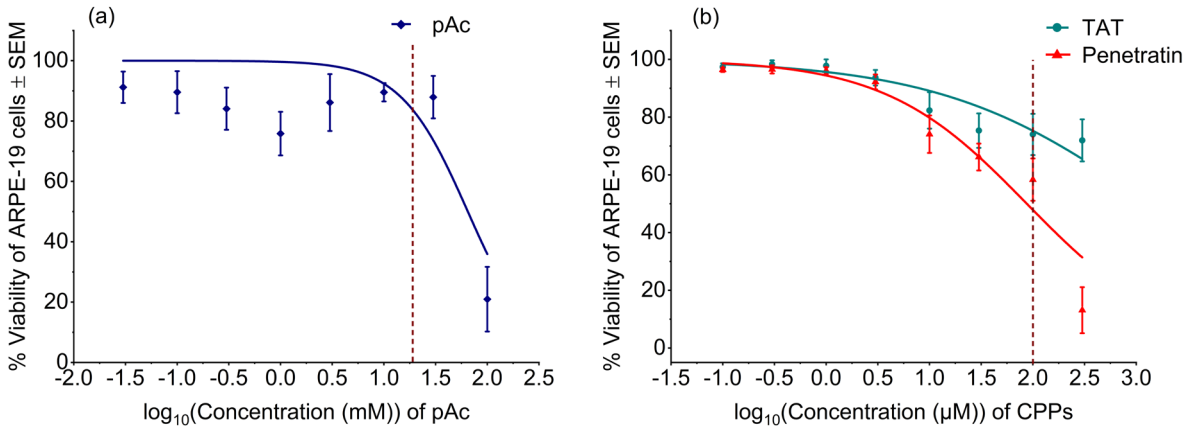

Figure 3.6: Dose response curves of (a) pAc and (b) CPPs TAT and penetratin showing percentage viability of ARPE-19 cells (Mean ± SEM; n = 3/PEA) against $\log_{10}$ concentration range of PEAs, from which IC_{50} values were calculated to evaluate their tolerance in-vitro. Dotted lines denote the concentrations used for ex-vivo permeability measurement.

3.4. Discussion

We aimed to increase DSP bioavailability in the posterior segment of the eye after topical administration using PEAs as a delivery agent. We showed that all three PEAs increased corneal permeability of DSP by several fold over control formulations. We also showed that none of the PEAs caused damage to the barrier integrity of the cornea by immunohistochemistry for tight junction proteins, including ZO-1 and occludin and PEAs had no effect on ARPE-19 cell viability at clinically relevant concentrations. These results suggest that PEAs increase DSP corneal permeability after topical application and may be used clinically to increase the bioavailability of DSP for the treatment of inflammatory conditions in the eye.

Dexamethasone is a corticosteroid used primarily in eye drop formulations to treat inflammation in the eye, particularly after cataract surgery (Karasu *et al.*, 2022). However, it is practically insoluble in aqueous systems, which limits its use for topical

ophthalmic administration (Shen *et al.*, 2021). Dexamethasone's hydrophilic prodrug – the sodium phosphate ester, DSP, is poorly permeable and hence efforts to overcome this limitation have been explored to allow local drug administration in posterior segment disease. For example, intravitreal implants of dexamethasone (Ozurdex) have been developed and used to treat diabetic macular oedema and macular oedema secondary to retinal vein occlusion and posterior uveitis, however, this requires intravitreal implantation and hence is an expensive treatment option, with a risk of complications that may worsen patients' vision (Massa, Georgoudis and Panos, 2019). Intracanalicular implants have also been tested for sustained release of dexamethasone to the ocular surface (Walters *et al.*, 2015; Lee and Blair, 2020). In clinical trials, one-off intracameral delivery of dexamethasone was preferred by patients undergoing cataract surgery and proved to be effective at reducing pain, inflammation, and thus improved visual acuity (Hovanesian and Donnenfeld, 2022). However, topical delivery of dexamethasone remains the most versatile, cheapest, and patient-friendly option for long-term treatment. Recent efforts in this regard have resulted in development of cyclodextrin – dexamethasone inclusion complexes micro-suspension which has been validated to achieve a therapeutic bioavailability of dexamethasone to the posterior segment by topical eye-drop instillation in an *in-vivo* animal model (Johannsdottir *et al.*, 2018), showed significant improvement in patients with diabetic macular oedema in a phase II randomised controlled trial (RCT) (Stefansson *et al.*, 2023), and is currently in phase III clinical trial (ClinicalTrials.gov ID NCT05066997; NCT06172257).

To measure the permeability of our PEAs and DSP formulations *ex-vivo* across cornea and sclera, we employed a novel diffusion model using porcine eye tissues owing to

easy availability as a meat industry excess and overwhelming anatomical similarities with human eye compared to other non-primate mammals. Porcine corneas are only slightly thicker than human corneas (Faber *et al.*, 2008; Blanch *et al.*, 2012), and the epithelium also has 1-2 extra cell layers (Ehlers, 1970) whereas porcine sclera is double in thickness to human sclera but has exactly similar composition and histology (Nicoli *et al.*, 2009). Normally, drugs applied on the ocular surface are instantly cleared from the tear film, showing a 100% surface elimination within a maximum 15-20 minutes (Balla *et al.*, 2022), thus limiting its precorneal residence time and bioavailability. Our rapid assay miniaturised model of permeability is favourable for shorter durations. The model only uses a 5mm biopsy of the tissue instead of whole cornea or sclera (Begum *et al.*, 2020, 2021), thus reducing the need for tissue and multiplexing its use by maximising the throughput in a 96-well microplate subscribing to the 3Rs principles of animal research (Graham and Prescott, 2015; Sneddon, Halsey and Bury, 2017) and the FDA modernisation act 2.0 (Stewart *et al.*, 2023).

PEAs were transported across both cornea and sclera at a rate much faster than fluorescein controls, with P_{app} values up to 5 times higher in cornea and 2X in sclera, however there were no differences between the corneal and scleral permeabilities of PEAs except for pAc. TAT is an 11 residue peptide derived from the 47-57 amino acid domain sequence of trans-activator of transcription (tat) protein in human immunodeficiency virus (HIV-1), penetratin or Antennapedia peptide (pAntp) is 16-18 residues corresponding to the third helix of Antennapedia homeodomain, a transcription factor found in *Drosophila melanogaster*, and the cellular internalization properties of both CPPs were discovered more than three decades ago (Frankel and Pabo, 1988; Green and Loewenstein, 1988; Derossi *et al.*, 1994; Derossi, Chassaing

and Prochiantz, 1998). Transport of CPPs across lipophilic and negatively charged corneal epithelium would necessarily be through transcellular means following either energy dependent endocytic pathways or passive direct translocation or a combination of both depending upon the concentration (Richard *et al.*, 2003; Lindgren *et al.*, 2004; Duchardt *et al.*, 2007; Lundin *et al.*, 2008) aided by their cationic and amphipathic nature, while hydrophilic stroma and endothelium should ideally offer no hinderance. A negatively charged and hydrophilic fluorescein sodium dye would be blocked transcellularly and only show low paracellular transport across the cornea due to its small molecular size, as is consistent with our results.

However, the sclera does not have distinct cell layers but instead is an aqueous matrix of collagen fibre bundles with embedded fibroblasts and negatively charged proteoglycans, and the permeability would depend on molecular weight and hydrodynamic radius of PEAs making their way through this matrix (Ambati *et al.*, 2000; Trier, 2005; Wen, Hao and Li, 2013), supporting the higher P_{app} of fluorescein across sclera compared to cornea whereas CPPs having a larger molecular radius show similar P_{app} in cornea and sclera. However, an amphiphilic pAc with a much smaller size would not face any resistance across sclera. The polycationic charge distribution and amphipathicity of TAT and penetratin would also depend on their spatial conformations in free form in solutions as well as in contact with phospholipids and proteoglycans on the ocular surface. A structural plasticity favours easier membrane insertion and determines the route of uptake as CPPs can modify and reorganize their hydrophilic-hydrophobic domains with a change in environment, for internalization through electrostatic and hydrophobic interactions with membrane lipids (Eiríksdóttir *et al.*, 2010). Conjugation of a fluorophore such as FITC to CPPs makes it easier to detect

in penetration assays, however it can also affect the membrane binding interactions of CPPs due to its polarity and hydrophilicity, restricting their ability to modulate secondary structures and conformations which can inhibit the enthalpy driven reactions that are necessary for fixation and internalization (Hedegaard *et al.*, 2018).

Permeability of DSP across cornea was enhanced more than of 6 times in combination with PEAs. DSP being a hydrophilic ester of dexamethasone, with a water/octanol partition coefficient (log P) value of 0.54 as opposed to 2.12 for dexamethasone, showed very low P_{app} values across cornea by itself, as it is obstructed by the corneal epithelium and paracellular transport is limited due to molecular size, but hydrolysis of some of DSP into dexamethasone alcohol by phosphatase enzymes on corneal surface would result in transcellular transport of lipophilic dexamethasone, which would then be limited by the corneal stroma (Baeyens *et al.*, 1997; Weijtens *et al.*, 2002; Civiale *et al.*, 2004). Therefore, there is also a difficulty in simultaneously estimating the concentrations of DSP and dexamethasone in the receptor chamber, depending upon the rate of hydrolysis, which needs to be addressed in future pharmacokinetic studies. However, a very high aqueous solubility of DSP would ideally cancel some of the hydrophilic limitations on permeability, as suspension particles are cleared much more quickly from the ocular surface (Schoenwald and Ward, 1978). pAc would ideally conjugate with DSP through the formation of an acyl hydrazone following dynamic covalent chemistry and DSP release can be triggered by hydrolysis of the acyl hydrazone at an acidic pH in endosomes in case of endocytic uptake (Priegue *et al.*, 2018; Ulrich, 2019). It is worth noting here that the CPPs are not covalently conjugated to DSP rather simply mixed to complex with DSP by non-covalent interactions and it has been shown that CPPs are highly effective in binding to a variety of cargoes non-

covalently and transporting them across cells without hampering the uptake efficacy of complexes by avoiding any chemical modification of the drug which preserves its pharmacological activity and does not cause unintended clearance, and this is also sometimes the preferred mode of combining cargoes to CPPs as it broadens the scope for their use (Hu *et al.*, 2009; Keller *et al.*, 2013).

The non-corneal route of drug transport to the posterior segment through conjunctiva-sclera-choroid has been suggested to contribute a major chunk of bioavailability in retina and other tissues, especially for hydrophilic drugs (Ahmed and Patton, 1987; Hughes *et al.*, 2005), thus it was important to test for scleral permeability alongside cornea though it will be more helpful in future studies to also study permeabilities across combined tissue membranes of conjunctiva, sclera, and RPE-choroid. As was evident from our results, we observed no enhancement in DSP transport across sclera with any of the PEAs, and all of them showed lower P_{app} of DSP compared to DSP itself – up to half its original value. At the same time, DSP on its own had a 10 times higher permeability across sclera compared to cornea, following on the above explanation that an aqueous sclera does not limit the transport of hydrophilic molecules. Moreover, it can be deduced that PEAs likely inhibited DSP penetration across sclera due to the formation of a complex of size much bigger than the radius of DSP, following that transport across sclera is dependent on the size of the molecules as a small molecule DSP would travel much faster across the scleral matrix than a larger PEA-DSP complex. It is important to note that in this study we have only used sclera *ex-vivo* after removing the vascular layers of conjunctiva and choroid, however the non-corneal absorption is limited by clearance via lymphatics and vasculature of conjunctiva and choroid, and further blocked by the tight retinal pigment epithelium

(RPE) and Bruch's membrane (Ahmed and Patton, 1987; Robinson *et al.*, 2006; Ranta *et al.*, 2010). Therefore, future studies should use a combined barrier system of conjunctiva-sclera-choroid.

Finally, TEER and immunostaining results showed that none of the PEAs caused any damage or structural toxicity to the corneal barrier integrity, as TEER is a very sensitive electrophysiological indicator of barrier function of corneal epithelium (Kusano *et al.*, 2010), while ZO-1 and occludin are cytoplasmic and transmembrane proteins respectively that constitute tight junctions between adjacent cells in the superficial layers of corneal epithelium and protect the barrier by regulating and restricting the paracellular transport of any foreign molecule (Sugrue and Zieske, 1997; Ban *et al.*, 2003; Contreras-Ruiz *et al.*, 2012). CPPs are often feared for inducing toxicity by causing cell membrane perturbations and leakage by pore formation (El-Andaloussi *et al.*, 2007; Eiríksdóttir *et al.*, 2010), However, our results showed no disruption to the structure of cell membranes of superficial epithelial cells by PEAs, and therefore, the cellular resistance was maintained in the apical layer of corneal epithelium. This was also corroborated by the IC_{50} values obtained from MTT assays on ARPE-19 cell line *in-vitro,* and any future *in-vivo* work could use a range of concentrations of PEAs below the obtained values. However, since the CPPs used in MTT assays were non-labelled, as opposed to FITC labelled ones in penetration assays, they might show different toxicities in either case, as fluorophore labelling can also affect the cytotoxicity of CPPs by modulating the net charge and hydrophobicity of the CPP (Birch *et al.*, 2017).

3.5. Conclusion

The PEAs tested, including penetratin, TAT, and pAc, are all capable of improving the corneal permeability of DSP by several fold and hence DSP bioavailability in the

posterior segment of the eye should be increased significantly after topical application. This would help reduce frequent intraocular injections and a higher treatment compliance by potentially allowing self-administered treatments for posterior segment degenerative and inflammatory conditions. Though even after corneal penetration, there are multiple other physiological dynamic barriers to overcome before reaching the retina, however the increased corneal permeability of hydrophilic drugs combined with the bioavailability from non-corneal transport would significantly increase the total drug penetration in the posterior segment. We have presented a more versatile alternative platform for topical ocular drug delivery to the retina using simple mixing of PEAs with drugs without the need of chemical modifications as compared to the more complicated synthesis and surface modification of nanoparticulate drug delivery systems. At the same time, it is evident from our work that these PEAs do not induce *in-vitro* cytotoxicity and do not cause any damage to the defensive barrier property of cornea *ex-vivo,* which is a common occurrence with nanoparticles. Further studies would be required to determine the therapeutic efficacy, safety, and pharmacokinetics-pharmacodynamics (PK/PD) of the formulations *in-vivo* in animal models. This proof of concept can then be used to deliver similar small molecule drugs and even macromolecular biologics to the posterior segment that can currently only be delivered through invasive intraocular administration.

3.6. References

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CHAPTER – 4

Assessing the in-vivo neuroprotective efficacy of intravitreal PLGA microsphere formulations containing vitamin-E and melatonin in a rat optic nerve crush model

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A version of this manuscript is in preparation for publication

Abstract

Degenerative diseases and damage in the ocular posterior segment are difficult to treat due to complications associated with repeated intravitreal injections, and vision restoration is usually not achieved due to inability of the visual neuronal system to regenerate. As such, prolonged release intravitreal dosage forms made of biodegradable and biocompatible microspheres containing neuroprotective drugs could provide relief from repeated injections and also promote regeneration of damaged tissue. Small-molecule endogenous antioxidants like vitamin-E (VE) and melatonin (MEL) have been known to provide neuroprotection in diseases of the central nervous system (CNS) and have been used in this study in slow release microsphere formulations. A biodegradable and non-toxic poly(lactic-co-glycolic acid) (50:50 PLGA) co-polymer was used for microencapsulation of VE, MEL and anti-inflammatory dexamethasone (DEX) in different combinations following an oil-in-water emulsion solvent extraction-evaporation protocol to form three different drug-loaded microsphere (MS) formulations with and without VE additive – MS-V (VE only), MS-MD (MEL and DEX), and MS-VMD (VE, MEL, and DEX) and their *in-vivo* efficacy was tested in an optic nerve crush (ONC) model of degenerative traumatic optic neuropathy (TON) in rats. Microspheres with diameters of 20 – 38 μm were obtained and 0.1 mg of each formulation was injected intravitreally in n=4 rats per treatment group, immediately after ONC to act as slow-release depots. Postmortem analyses of neuroprotective, anti-inflammatory and regenerative effects of the formulations were done by measuring retinal ganglion cell (RGC) survival, and glial activation of astrocytes, Müller cells, and microglia in the retinas, and axonal regeneration was measured in the optic nerves in immunostained tissue sections. None of the

formulations provided any neuroprotection and drug loaded formulations showed further RGC loss as was evident from an almost three times $RBPMS^+$ RGC counts in blank microsphere (MS-B) treated retinas at 7.25 ± 2.48, compared to drug loaded formulations 2.49 ± 0.22, 2.14 ± 0.18, and 2.15 ± 0.22 for MS-V, MS-MD, and MS-VMD respectively, although non-significantly. Furthermore, a non-significant increase in activation of all $GFAP^+$ astrocytes and Müller glia, and $OX\text{-}42^+$ microglia in the retina was observed with MS-V, MS-MD, and MS-VMD compared to MS-B, and a significant increase over intact uninjured contralateral eyes, suggesting a chronic inflammatory response worse in the drug-loaded formulations. However, VE containing formulations MS-V and MS-VMD showed non-significant increase in axonal regeneration over MS-B up to a distance of 1200 µm distal to the injury site possibly due to independent pathways for RGC survival and axonal regeneration. The results could be explained partly due to the inability of slow-release formulations to cope with the rapidly developing pathophysiology of the model characterised by exponential RGC loss very early after the injury and it is recommended to pre-treat the animals with intravitreal injections of microsphere formulations a few weeks before the injury in future studies with this model to provide a neuroprotective effect.

4.1. Introduction

Diseases of the ocular posterior segment are clinically managed by repeated intravitreal injections (World report on vision, 2019), which can present adverse local as well as systemic complications (Falavarjani and Nguyen, 2013), and lead to inadequate treatment adherence and poor clinical outcomes (Ehlken *et al.*, 2020). Sustained release drug delivery systems for the posterior ocular segment diseases have made considerable progress in the recent decades, both clinically and commercially. These platforms can release and maintain an effective minimum therapeutic concentration of drugs with short half-lives in the vitreous or small therapeutic windows, thus avoiding drug loss to systemic or non-target absorption and providing a prolonged therapy without the recurring need of frequent invasive interventions and associated complications (Thrimawithana *et al.*, 2011; Kang-Mieler, Osswald and Mieler, 2014).

One such approach of controlled intraocular drug delivery is by intravitreal depots of biodegradable polymeric microspheres loaded with drug molecules. Injectable microspheres composed of poly(lactic-*co*-glycolic acid) (50:50 PLGA) co-polymer are the most commonly used microspheres for intravitreal drug delivery (Herrero-Vanrell and Refojo, 2001; Herrero-Vanrell and Molina-Martinez, 2007; Herrero-Vanrell *et al.*, 2014), primarily due to their *in-vivo* biocompatibility and homogeneous hydrolytic biodegradation into non-toxic metabolites over long periods of time (Anderson and Shive, 1997), thus enabling a long duration sustained release of the encapsulated drugs. Their high encapsulation efficiencies, up to 100%, coupled with the ability to encapsulate both hydrophilic (Gavini *et al.*, 2004) as well as lipophilic drugs (Barcia *et al.*, 2009), small molecule drugs (Herrero-Vanrell and Ramirez, 2000) as well as

macromolecular biologics (Ye *et al.*, 2015) are a great advantage for intravitreal drug delivery.

Among multiple factors involved in the pathogenesis of major diseases of the posterior segment that constitute the leading cause for irreversible blindness, endogenous oxidative stress induced via elevated production of mitochondrial reactive oxygen species (ROS) including superoxide anion ($O_2^{-\bullet}$), hydroxyl radical ($OH^{\bullet}$), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$), has been shown to be damaging in more ways than one (Nita and Grzybowski, 2016).

In glaucoma patients, ROS cause damage and remodelling of trabecular meshwork resulting in increased intra-ocular pressure (IOP), which in turn stimulates further ROS production, and that leads to impaired autophagy and glaucomatous neurodegeneration in the retina and optic nerve following progressive death of retinal ganglion cell (RGC) neurones by apoptosis (Izzotti, Bagnis and Saccà, 2006; Tezel, 2006; Nita and Grzybowski, 2016). ROS overproduction in traumatic optic neuropathy (TON) leads to RGC apoptosis and secondary degeneration in intact optic nerve axons (O'Hare Doig *et al.*, 2014; Nita and Grzybowski, 2016).

In diabetic retinopathy, hyperglycaemia inhibits endogenous scavengers of ROS like glutathione, resulting in ROS accumulation, and chronic oxidative and hypoxic stress in the retina. This triggers generation of inflammatory and angiogenic mediators including tumour necrosis factor alpha (TNF-α) and vascular endothelial growth factor (VEGF), and promotes apoptosis and senescence of endothelial cells in the capillaries of retinal microvasculature via mitochondrial dysfunction, that finally lead to the breakdown of blood-retinal barrier, pathological angiogenesis, vascular leakage,

microvascular lesions, and macular oedema (Kowluru and Chan, 2007; Nita and Grzybowski, 2016).

In ageing adult retinas, oxidative stress, due to deficient anti-oxidative mechanisms and ROS accumulation, causes mitochondrial DNA damage and pathophysiological parainflammation, leading to apoptosis in retinal pigment epithelium (RPE) cells and neurodegeneration of photoreceptors in both dry as well as wet age-related macular degeneration (AMD) (Beatty *et al.*, 2000; Nita and Grzybowski, 2016). It also causes autophagy dysfunction which results in pathological angiogenesis leading to the characteristic choroidal neovascularisation (CNV) in exudative or wet AMD (Beatty *et al.*, 2000; Nita and Grzybowski, 2016).

Excessive ROS levels in the retina also cause neurodegeneration by neuronal apoptosis via degradation of synaptic transmitter proteins like synaptophysin and neurotrophic factors like brain-derived neurotrophic factor (BDNF), and by glutamate induced neuronal excitotoxicity due to glial dysfunction (Kowluru and Chan, 2007; Sasaki *et al.*, 2010; Nita and Grzybowski, 2016).

Small molecule endogenous anti-oxidants have been presented as both a preventive as well as a therapeutic modality for remediating the oxidative stress in diseases of the posterior segment (Wang, Chin and Almeida, 2021). α-tocopherol (vitamin E) is an endogenous ROS scavenger which has been known to prevent oxidative damage by inhibiting lipid peroxidation of polyunsaturated fatty acids (PUFA) and accumulation of lipofuscin in the cell membranes of retinal cells in case of injury or disease (Farnsworth and Dratz, 1976; Siu, Reiter and To, 1998; Aydemir *et al.*, 2004; Yülek *et al.*, 2007).

Melatonin, an endogenously produced neurohormone in the photoreceptors of the retina, is another small molecule anti-oxidant that has been shown to slow down the progression of retinal diseases by its direct free radical scavenging actions to inhibit lipid peroxidation, in combination with its indirect effects including upregulation of glutathione production, preventing apoptosis of retinal cells via upregulation of autophagy, and attenuation of oxidative stress and inflammation by receptor mediated signalling effects (Siu, Reiter and To, 1998; Marchiafava and Longoni, 1999; Stefanova *et al.*, 2013; Jiang *et al.*, 2016; Chang *et al.*, 2018). Melatonin has also been shown to suppress elevated expression of VEGF, glutamate, and caspases in the retina, thereby preventing angiogenesis and preserving the integrity of blood-retinal barrier, while providing neuroprotection in the neural retina (Belforte *et al.*, 2010; Kaur *et al.*, 2013; Jiang *et al.*, 2016). Multiple studies have proven that physiological deficiencies of vitamin E and melatonin are directly associated with age related degenerative diseases of the retina (Hayes, 1974; Robinson, Kuwabara and Bieri, 1979; Rosen *et al.*, 2009; Ko *et al.*, 2010; Buonfiglio *et al.*, 2011).

In this chapter, we investigated the *in-vivo* therapeutic efficacy of intravitreal depots of biodegradable 50:50 PLGA microspheres co-encapsulated with anti-oxidants α-tocopherol (vitamin E) and melatonin, and an anti-inflammatory corticosteroid, dexamethasone, in a rat model with optic nerve crush (ONC) injury (Berry, Carlile and Hunter, 1996; Berry *et al.*, 1999; Ahmed, Dent, Leadbeater, *et al.*, 2005; Vigneswara *et al.*, 2014; Ahmed *et al.*, 2019; Thompson *et al.*, 2019) mimicking the pathophysiology of neurodegeneration in TON and glaucoma. Dexamethasone has been extensively used in the treatment of retinal oedema (Sarao *et al.*, 2014; Dugel, Bandello and Loewenstein, 2015) and other inflammatory conditions like uveitis (Saincher and

Gottlieb, 2020), and has also shown some neuroprotective effects in diseases and injuries of central nervous system (CNS) (Felszeghy *et al.*, 2004; Pereiro *et al.*, 2018). The encapsulated microspheres were prepared using an oil-in-water emulsion solvent extraction/evaporation technique (Barcia *et al.*, 2009; Arranz-Romera *et al.*, 2019) and injected intravitreally in ONC rats allowing a sustained release of active substances for 21 days. Postmortem immunohistochemical analyses in the eyes and optic nerves were carried out to determine $RBPMS^+$ RGC survival and $GAP43^+$ axonal regeneration, $GFAP^+$ Müller cell gliosis and $OX\text{-}42^+$ microglial activation for neuroinflammation, and $CD\text{-}68^+$ macrophage migration for immune response.

4.2. Materials and Methods

4.2.1. Microspheres synthesis and characterisation

Microencapsulation was performed to fabricate drug-loaded microspheres following an oil-in-water emulsion solvent extraction-evaporation protocol described previously (Barcia *et al.*, 2009; Arranz-Romera *et al.*, 2019). All the solvents, reagents, and drugs were procured from Sigma-Aldrich, Spain, unless otherwise specified. Briefly, 50:50 PLGA co-polymer (MW = 35000 g/mol; Evonik España, Granollers, Spain) with equal proportions of lactic acid and glycolic acid repeating units, was dissolved in methylene chloride and mixed with a solution of melatonin (MEL) and dexamethasone (DEX) in ethanol. α-tocopherol acetate (vitamin E; VE) was then added as the oily additive and the mixture was sonicated followed by emulsification in a solution of 1% w/v polyvinyl alcohol (PVA; M_w: 67,000 g/mol) surfactant in water in a homogeniser at 8500 rpm for 2 minutes. The organic solvents were evaporated by magnetic stirring for three hours in a 0.1% w/v aqueous solution of PVA, and the PVA was removed by repeated washes in H_2O. Microspheres were hardened for three hours, filtered through sieves, and

freeze-dried. Four formulations were prepared for *in-vivo* testing: blank PLGA microspheres (MS-B) as vehicle or negative control; PLGA MS with VE additive (MS-V); PLGA MS loaded with MEL and DEX (MS-MD); and PLGA microspheres with VE additive, loaded with MEL and DEX (MS-VMD). All the solvents and reagents were sterile filtered, and the microencapsulation experiments were performed in an aseptic manner.

Microsphere size and morphology was determined using laser diffraction (LD) / static light scattering (SLS) (Microtrac® S3500 Series Particle Size Analyzer, Montgomeryville, PA, USA) and scanning electron microscopy (SEM, Jeol, JSM-6335F, Tokyo, Japan). Encapsulation efficiencies for MEL and DEX were evaluated by dissolving the microspheres in methylene chloride and addition of methanol to extract the drugs and precipitate the polymer. I*n-vitro* release studies carried were out in phosphate buffered saline (PBS) at 37 ºC for 21 days, and drug estimation was done using high-performance liquid chromatography (HPLC) (Brugnera *et al.*, 2022).

4.2.2. Animals

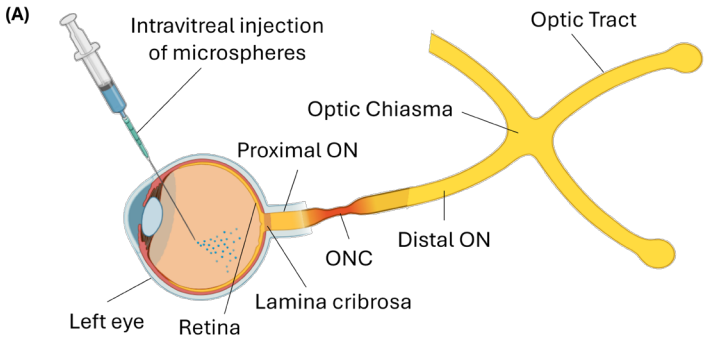
For *in-vivo* experiments, sixteen adult Sprague-Dawley rats (both male and female, 6-8 weeks old, 180-220 g; Charles River, Margate, UK) were randomly assigned to four treatment groups, n = 4 each for MS-B, MS-V, MS-MD, and MS-VMD, and the investigators were masked to the treatments (Figure 4.1). Animal numbers were determined through power calculations using the NC3Rs Experimental Design Assistant set up to provide a power of 0.8 and alpha of 0.05. Effect sizes and standard deviations were determined from previous published studies using the same ONC model in similar experiments (Ahmed, Dent, Suggate, *et al.*, 2005; Ahmed *et al.*, 2006). Animals were housed at 21 ºC and 55% relative humidity in a 12-hour light-dark cycle

with ad libitum access to food and water. Optic nerve crush (ONC) was insulted unilaterally in the left eyes of all animals and formulations were intravitreally injected ipsilaterally, with contralateral right eye intact controls as a refinement of the model in accordance with the 3R's principles of animal research, and the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals in ophthalmic and vision research. All surgeries were performed in strict compliance with the UK home office regulations for the care and use of experimental animals and the UK Animals (Scientific Procedures) Act 1986, licensed by the UK home office and approved by the University of Birmingham Animal Welfare and Ethical Review Board. All *in-vivo* experiments also conformed to Animal Research: Reporting of In-Vivo Experiments (ARRIVE) guidelines (du Sert, Hurst, *et al.*, 2020). Pain was controlled through use of routine analgesia peri- and post-operatively and as advised by the named veterinary surgeon.

4.2.3. Optic Nerve Crush

Rats were anaesthetised by isoflurane inhalation anaesthesia (Janssen Pharmaceuticals), left ONs were exposed through a supra-orbital approach and after severing the meninges, ONs were axotomized intraorbitally 2 mm posterior to the lamina cribrosa for 10 s using a calibrated watchmaker's forceps, as described previously (Berry, Carlile and Hunter, 1996; Berry *et al.*, 1999; Ahmed, Dent, Leadbeater, *et al.*, 2005; Vigneswara *et al.*, 2014; Ahmed *et al.*, 2019; Thompson *et al.*, 2019) (Figure 4.1). Complete severance of the ON was confirmed after releasing the forceps, by absence of optic nerve tissue within the translucent dura sheath at the injury site. ONs were then defined in two segments: proximal segment from the retina to the crush site, and distal segment from the crush site to the optic chiasma. Care was

taken to prevent any damage to the central retinal artery and thus preserving the retinal vascular supply. Animals were observed by an ophthalmoscope post-surgery to detect any damage to the lens or development of cataracts, and none of the animals developed such complications.



(B)

Treatments (left eyes)	n = rats
ONC + Blank Microspheres (MS-B)	4
ONC + MS with VE (MS-V)	4
ONC + MS with MEL and DEX (MS-MD)	4
ONC + MS with VE, MEL and DEX (MS-VMD)	4

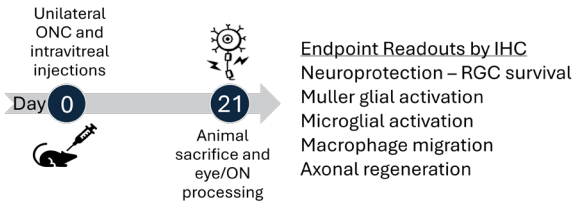


Figure 4.1: Optic nerve crush (ONC) model and experimental design. (A) Illustration of the in-vivo model of rat ONC showing the injury site in the left ON 2mm posterior to lamina cribrosa, dividing the nerve into proximal segment from the retina to the injury site and distal segment from the ONC site to the optic chiasma, and (B) a schematic of experimental design showing the treatment paradigms and the timeline for in-vivo studies with blank (MS-B) and drug loaded microspheres containing Vitamin-E (VE): MS-V, Melatonin (MEL) and Dexamethasone (DEX): MS-MD, and combination of VE, MEL and DEX (MS-VMD).

4.2.4. Intravitreal Injections

Microsphere formulations were injected unilaterally in the left eyes (n = 4 rats/formulation), immediately after ONC surgeries. MS-B, MS-V, MS-MD, and MS-VMD microspheres were dispersed in sterile PBS (Dulbecco's; Cat no. 14190094, ThermoFisher, Hampshire, UK) at 50 mg/mL concentration. 2 µL of this suspension containing 0.1 mg of microspheres were injected intravitreally through the sclera

immediately posterior to the limbus using glass micropipettes, ensuring complete care to not perforate the lens, which was subsequently confirmed by absence of cataract formation. MS-MD and MS-VMD formulations contained 3.5 µg DEX and 6 µg MEL each.

4.2.5. Tissue Preparation

Animals were sacrificed 21 days post ONC injury (DPI) and intravitreal injections, by rising concentrations of CO_2 and perfused transcardially with 4% paraformaldehyde (PFA; BioReagent, ≥ 36.0%; Cat no. 47608, Sigma-Aldrich, Poole, Dorset, UK). Eyes were enucleated and ONs were dissected out till the chiasma. The tissues were immersion-fixed in 4% PFA for 2 hours at 4 ºC followed by cryoprotection in increasing concentrations of sucrose (≥ 99.5%; Cat no. S9378, Sigma-Aldrich, Switzerland) solutions – 10%, 20%, and 30% for 8 – 14 hours each, and finally embedded in moulds with optimal cutting temperature compound (OCT; Cat no. 12678646, Fisher Scientific), frozen on dry ice and stored at -80 ºC. 15 µm parasagittal and longitudinal sections were taken of eyes and optic nerves respectively at -20 ºC using a cryostat (Brights Instruments, Huntingdon, UK) and collected on SuperFrost Plus adhesion glass microscope slides (Cat no. 10149870, Fisher Scientific, Ashford, UK). The sections were allowed to settle overnight at RT before storing at -20°C until use. Sections of ON containing a well-defined ONC site and radial ocular sections of eyes taken through the ON head were selected for further analysis.

4.2.6. Immunohistochemistry

Eye and ON sections were immunostained for different markers to analyse RGC survival, axonal regeneration, gliosis, inflammation, and immune response. Frozen sections were thawed for 30 minutes at room temperature (RT), washed twice in PBS

for 5 minutes, and permeabilised for 10 minutes in 0.1% triton X-100 (Cat no. T8787, Sigma-Aldrich, USA) in PBS. After washing again twice in PBS for 5 minutes at RT, sections were blocked in 0.5% bovine serum albumin (BSA; Cat no. A3311, Sigma-Aldrich) + 0.1% triton X-100 in PBS for 30 min at RT to prevent any non-specific binding. Sections were then incubated with appropriate primary antibodies as listed in Table 4.1, diluted in PBS containing 0.5% BSA + 0.05% tween-20 (Cat no. 663684B, VWR) and left overnight at 4°C in a humidified chamber. Sections were washed thrice for 5 minutes in PBS the following day, then incubated with fluorescently labelled secondary antibodies (Table 4.1) diluted in PBS containing 0.5% BSA + 0.05% tween-20 for 1 hour at RT, washed again thrice for 5 minutes in PBS, and finally mounted with coverslips in Vectashield Plus antifade mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) (Cat no. H2000, Vector Laboratories, Peterborough, UK). Mounted slides were imaged under fluorescence settings on an Axioplan-2 upright microscope (Carl Zeiss Ltd, Cambridge, UK). Images were captured with Axiovision software (Carl Zeiss Ltd) in AxioCam HRc microscope camera, using a 20X objective lens (200X magnification; Carl Zeiss) and appropriate colour filters according to the fluorophores, and analysed on ImageJ (NIH, USA) (Schneider, Rasband and Eliceiri, 2012), by an experimenter masked to the treatment conditions. A minimum of four sections (technical replicates) were analysed from each eye and optic nerve.

Table 4.1: List of antibodies used for immunohistochemistry in this study.

Antibody	Target	Dilution	Cat No. / Supplier
Primary antibodies			

Rabbit anti-RBPMS polyclonal	RGCs	1:500	PA531231 / ThermoFisher UK
Rabbit anti-GFAP polyclonal	Astrocytes and Müller glia	1:500	PA516291 / ThermoFisher UK
Mouse anti-Rat CD11b/c monoclonal OX-42	Microglia	1:200	MA181606 / ThermoFisher UK
Rabbit anti-CD68 polyclonal	Macrophages	1:200	AB125212 / Abcam UK
Mouse anti-GAP43 monoclonal 7B10	Regenerating ON axons	1:500	335000 / ThermoFisher UK
Rabbit anti-Laminin polyclonal	ONC site	1:500	PA116730 / ThermoFisher UK
Secondary antibodies			
Goat anti-Rabbit polyclonal Alexa Fluor Plus 488	Rabbit Primary	1:500	A32731 / ThermoFisher UK
Donkey anti-Mouse polyclonal Alexa Fluor Plus 594	Mouse Primary	1:500	A32744 / ThermoFisher UK

4.2.7. Assessment of RGC Survival

RGC survival was assessed as a measure of neuroprotective effect of the formulations, as described previously (Ahmed *et al.*, 2019; Thompson *et al.*, 2019). Frozen eye sections were stained with an antibody against RBPMS, which is an RGC specific phenotypic marker, and $RBPMS^{+}$ live RGCs were counted over a standard 250 µm linear strip of the ganglion cell layer (GCL) in radial sections using ImageJ in five different images taken on either side of the ON head by an experimenter masked to the treatments (four radial sections/retina, n = 4 eyes/treatment group). The results

were expressed as numbers of RBPMS$^+$ RGCs/250 µm GCL. This method of counting surviving RGCs in frozen radial sections in rats produces similar results to freshly prepared retinal wholemounts back-labelled with a fluorogold tracer retrogradely injected into the optic nerve before animal sacrifice (Mead *et al.*, 2014) and is therefore an equally reliable method which conserves a large amount of tissue by maximising measurements on individual sections rather than using the whole retina.

4.2.8. Assessment of Glial Activation and Immune Response

Activation of retinal astroglia in the GCL/nerve fibre layer (NFL), and Müller glial processes and somata were identified after staining with an antibody against filament glial fibrillary acidic protein (GFAP) in radial retinal sections and quantified as previously defined (Ahmed *et al.*, 2019; Thompson *et al.*, 2019). Briefly, GFAP$^+$ Müller glial processes intersecting a 250 µm horizontal sampling line passing through inner plexiform layer (IPL) were counted in the middle of GCL and inner nuclear layer (INL) by a masked experimenter using ImageJ in five different images taken on either side of the ON head (four radial sections/retina, n = 4 eyes/treatment group).

Similarly, retinal microglia were immunostained for an integrin alpha-M clone OX-42 specific to rat CD11b and OX-42$^+$ reactive microglia were identified based on morphological differences from resting cells and their numbers were counted in standard 250 µm linear strips of GCL/NFL and IPL in radial retinal sections in five different images taken on either side of the ON head (four radial sections/retina, n = 4 eyes/treatment group).

Macrophage migration into the retina as an overt immune response was quantified by staining with a CD68 antibody and numbers of CD68$^+$ macrophages were counted, and

their localisation was observed in radial retinal sections in five different images taken on either side of the ON head (four radial sections/retina, n = 4 eyes/treatment group).

4.2.9. Assessment of Axonal Regeneration

Regenerating axons in the distal ON section were quantified in longitudinal ON sections after staining for growth associated protein 43 (GAP43) neuromodulin as previously described (Vigneswara *et al.*, 2013; Ahmed *et al.*, 2019). Briefly, composite images of ON sections were constructed using Adobe Photoshop software (Version 24.6.0, 2023) and the ONC site was identified by laminin^{+} immunostaining, following which the number of GAP43^{+} regenerating axons was counted intersecting linear vertical sampling lines across the width of ON at distances extending 100, 200, 400, 800, and 1200 µm distal to the injury site using ImageJ by an experimenter masked to the treatments (four longitudinal sections/ON, n = 4 ON/treatment group). Postmortem GAP43 immunostaining of ON has been proven to produce similar counts of regenerating axons as by pre-sacrifice labelling with Rhodamine B anterograde tracer and is thus a more specific gold-standard method of quantifying RGC axonal regeneration in distal segment (Vigneswara *et al.*, 2014). The cross-sectional width in µm (w) of the ON was also measured at all distances where the mean number of GAP43^{+} regenerating axons (a) were counted, to quantify the total number of regenerating axons ($\sum a_d$) across the whole area of the ON cross section with a radius r, where t (15 µm) is the thickness of the longitudinal section:

$$\sum a_d = \pi r^2 \times {}^{(a}/_{w)}/_{t}$$

4.2.10. Statistical Analysis

OriginPro software (Version 2021b SR1, OriginLab Corporation, Northampton, MA, USA) was used to analyse data. All data values are expressed as mean ± standard

error of mean (SEM). Distribution of data from IHC analyses was tested for normality using Shapiro-Wilk test and upon confirmation of a parametric distribution, one-way ANOVA with post-hoc Bonferroni's multiple comparisons correction was used to compare the means among different treatment groups. A p value of < 0.05 was the designated threshold for differences to be statistically significant.

4.3. Results

4.3.1. Microsphere Characteristics

Microsphere diameter in a range of 20 – 38 μm with a unimodal distribution was determined using laser diffraction. SEM imaging revealed a porous structure and golf-ball like morphology for microspheres with VE additive due to a slower rate of diffusion of the organic solvent from the internal to external phase, whereas microspheres without VE were smooth (Figure 4.2). A total amount of 35 μg MEL and 60 μg DEX were encapsulated per mg of PLGA microspheres in both MS-MD and MS-VMD formulations with encapsulation efficiency of about 60% for MEL and 80% for DEX, as estimated by instant dissolution of microspheres and further extraction of encapsulated drugs. In *in-vitro* release studies over 21 days in PBS, there was an initial burst release over the first 24 hours for both MEL and DEX in MS-MD and MS-VMD formulations, followed by biphasic release kinetics leading to cumulative release amount of 31.05 ± 0.12 μg MEL and 20.76 ± 0.06 μg DEX per mg MS-MD, and 32.45 ± 0.11 μg MEL and 18.86 ± 0.05 μg DEX per mg MS-VMD formulations in 21 days.

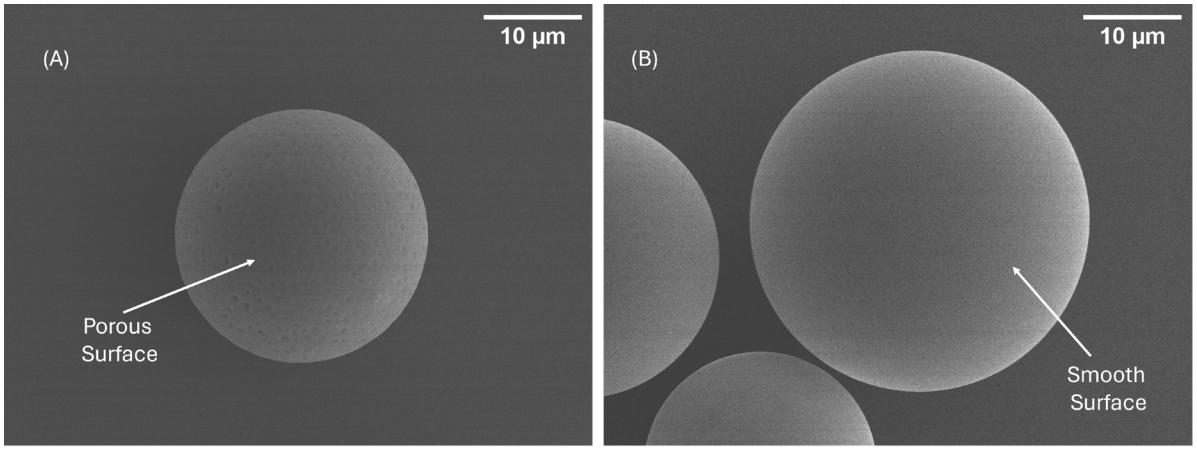
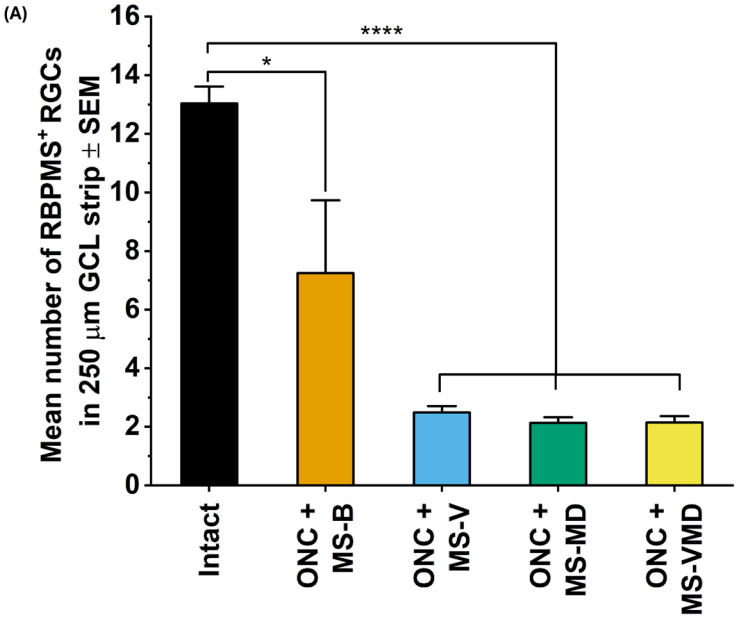


Figure 4.2: Scanning electron microscope (SEM) micrographs at 2000X of PLGA microspheres (A) with vitamin-E (VE) additive showing a porous surface with golf-ball like morphology, and (B) without VE additive having a smooth surface. Scale = 10 µm

4.3.2. None of the microsphere formulations provide RGC neuroprotection after ONC

We counted $RBPMS^+$ surviving RGCs in the GCL at 21 DPI and observed that none of the three drug-loaded formulations (MS-V, MS-MD, MS-VMD) provided any protection from cell death after ONC injury (Figure 4.3). The surviving RGC counts in uninjured retinas (13.04 $\pm$ 0.58), as expected, were significantly higher than MS-B (7.25 $\pm$ 2.48, *p = 0.0290 vs intact) and all the drug loaded formulations MS-V (2.49 $\pm$ 0.22), MS-MD (2.14 $\pm$ 0.18), and MS-VMD (2.15 $\pm$ 0.22) (****p < 0.0001 for all three vs intact). Though there was no significant difference in RGC survival among the ONC retinas treated with the microsphere formulations, the blank controls showed almost three-fold higher cell counts than any of the drug-loaded microsphere formulations which indicates a rather deleterious effect of drugs on survival of injured RGCs.


(A)
Mean number of RBPMS+ RGCs in 250 μm GCL strip ± SEM
16
14
12
10
8
6
4
2
0
****
*
Intact
ONC + MS-B
ONC + MS-V
ONC + MS-MD
ONC + MS-VMD

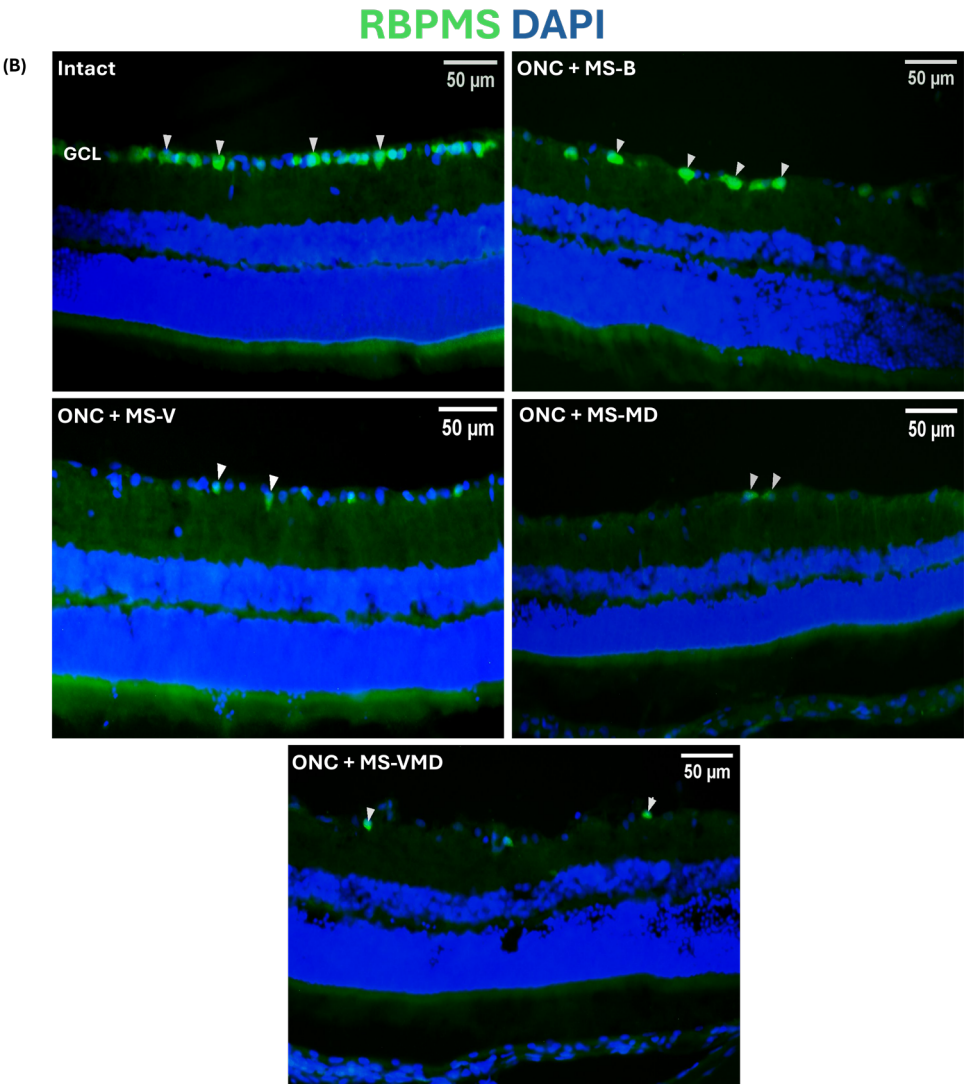

RBPMS DAPI
(B)
Intact
50 μm
GCL
ONC + MS-B
50 μm
ONC + MS-V
50 μm
ONC + MS-MD
50 μm
ONC + MS-VMD
50 μm

Figure 4.3: Retinal ganglion cell (RGC) survival in intact retinas and optic nerve crush (ONC) injured retinas treated with blank (MS-B) and drug loaded microspheres containing vitamin-E (VE): MS-V; melatonin (MEL) and dexamethasone (DEX): MS-MD; and combination of VE, MEL and DEX (MS-VMD). (A) Bar graph representing mean ± standard error of mean (SEM) numbers of $RBPMS^+$ RGC counts in 250 µm standard sampling strip of ganglion cell layer (GCL) and the statistical difference between different treatment groups estimated using one-way ANOVA and post-hoc Bonferroni's correction (*p = 0.0290, ****p < 0.0001). (B) Fluorescent microscopy images at 200X magnification of immunostained 15 µm thick radial sections of retina showing $RBPMS^+$ surviving RGC somata in green (Alexa Fluor 488) and indicated by white arrowheads in the GCL, and counterstained with DAPI nuclear stain in blue showing distinct histological layers in the retina. Scale bar = 50 µm. Note that none of the drug loaded formulations exhibit any neuroprotective effects on RGC survival, and instead seem to have detrimental effects as is evident from a lower cell count in MS-V, MS-MD, and MS-VMD formulations than the control MS-B. Data in (A) has been obtained from analysis of images in (B) and are both representative of measurements taken in 5 different images taken on either side of the ON head in 4 radial sections of retina in n = 4 rats/eyes per treatment group 21 days post injury.

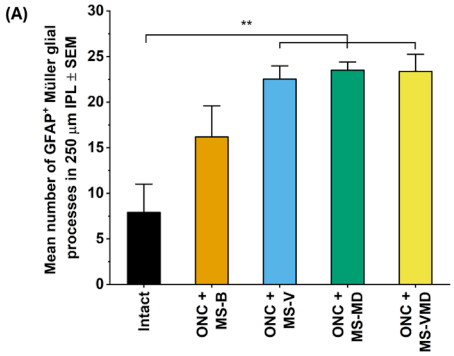
4.3.3. Drug loaded microsphere formulations exacerbate retinal gliosis after ONC injury

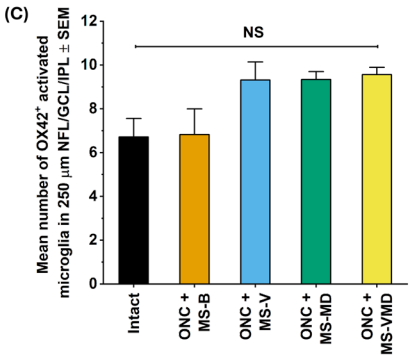
In anti-GFAP stained radial sections of the retina, drug loaded microsphere treatments (MS-V, MS-MD, and MS-VMD) showed higher levels of astrocyte and Müller glial activation in GCL/NFL and IPL respectively at 21 DPI in ONC eyes compared to the blank and uninjured group (Figure 4.4 (A) and (B)). GFAP upregulation was evident by significantly higher number of $GFAP^+$ Müller cell processes passing through the IPL in ONC retinas treated with MS-V (22.54 ± 1.44, **p = 0.0051), MS-MD (23.51 ± 0.90, **p = 0.0029), and MS-VMD (23.38 ± 1.88, **p = 0.0031), and an insignificant increase in MS-B (16.19 ± 3.40, p = 0.2486) compared to the intact retinas (7.9 ± 3.10). Even though we did not observe any significant difference in Müller cell activation amongst the ONC retinas treated with microsphere formulations, a higher activation of Müller glia in retinas treated with drug loaded microspheres than in the retinas treated with blank controls, points towards an exacerbation of Müller cell gliosis promoted by the

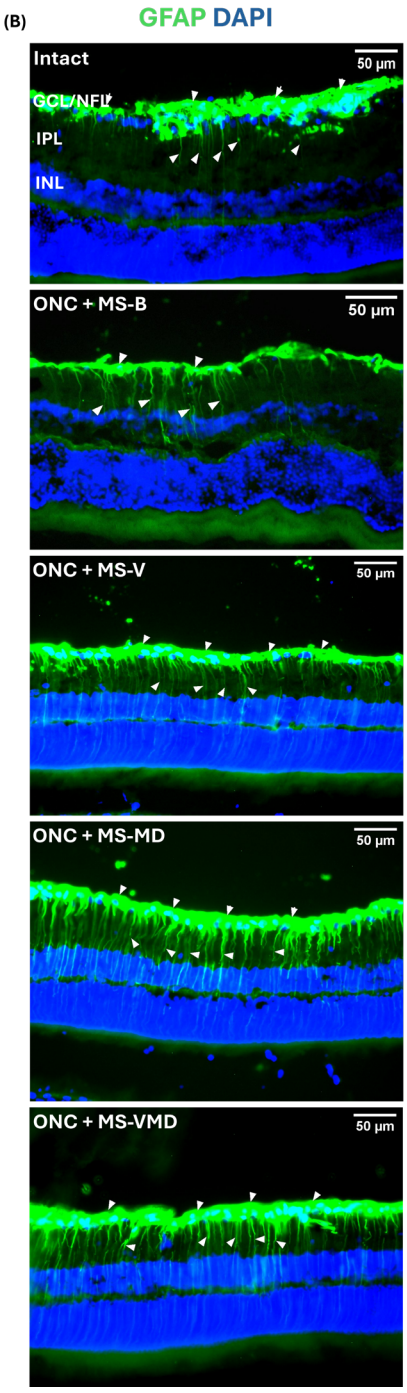
drugs in formulations. It was also observed that retinas treated with drug loaded MS exhibited a stronger expression of $GFAP^+$ hypertrophy and proliferation of the Müller glial fibrous processes extending from GCL/NFL to the somata in INL compared to the blank and intact controls.

Anti-OX42 staining revealed similar trends of activated microglial populations in NFL/GCL and IPL (Figure 4.4 (C) and (D)). Retinas treated with drug loaded microsphere formulations MS-V (9.31 ± 0.83), MS-MD (9.34 ± 0.36), and MS-VMD (9.56 ± 0.33) had higher counts of $OX42^+$ activated microglia compared to the MS-B controls (6.83 ± 1.17) and uninjured group (6.71 ± 0.85), however, none of the groups had any significant difference in the numbers of activated microglia. The activated reactive microglia were identified from their distinct amoeboid or bipolar/rod-like or hyper-ramified morphologies compared to a ramified structure of resting microglia. It was also observed that the intact group had mainly ramified microglia in the NFL/GCL and IPL, whereas in the ONC groups, the reactive rod shaped, amoeboid, and hyper-ramified microglia localised mainly in the NFL and GCL. These results also corroborate our assumption that the drugs caused detrimental gliosis effects after ONC.

There were no macrophages detected in CD-68 stained retinal sections in any of the treatment groups. This confirms that there was no migration of $CD68^+$ macrophages into the retina and thus none of the formulations triggered an overt immune response after ONC.


(A)
Mean number of GFAP+ Müller glial processes in 250 µm IPL ± SEM
30
25
20
15
10
5
0
**
Intact
ONC + MS-B
ONC + MS-V
ONC + MS-MD
ONC + MS-VMD


(C)
Mean number of OX42+ activated microglia in 250 µm NFL/GCL/IPL ± SEM
12
10
8
6
4
2
0
NS
Intact
ONC + MS-B
ONC + MS-V
ONC + MS-MD
ONC + MS-VMD


(B)
GFAP DAPI
Intact
GCL/NFL
IPL
INL
50 µm
ONC + MS-B
ONC + MS-V
ONC + MS-MD
ONC + MS-VMD

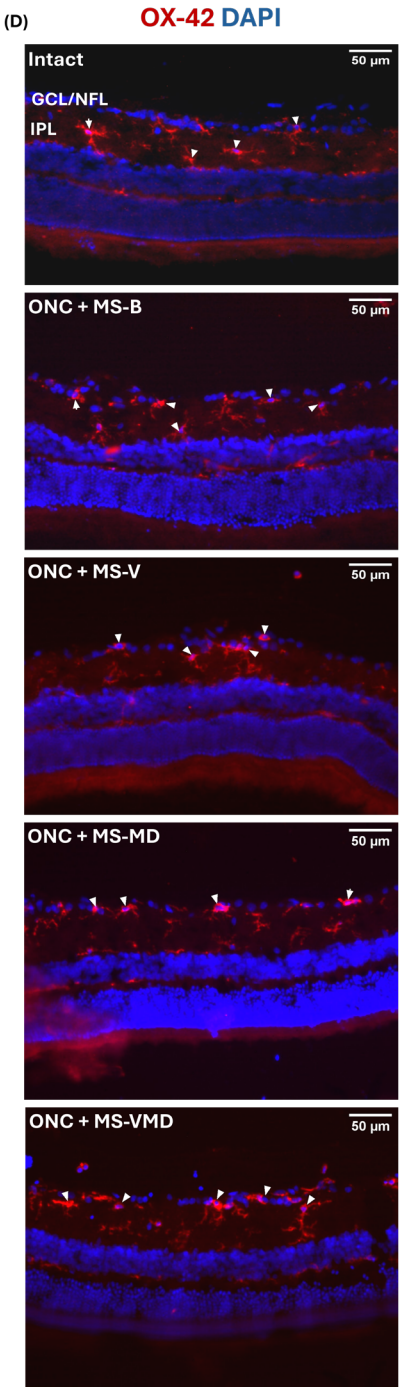
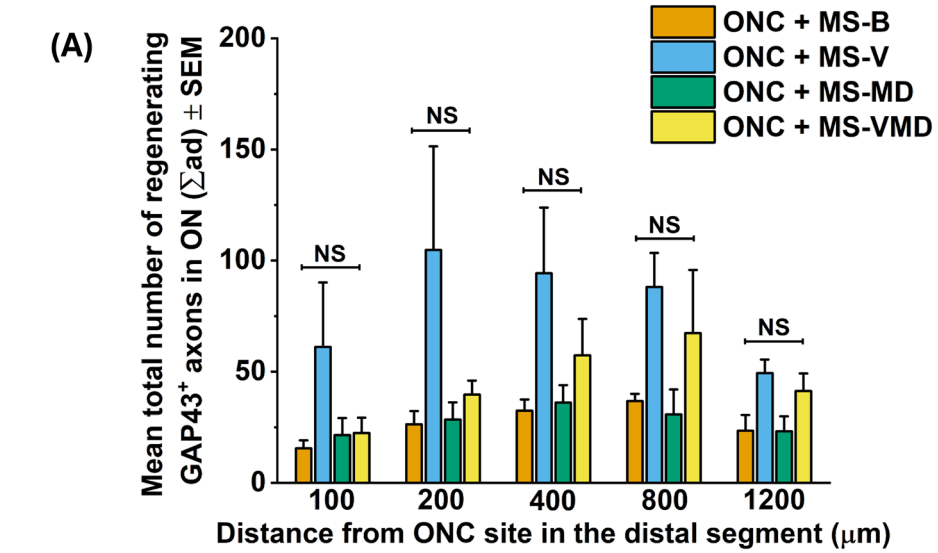

(D)
OX-42 DAPI
Intact
GCL/NFL
IPL
50 µm
ONC + MS-B
ONC + MS-V
ONC + MS-MD
ONC + MS-VMD

Figure 4.4: Activation of retinal glial cells in intact retinas and optic nerve crush (ONC) injured retinas treated with blank (MS-B) and drug loaded microspheres containing vitamin-E (VE): MS-V; melatonin (MEL) and dexamethasone (DEX): MS-MD; and combination of VE, MEL and DEX (MS-VMD). (A) and (C) Bar graphs representing mean ± standard error of mean (SEM) numbers of (A) $GFAP^+$ Müller glial processes intersecting a 250 µm horizontal sampling line passing through inner plexiform layer (IPL), and (C) $OX\text{-}42^+$ activated microglia in standard 250 µm linear strips of nerve fibre layer (NFL) ganglion cell layer (GCL), and IPL, and the statistical difference between different treatment groups estimated using one-way ANOVA and post-hoc Bonferroni's correction ((A): **p = 0.0051 for MS-V, **p = 0.0029 for MS-MD, **p = 0.0031 for MS-VMD vs intact group, and (C): NS = no significant difference within groups). (B) and (D) Fluorescent microscopy images at 200X magnification of immunostained 15 µm thick radial sections of retina showing (B) $GFAP^+$ astrocytes indicated by white arrowheads in the NFL and filopodia like fibrous Müller glial processes indicated by white arrowheads in the IPL, extending from GCL into the IPL and to the somata in inner nuclear layer (INL) in green (Alexa Fluor 488), and (D) $OX\text{-}42^+$ microglia in red (Alexa Fluor 594) and indicated by white arrowheads in the NFL/GCL and IPL, both (B) and (D) counterstained with DAPI nuclear stain in blue showing distinct histological layers in the retina. Scale bar = 50 µm for both (B) and (D). A hypertrophy and proliferation of filamentous $GFAP^+$ Müller glial processes can be seen in the ONC groups treated with drug loaded formulations in (B) compared with intact and blank controls. In (D), resting microglia can be differentiated from the reactive microglia based on the distinct hyper-ramified, amoeboid or bipolar/rod-like morphologies that microglia assume on activation compared to a ramified structure of resting microglia, where the intact group mainly shows ramified microglia in the NFL/GCL and IPL, whereas in the ONC groups, the reactive rod shaped, hyper-ramified, and amoeboid microglia localised mainly in the NFL and GCL. These results clearly suggest exacerbation of gliosis in ONC retinas treated with drug loaded formulations. Data in (A) and (C) have been obtained from analysis of images in (B) and (D) respectively and are both representative of measurements taken in 5 different images taken on either side of the ON head in 4 radial sections of retina in n = 4 rats/eyes per treatment group 21 days post injury.

4.3.4. Vitamin-E containing formulations promoted axonal regeneration at all distances distal to the ONC

ON sections stained for GAP43 after ONC revealed that regenerating axons were mainly concentrated in the proximal stump and few of them traversed across the injury site into the distal segment and their numbers were measured across set distances from the lesion (Figure 4.5(B)). We observed that only the microsphere formulations containing VE additive (MS-V and MS-VMD) showed consistent axonal regeneration

across all distances where measurements were taken, however, there was no significant difference in the number of regenerating axons within any of the treatment groups at any distance (Figure 4.5(A)). In terms of total number of regenerating axons after ONC, ONs treated with MS-V showed increasing mean $\sum a_d$ values from 61.13 ± 28.99 at 100 µm to 104.78 ± 46.65 at 200 µm, then decreasing further distal to the ONC to 94.36 ± 29.45 at 400 µm, 88.08 ± 15.33 at 800 µm, and finally going down to its lowest 49.39 ± 6.04 at 1200 µm distal to the injury site. In MS-VMD treated ONs, the mean $\sum a_d$ values for axonal regeneration gradually increased from 22.47 ± 6.83 at 100 µm to 39.73 ± 6.30 at 200 µm, then further increasing to 57.42 ± 16.33 at 400 µm, 67.35 ± 28.42 at 800 µm, and finally decreasing to 41.31 ± 7.98 at 1200 µm. It is evident that both MS-V and MS-VMD showed increased axonal regeneration, though non-significantly, at all distances compared to blank controls and the corresponding $\sum a_d$ values for MS-B were 15.58 ± 3.55 at 100 µm, 26.32 ± 5.98 at 200 µm, 32.45 ± 5.03 at 400 µm, 36.83 ± 3.23 at 800 µm, and 23.44 ± 7.05 at 1200 µm. On the other hand, MS-MD formulations showed similar axonal regeneration at all distances compared to the blank controls, and $\sum a_d$ values for MS-MD were 21.49 ± 7.69 at 100 µm, 28.46 ± 7.71 at 200 µm, 36.08 ± 7.93 at 400 µm, 30.78 ± 11.24 at 800 µm, and 23.20 ± 6.67 at 1200 µm. Since, both MS-V and MS-VMD show increased axonal regeneration than the MS-B control at all distances, and MS-MD doesn't show any increased regeneration, it can be concluded that it is the presence of VE additive in the microsphere formulations that is promoting axonal regeneration. Also, since MS-VMD treated ONs showed lower axonal regeneration compared to the MS-V treated ONs, we can assume that either MEL or DEX or both are in fact detrimental to the regeneration effects of VE in MS-VMD.


(A)
ONC + MS-B
ONC + MS-V
ONC + MS-MD
ONC + MS-VMD
Mean total number of regenerating GAP43+ axons in ON (∑ad) ± SEM
200
150
100
50
0
NS
NS
NS
NS
NS
100
200
400
800
1200
Distance from ONC site in the distal segment (μm)

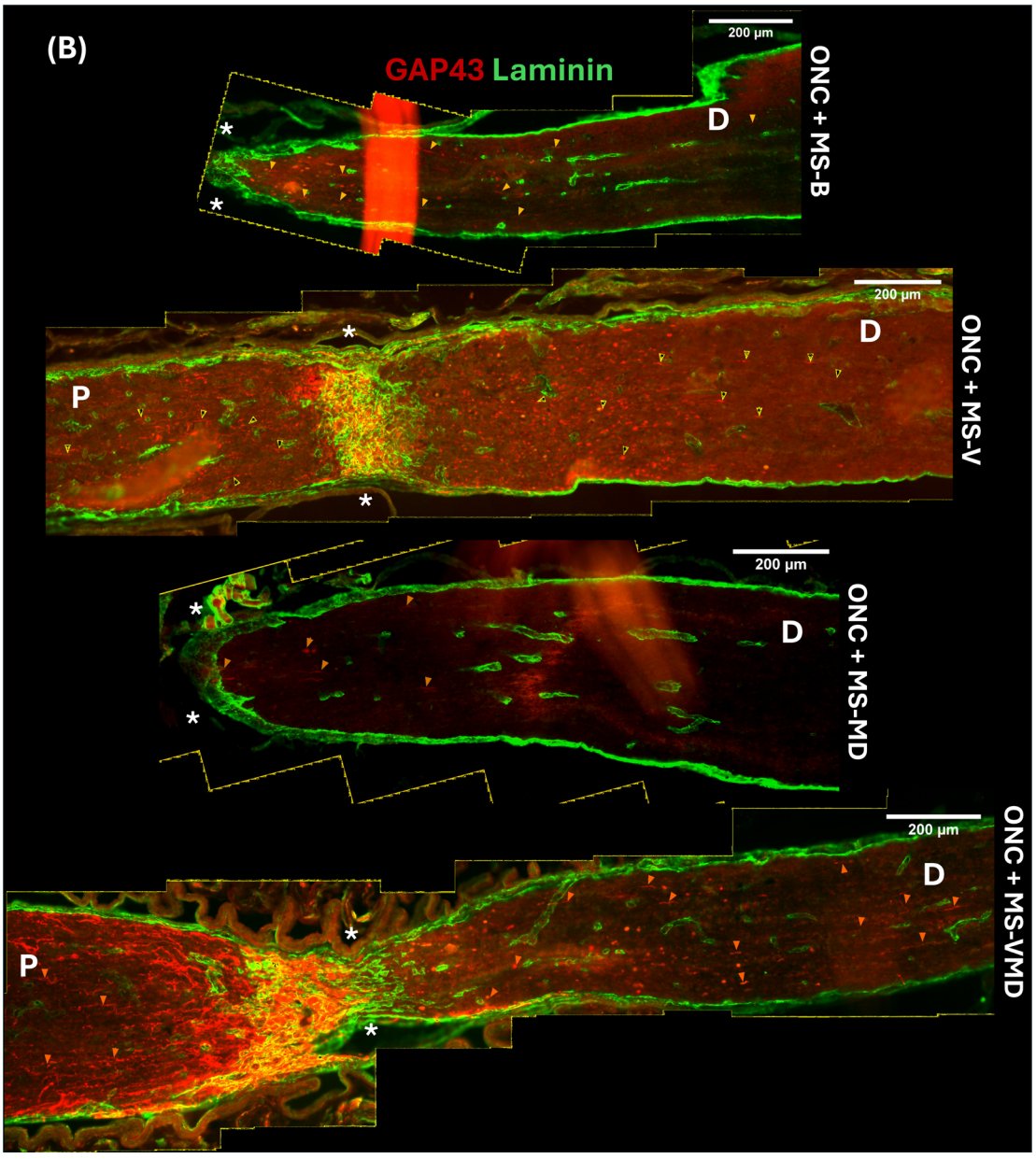

(B)
GAP43 Laminin
200 μm
D
ONC + MS-B
P
ONC + MS-V
ONC + MS-MD
ONC + MS-VMD

Figure 4.5: Regeneration of retinal ganglion cell (RGC) axons in the optic nerve (ON) after optic nerve crush (ONC) injury and treatment with blank controls (MS-B) and drug loaded microspheres containing vitamin-E (VE): MS-V; melatonin (MEL) and dexamethasone (DEX): MS-MD; and combination of VE, MEL and DEX (MS-VMD). (A) Bar graph representing mean ± standard error of mean (SEM) of total numbers of $GAP43^+$ regenerating axons $\sum a_d$ across the entire cross-sectional width of ON at 100, 200, 400, 800 and 1200 µm distances from the ONC injury site in the distal stump of ON and the statistical difference between different treatment groups at all distances estimated using one-way ANOVA and post-hoc Bonferroni's correction (NS = no significant difference within groups). It can be noted that only vitamin-E containing formulations MS-V and MS-VMD actually show increased axonal regeneration across all distances compared to the MS-B controls, and that MS-MD shows no such increase. (B) Fluorescent microscopy composite images created using Photoshop, at 200X magnification of immunostained 15 µm thick longitudinal sections of ON, showing $GAP43^+$ regenerating axons in red (Alexa Fluor 594) indicated by yellow arrowheads in the proximal (P) and distal (D) segments formed after ONC, and co-stained with laminin in green (Alexa Fluor 488) showing continuous $laminin^+$ in the connective tissue sheath and the vascular lamina in the ON and to identify ONC injury site indicated by asterisks with dense $laminin^+$ staining of the blood vessels and matrix deposition in the scar. Scale bar = 200 µm. Very high $GAP43^+$ expression can be seen in the proximal stumps as the RGC axons extending from retina through the lamina cribrosa to the crush site are trying to regenerate after crush but only few could regenerate across the transection site into the distal stump where measurements are taken. Data in (A) has been obtained from analysis of images in (B) and are both representative of measurements taken in 4 longitudinal sections of ON in n = 4 rats/ONs per treatment group 21 days post injury.

4.4. Discussion

This study aimed to determine the *in-vivo* efficacy of controlled release PLGA microsphere formulations loaded with different combinations of natural anti-oxidants vitamin-E and melatonin, along with a glucocorticoid dexamethasone, in preventing neurodegeneration in the retina and promoting regeneration in axons of damaged optic nerves when injected intravitreally for a prolonged delivery to treat neurodegenerative diseases and damage in the posterior ocular segment in order to avoid frequent injections that are usually required in clinical management of such diseases. To the contrary, none of the drug-loaded microsphere formulations provided any *in-vivo* relief from RGC cell death after ONC and there was no increase observed in RGC survival

over blank controls, instead they seemed to have some deleterious effect on RGC survival as there were almost three-fold higher RGC counts in blank controls than the drug-loaded formulations. Similarly, there was exacerbated gliosis in the retina by increased activation of retinal glial cells including astrocytes, Müller glia, and microglia in the retinas treated with drug-loaded formulations than the blank controls. However, the formulations containing vitamin-E additive promoted *in-vivo* axonal regeneration up to 1200 µm in the distal stump after ONC.

The optic nerve crush model of traumatic optic neuropathy has been extensively validated in multiple studies within our group previously characterised by a rapid RGC apoptosis and complete transection of all RGC axons upon axotomy followed by secondary neurodegeneration over weeks in the retina and an inability of the RGC axons to regenerate, partly due to lack of essential neurotrophic factors (Berry, Carlile and Hunter, 1996; Berry *et al.*, 1999; Ahmed, Dent, Leadbeater, *et al.*, 2005; Vigneswara *et al.*, 2013, 2014; Ahmed *et al.*, 2019). It has also been found that RGC cell death after ONC involves ROS signalling, which has been proven by increased survival of RGCs upon ROS inhibition or by changing the redox state of cellular environment to mildly reduced after ONC (Swanson *et al.*, 2005; Zuo *et al.*, 2013). This make ONC an attractive model to study the efficacy of anti-oxidants in providing neuroprotection against RGC apoptosis and to promote axonal regeneration.

There are multiple ROS signalling pathways that affect RGC survival in the retina after axonal injury. Axotomy is believed to trigger a superoxide burst from the mitochondria of RGCs which signals apoptosis (Kanamori *et al.*, 2010). Oxidative stress also leads to excess glutamate production in the retina causing excitotoxicity leading to degeneration of RGCs mediated by overactivation of N-methyl-D-aspartate receptors

(NMDAR) (Namekata *et al.*, 2013). This glutamate excitotoxicity also inhibits the transport of neurotrophic factors like brain-derived neurotrophic factor (BDNF), which in-turn prevents the phosphorylation of tropomyosin-related kinase receptor B (TrkB) and TrkB signalling inactivation inhibits further downstream intracellular signalling pathways of Akt and the mitogen-activated protein kinases / extracellular signal-regulated kinases 1 and 2 (MAPK/Erk1/2), which are essential for the survival of RGCs (Gupta *et al.*, 2013).

To attenuate these effects of oxidative stress mediated neurodegeneration, we formulated intravitreal microspheres loaded with small molecule endogenous ROS scavengers like vitamin-E and melatonin which have previously shown to be neuroprotective in neurodegenerative retinal disorders (Hayes, 1974; Siu, Reiter and To, 1998; Belforte *et al.*, 2010; Ko *et al.*, 2010; Kaur *et al.*, 2013; Wang, Chin and Almeida, 2021). However, we obtained counterintuitive results with all the three drug-loaded formulations showing much lower RGC survival than blank controls leading us to believe that these formulations instead promote degeneration of the RGCs. This could be attributed to few reasons. Firstly, this is an acute model of RGC degeneration and cell death is exponential in absence of an intervention and up-to 50-70% RGCs are lost within 7 days of axotomy (Nadal-Nicolás *et al.*, 2015; Sánchez-Migallón *et al.*, 2018), and it is possible this is too aggressive for the slow release formulations as they might not have released a therapeutic concentration in the vitreous to cope with the cell death, even though in *in-vitro* release studies, the formulations are shown to have released majority of the encapsulated drug within 21 days which is the same period as our *in-vivo* studies. Also, this is the first time in our knowledge that vitamin-E and melatonin slow release formulations have been tested intravitreally to provide

neuroprotection in an ONC model, there are no available values for an optimum therapeutic dosage in the vitreous in published literature that we know of. To overcome this limitation, it would be more reasonable in future studies to pre-treat the rats with intravitreal microsphere formulations a few weeks before axotomy in order to maintain a therapeutic concentration of anti-oxidants that can provide neuroprotection before permanent loss of RGCs. This coupled with an estimation of *in-vivo* release profiles and pharmacokinetic/pharmacodynamic (PK/PD) parameters will greatly benefit future studies in determining an effective therapeutic dose.

Majority of the studies reporting a neuroprotective effect of VE and MEL on RGCs are either done on mixed retinal cell cultures *in-vitro* or *in-vivo* as a dietary supplement, systemic delivery, or on their endogenous effects, and thus do not replicate the effect of an intravitreal administration. Those that have injected intravitreally either directly or in controlled release formulations have done in milder models of retinal injury. In a previous *in-vivo* study, PLGA microspheres containing vitamin-E and glial cell line-derived neurotrophic factor (GDNF) were injected intravitreally in a rat model of chronic intraocular hypertension achieved by intraocular pressure (IOP) elevation for glaucomatous neurodegeneration, to determine their neuroprotective efficacy, which shows that only GDNF formulations showed RGC neuroprotection, while vitamin-E formulations showed same RGC numbers as blank controls (Checa-Casalengua *et al.*, 2011).

However, this still does not explain the reduced RGC survival in all drug-loaded treatment groups compared to the blanks. Another limitation of this study is even though we have used blank microspheres as negative controls for a zero response, there is absence of no treatment ONC group as true negative controls to account for

the natural response in the injured tissues after ONC and the comparative effect of blank PLGA microspheres on RGC survival and axonal regeneration. Theoretically, the ONC + MS-B group should not provide any neuroprotective or regenerative effect, however, due to a detrimental response of all the drug-loaded treatments in ONC group, it is difficult to understand or explain the effect without making undue assumptions. In future studies, we should also test formulations of each drug individually to test its effect on the model as these antioxidants can be harmful at supraphysiological concentrations (Tao *et al.*, 2020; Ryan, Rich and Reilly, 2023), whereas intravitreal corticosteroids are shown to exacerbate RGC loss after ONC injury in rats (Steinsapir *et al.*, 2000; Huang *et al.*, 2011) and can actually be harmful in clinical use for TON (Steinsapir, 2006). This can explain our results in case of an instant burst release of drugs from the formulations *in-vivo* in the rat eyes after ONC but only as an assumption until a corresponding *in-vivo* release profile is obtained.

We have used an antibody against RNA-binding protein with multiple splicing (RBPMS) to stain the surviving RGCs after ONC instead of Brn3a antibody or anterograde neural tracers like horseradish peroxidase (HRP) or Fluoro-Gold, and in radial sections instead of retinal wholemounts, as this is now established as the gold standard method for estimating RGC survival after axotomy and stains all the RGCs in the GCL consistently and also conserves tissue by multiplying measurements on single retina while yielding similar scores to earlier methods (Mead *et al.*, 2014; Rodriguez, de Sevilla Müller and Brecha, 2014). In this study, only left optic nerves were crushed while using the contralateral right eye as intact controls as a refinement of the model and reducing animal use as bilateral intact controls in accordance with the 3R's principles of animal research, and the ARVO statement for the use of animals in

ophthalmic and vision research. This approach has been shown to elicit very minor responses in the contralateral eye and ON in the form of activation of transcription factors in the RGCs, glial activation in astrocytes, Müller cells, and microglia, and axonal regeneration due to crossing of ONs at the optic chiasma, presence of a small number of RGC axons connecting the two retinas called retino-retinal projections, and the possibility of a vascular response due to lesioning of ON microvasculature at the ONC site (Bodeutsch *et al.*, 1999).

There was consistently increased gliosis in all glial cell types in the ONC retinas treated with drug loaded formulations. $GFAP^+$ reactive astroglia in the NFL/GCL assumed cellular hypertrophy and massive GFAP upregulation was observed in the ONC groups. Reactive astrogliosis is primarily triggered by activated microglia promoting production and upregulation of certain growth factors and cytokines including transforming growth factor (TGF)-β1, interleukin (IL)-1β, ciliary neurotrophic factor (CNTF), tumour necrosis factor (TNF)-α, IL-6, etc. and by other signalling molecules including epidermal growth factor receptor (EGFR), endothelin (ET)-1, calcium, nuclear factor (NF)-κB, STAT3, toll-like receptor 2 (TLR2) , B-cell CLL/lymphoma 2 (BCL-2), etc (Battisti *et al.*, 1995; James and Butt, 2001; Prasanna *et al.*, 2002; Liu *et al.*, 2006; Yang *et al.*, 2015; Xu *et al.*, 2017; Wagner *et al.*, 2021; Wang *et al.*, 2022).

The dominant glial cells of the retina, Müller glia, also showed increased $GFAP^+$ expression in the drug loaded treatment groups characterised by hypertrophy and proliferation of the fibrous processes from the soma in the INL to the NFL. Müller cell gliosis in combination of reactive astrogliosis can exert both good and harmful effects on the survival of RGC after ONC. At the early stages of degeneration after axotomy, the gliotic response acts as neuroprotective in order to limit the neuronal damage by

sequestration of elevated potassium levels, removal of glutamate, and release of antioxidants like metallothioneins, lysozyme, the ferroxidase ceruloplasmin, and heme oxygenase, etc., and neurotrophins, growth factors, cytokines, and phagocytosis of foreign substances and cell debris (Wen *et al.*, 1995; Chen and Weber, 2002; García and Vecino, 2003; Bringmann *et al.*, 2009). However, at the same time, gliosis can lead to neurodegeneration of RGCs by downregulation of glutamine synthetase causing glutamate excitotoxicity , release of pro-inflammatory cytokines like TNF, excess production of NO causing protein nitrosylation by toxic nitrogen radicals, and prevention of axonal regeneration by formation of glial scars (De Kozak *et al.*, 1997; Bringmann *et al.*, 2006, 2009). In our results, it clearly seems to be a neurodegenerative response corelated with pronounced RGC death in agreement with the results. GFAP is an intermediate filament expressed in Müller cell endfeet and fibrous processes and in the astroglia and is the most favourable marker for an ongoing inflammatory response in an injury and is a gold standard indicator for retinal neuropathy where its expression increases faster than the RGC loss in ONC injury. RGC cell loss further signals increase in its expression as the Muller cells can sense a reduction in the neuronal activity from their processes in the NFL/GCL. The long-term expression of GFAP is modulated and signalled by neurotrophic and growth factors from the RGC and glia including fibroblast growth factor (FGF), CNTF, and TGF (Chen and Weber, 2002).

Microglia are the resident phagocytic immune cells and get activated immediately after optic nerve injury. In our study, OX-42^{+} microglia appeared in different morphologies including majority resting microglia in the intact group showing a ramified structure with elongated fibrous branches extending to NFL/GCL and INL from the somata mainly

located in IPL. The activated forms mainly found in the ONC groups showed multiple changes in morphology. There were hyper-ramified microglia with small somata mainly located in NFL/GCL and IPL and hyper-branched extensions that were indefinitely long and extending to different layers of the retina including NFL/GCL, IPL and INL. Then there were amoeboid microglia with a bigger rounded soma, and another bipolar/rod like cells with a flattened soma and very long processes from either end, both mainly localised in the NFL and GCL. Our results showed increased microgliosis in the retinas treated with drug-loaded formulations suggesting a detrimental effect in RGC survival. Activated microglia, like other glial cells after ONC injury are associated with both survival of RGCs by releasing neurotrophic factors like CNTF, and also their death by releasing neurotoxic pro-inflammatory cytokines like IL-1β, IL-6 and TNF-α upon prolonged activation, and can promote axonal regeneration in ON by Wallerian degeneration of myelin debris at the injury site after loss of oligodendrocytes, and can also exacerbate the damage to axons (Salvador-Silva, Vidal-Sanz and Villegas-Pérez, 2000; Mac Nair *et al.*, 2016; Heuss *et al.*, 2018; Au and Ma, 2022). However, it has also been observed that microglia are not as relevant in the RGC degeneration and axonal regeneration as other retinal glial cells (Hilla, Diekmann and Fischer, 2017).

It is clear that inflammatory gliosis triggered by injured RGCs in the retina after ONC can be a double-edged sword. Acute inflammation immediately after injury can be neuroprotective to RGCs, however a chronic gliosis leads to secondary degeneration resulting in increased RGC loss and also prevents axonal regeneration by gliotic scarring. Our drug-loaded treatments in the ONC eyes seem to somehow promote a chronic gliosis which is proving to be neurotoxic to RGCs. We have previously shown in our group that acute inflammation produced by intravitreal injections of zymosan

immediately after ONC and showed increases RGC survival and axonal regeneration in the ON at 10 DPI (Ahmed *et al.*, 2010).

The failure of optic nerve axons to regenerate spontaneously after injury, like any other CNS nerve, can be attributed to a number of intrinsic and extrinsic factors including inhibitory substrates in the injury environment like myelin-associated and oligodendrocyte myelin glycoproteins, gliotic scarring, lack of an intrinsic regenerative ability in RGCs, inhibition of growth-cone formation in actin cytoskeleton and microtubules, and a lack of neurotrophic factors (Schaden, Stuermer and Bähr, 1994; Berry, Carlile and Hunter, 1996; Berry *et al.*, 1999; Williams *et al.*, 2020). Our results show that vitamin-E promotes axonal regeneration distally up to 1200 µm after ONC as was evident by increased $GAP43^{+}$ axons in the distal ON segment in the ONC rats treated with MS-V and MS-VMD. However, MEL and DEX were attenuating the regenerative effects of VE, as there was no regeneration in these groups and a reduced regeneration in the MS-VMD group compared to the MS-V. Even though these results do not corelate with RGC survival, but it is possible that the RGCs that survived after ONC could regenerate their axons promoted by VE, as firstly, RGCs are a heterogeneous population and some sub-types are more resilient to injury than others, and secondly, RGC neuroprotection and axonal regeneration are supposed to follow different pathways (Chierzi *et al.*, 1999; Goldberg *et al.*, 2002; Ahmed *et al.*, 2019; Tapia, Nascimento-dos-Santos and Park, 2022). GAP43 is an exceptional marker for regenerating axons as it only stains the axons from competent RGCs because not all sprouting axons sustain the growth and only around 10% of the surviving RGCs show axonal regeneration (Schaden, Stuermer and Bähr, 1994).

4.5. Conclusion

Our microsphere formulations containing endogenous antioxidants for neuroprotection in an ONC model, could not provide a therapeutic efficacy in preventing rapid exponential RGC death early on in the injury that is characteristic of the model, and a chronic inflammatory response due to gliosis in the retina caused secondary degeneration. It is also possible that a complete burst-release of the drugs from the microspheres caused detrimental effects on RGC survival, however this is left to assumptions until we have more *in-vivo* release data and PK/PD in rats. Therefore, it is recommended that a pre-treatment with intravitreal microspheres a few weeks before ONC could show favourable neuroprotective effects in future studies. However, vitamin-E containing treatments showed increased growth of surviving RGC axons in the optic nerve possibly due to independent pathways for neuroprotection and regeneration.

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CHAPTER – 5

GENERAL DISCUSSION

5.1. Main findings and future work

Degenerative diseases of the posterior segment cause highest cases of permanent blindness globally. This thesis attempted to innovate polymer based novel drug delivery technologies to achieve a therapeutic bioavailability of neuroprotective drugs to the fundus oculi including retina and optic nerve. By means of our experimental approach we were able to investigate two different modalities of drug delivery – topical and sustained-release intravitreal. The topical formulations were able to achieve our aim of increasing bioavailability of a hydrophilic drug across lipophilic cornea significantly up to 5-7 times, however, the sustained-release formulations worked counterintuitive to our hypothesis due to the experimental design as the *in-vivo* model was probably too aggressive.

By means of a systematic review in chapter – 2, we were able to look at all the available literature on PEAs to selectively identify the best previously used PEAs that could deliver our drug across the cornea by means of simple self-administered topical eye-drop delivery which happens to be the most convenient way of drug application on the eye. We found that cationic and amphipathic CPPs like TAT and Penetratin were the most suitable not only because they showed increased permeability across barriers of the eye but also because of their safety profile and very low required concentrations to be effective.

To further exploit these CPPs in our work in chapter – 3, we synthesised a library of our own polymer based PEA – pAc which not only proved effective in increasing the permeability of DSP across cornea by several fold but was also very well tolerated. Though just crossing the cornea would not achieve a therapeutic bioavailability in the posterior segment, it certainly is a remarkable advancement on the currently available

topical formulations as also cornea is the most impermeable barrier for topical corneal delivery. Future work in this direction in advancing this topical formulation would involve first to investigate their *in-vivo* PK/PD profiles in small animals like a rat or rabbit, more preferably rabbit due to its morphological similarities with human eye, to replicate the *ex-vivo* results and also investigate the route of transport of the PEA formulations to the retina as presence of multiple barriers and fluid flows in the eye would give a correct estimate of bioavailability, and possibly replicate it with larger neuroprotective drugs like neurotrophins, si-RNA to caspases, etc. and if a therapeutic bioavailability is established, then those formulations should be studied for neuroprotective therapeutic efficacy in the ONC rat model. Our group has previously demonstrated delivery of si-RNAs against CASP2 and CASP6 using conjugation with a penetratin analogue in a rat ONC model which showed promising results by suppression of these caspases and promoting RGCs from apoptosis and also promoting axonal regeneration (Vigneswara *et al.*, 2014).

The PLGA formulations failed to provide any neuroprotection partly due the rapid exponential RGC apoptosis in the ONC model and indicating neurotoxic chronic inflammation in the retina. Therefore, the ideal next step in this direction would be to inject the microspheres in advance of the ONC injury to allow a therapeutic half-life of drugs in the retina via controlled-release. However, these microsphere formulations are still not suitable for clinical management of TON injury as it will have the same pathophysiology as in the ONC model and already has poor prognosis (Yu-Wai-Man, 2015). It would be wise be to test these formulations in a milder model of degeneration like chronic ocular hypertension models of glaucomatous neurodegeneration (Johnson and Tomarev, 2010).

5.2. Clinical translation potential of the technologies developed

Though the research involved in this thesis to develop two distinct drug delivery platforms is inherently translational in nature, it would require replicating in more robust *in-vitro, ex-vivo, and in-vivo* models to meet the clinical needs and become a market product in a competitive landscape through regulatory guidelines for successful translation (Mann *et al.*, 2018). This work would require working with cross-sectional multidisciplinary teams of academics, clinicians, and industry specialists. The unmet clinical need to tackle the ever-increasing numbers of preventable global blindness due to diseases of the posterior segment of the eye, has been identified at multiple points in this thesis (Steinsapir and Goldberg, 1994; Prokofyeva and Zrenner, 2012; Yau *et al.*, 2012; Wong *et al.*, 2014; Colijn *et al.*, 2017; World report on vision, 2019; Allison, Patel and Alabi, 2020).

5.2.1. Validation in pre-clinical models before clinical trials

Prototypes developed in this thesis for both the topical and intravitreal drug delivery platforms must be validated in relevant pre-clinical models to demonstrate their safety and efficacy before they can be tested in humans. The regulatory bodies mainly require pharmacological and toxicological testing to validate safety standards for human use; however, they do not list any specific models to be used. Therefore, the choice of these models depends on individual drugs and formulations like the active pharmaceutical ingredient (API), route of administration, and the pathophysiology of the disease corresponding to humans, and hence, standardised models have been developed for testing all these parameters.

5.2.1.1. Bench-top physicochemical testing

Non-biological bench-top models are the first line testing methods for assessment of the physico-chemical stability and release properties of the formulations. The microsphere formulations have been very robustly tested in this regard including their *in-vitro* release profiles and drug-loading efficiencies. The PEA-DSP formulations would require some additional physico-chemical characterisations in respect of the drug binding capacities of PEAs and their stabilities in different simulated physiological media of ocular microenvironment like tear fluid, aqueous, and vitreous humour.

5.2.1.2. In-vitro and ex-vivo testing

Introducing biology via *in-vitro* and *ex-vivo* models, helps understanding the real-world physiological effects of the formulations. Though, we have tested the permeability of PEA-DSP formulations in a validated *ex-vivo* model of porcine cornea and sclera, their transport across a cellular barrier can be modelled through the tight junctions in corneal and conjunctival epithelial layers in organotypic cell culture models like this model of a human corneal construct incorporating immortalised primary human corneal epithelial cells (Reichl, Bednarz and Müller-Goymann, 2004) and/or in the primary conjunctival epithelial cells from rabbits (Saha, Kim and Lee, 1996). These same cell cultures can be used to test the cytotoxicity and tolerance of the PEA-DSP formulations to test for their safety on the ocular surface, as they have been tested in the ARPE-19 cell line for intra-ocular tolerance. Similarly, both the PEA-DSP and PLGA microsphere formulations can be tested for additional safety and efficacy in healthy as well as neurodegeneration rat primary mixed retinal culture models of RGC neurones for neuroprotection in degenerative diseases like this model of laser-induced ocular hypertension (Bull *et al.*, 2011). Immunocytochemistry of neuronal cells cultured from

such models can reveal outgrowths in the dendrites and axons as a measure of neuroprotection and regeneration.

The permeability of PEA-DSP formulations has been tested in a novel, rapid and medium throughput *ex-vivo* model across cornea and sclera (Begum *et al.*, 2020; Thareja *et al.*, 2024), in pig eyes as they the most similar anatomically and physiologically to a human eye compared to other non-primate mammals (Ehlers, 1970; Faber *et al.*, 2008; Blanch *et al.*, 2012). However, the sclera was not combined with the bulbar conjunctiva layer thar usually lines the human eyes in the anterior segment next to corneal limbus. Therefore, it would benefit to test in a combined conjunctiva-sclera tissue to measure real permeability as non-corneal transport of drugs though the conjunctiva-sclera pathway contributes a major proportion of drug bioavailability to the posterior segment, and conjunctival epithelium is the main limiting barrier in this route of drug transport as the aqueous sclera offers only negligible barrier to hydrophilic small molecules and would only limit larger molecules (Ahmed and Patton, 1987; Ahmed *et al.*, 1987; Hughes *et al.*, 2005; Robinson *et al.*, 2006; Ranta *et al.*, 2010). The pre-corneal residence time of topically applied formulations is limited by tear-film stability and tear-turnover and drainage into the nasolacrimal ducts (Balla *et al.*, 2022). Even though, it was not an aim of the thesis to increase the pre-corneal residence time in our topical formulations, and these are meant to be absorbed quickly and, in enough amounts, to have a therapeutic bioavailability in the posterior segments, testing them in more complex *ex-vivo* models with a simulated tear-flow (Richman and Tang-Liu, 1990) can give us an idea of how much of the formulation is lost before corneal diffusion. Moreover, it also possible to test the permeabilities of the formulations *ex-vivo* in surplus human donor corneas in collaboration with transplant

clinics, however, they are not always available and is not possible to test multiple formulations due to restricted use of human tissue.

Furthermore, topically instilled formulations need to be tested for irritation of the mucous membranes on ocular surfaces, as any irritable compound would reduce patient compliance and also lead to increased drug loss from the surface due to increased tear production. This can be tested *in-ovo* via hen's egg test on the chorioallantoic membrane (HET-CAM), which has been shown to give equivalent irritancy scores to the controversial "gold standard" Draize rabbit eye test (Luepke and Kemper, 1986; Steiling *et al.*, 1999).

5.2.1.3. ***In-vivo* evaluation**

In-vivo evaluation of the pharmacokinetic/pharmacodynamic parameters of both the formulations is necessary to measure the real-time biodistribution of the drug in various tissues after administration as the efficacy of a formulation is entirely dependent on the real time bioavailability of the molecule at the diseased site. This also determines the effects of various dynamic barriers like tear flow, systemic clearance, and the physiological outflows in the aqueous and vitreous which is hard to mimic in any ex-vivo or in-vitro model. Furthermore, the chosen animal model has to be specific to the ocular disease to closely mimic the pathophysiology in the human eyes, which is very important to extrapolate the dosages for human from animal results. This also means that how the disease is induced in the animal model and how it presents phenotypically and its progression, has to be very similar to the humans as there can be variations in physiological responses to a treatment in a healthy vs diseased environment (Shen *et al.*, 2014). Efficacy and ocular toxicity are the most important parameters for the successful translation of a formulation to the clinic and thus require suitable animal

models. Rabbit models are very advantageous over rodent models in this regard owing to their higher similarities to the anatomy and physiology of a human eye in terms of size, intravitreal volume, internal retinal structure, and similar pathways and pharmacokinetics for topically applied drugs as well as intravitreally injected formulations (Owen *et al.*, 2007; del Amo and Urtti, 2015; Y. Zernii *et al.*, 2016; Rodrigues *et al.*, 2018).

PEA-DSP formulations can be tested for their *in-vivo* PK/PD, ocular tolerance, and efficacy in a rabbit model od VEGF induced angiogenesis and vascular leakage leading to the breakdown of blood-aqueous and blood-retinal barriers (Edelman, Lutz and Castro, 2005), which mimics oedematous vascular leakage in macular oedema secondary to diabetic retinopathy in humans also known as diabetic macular oedema (DMO). The microsphere formulations failed to show any efficacy in the ONC rat model in this thesis due to incompatibility of the slow release formulations to the very rapid and aggressive RGC neurodegeneration pathophysiology of the ONC-TON model. Therefore, they can be tested in a chronic high intraocular pressure model in rabbits leading to optic nerve degeneration as a phenotypic model of progressive secondary glaucomatous neurodegeneration in open-angle glaucoma (Shimizu *et al.*, 2024).

5.2.2. Clinical trials and commercialisation

Once all the regulatory guidelines have been met and the pre-clinical data has been validated in accordance with standardised methodologies, the formulations will be ready to be sent for clinical trials. This can be done in partnership with big-pharma or as a funded collaboration with clinical specialists. The UoB clinical trials unit can help with planning the first-in-human trials, and the various incubator facilities at the

university can help with commercialisation as a spin-out and protecting the Intellectual Property (IP) rights of the marketable end-product.

5.3. References

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