BIO-ADHESIVE POLYMERS CONTAINING LIPOSOMES FOR DED TREATMENT

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LIPOSOMAS Y POLÍMEROS BIOADHESIVOS PARA EL TRATAMIENTO DE LA ENFERMEDAD DE OJO SECO

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SUMMARY

Bio-adhesive polymers containing liposomes for DED treatment

Duygu Acar, 2019

Introduction

Dry eye disease (DED) is a prevalent and multifactorial pathology of the ocular surface affecting 5-50% of the world's population.[1] The disease is characterized by a loss of homeostasis of the tear film and it may manifest with ocular symptoms such as dryness and burning.[2,3] Tear hyperosmolarity and tear film instability constitute the core mechanisms in DED pathophysiology.[4] Recently, neutrophil extracellular traps (NETs) have also been postulated to play a role in the pathogenesis of the disease.[5,6] Artificial tears constitute the first-line therapy for mild dry eye.[7] Nonetheless, most of these ophthalmic formulations in the market supplement only the aqueous phase of the tear film, providing primarily symptomatic relief. Furthermore, artificial tears have drawbacks such as short ocular residence time.[8]

Objectives and Results

Objective-1: Development of artificial tear formulations containing novel components, with suitable physicochemical properties, optimum tolerance, and extended ocular residence time. Liposomes were utilized to replenish the lipids of the tear film and bioadhesive and/or *in situ* gelling polymers (hydroxypropyl methylcellulose and/or gellan gum, respectively) to extend the ocular residence time of the formulations. In addition, antioxidants (vitamins A, C, and E), osmoprotectants (levocarnitine and trehalose), or antidessicants (trehalose) were incorporated into the formulations for their potential beneficial effects in DED treatment. The neutral to slightly alkaline pH (7.0–7.6), surface tension (44.6–48.7 mN/m), viscosity (3.6–12.1 mPa.s), slight hypotonicity (222.7–272.7 mOsm/L), and liposome size (191.0–200.1 nm) exhibited by the formulations in the physicochemical characterization were considered suitable for topical ophthalmic administration. Formulations containing gellan gum produced viscoelastic gels with shear-thinning properties and suitable viscosities (2.8–3.0 mPa.s). *In vivo* tolerance studies (in rabbits) showed that the formulations resulted well tolerated. Objective-2: Development of novel tools to evaluate the efficacy of the artificial tear formulations. An *in vivo* dry eye model and an *ex vivo* dry eye model were developed. Dry eye was induced in rabbits with topical administration of the preservative benzalkonium chloride (BAK) at 0.1% for 15 days. Dry eye signs including corneal damage, goblet cell loss, and impairments in the conjunctival epithelium were observed in animals after the BAK treatment. Furthermore, a complete *ex vivo* porcine anterior eye model (Aston Biological Anterior Eye Model) was optimized by adjusting the irrigation frequency of the eyes with a tear

analogue. Objective-3: Efficacy studies of the novel artificial tear formulations. Preliminary studies. Preliminary efficacy studies of one of the novel formulations, composed of an *in situ* gelling polymer (gellan gum 0.25%), a bioadhesive polymer (hydroxypropyl methylcellulose 0.12%), osmoprotectants (levocarnitine 0.4% and trehalose 1.68%), an antioxidant (vitamin C 0.01%), electrolytes (sodium chloride 0.05% and potassium chloride 0.05%), and borates (boric acid 0.84% and sodium tetraborate anhydrous 0.08%), were performed in the previously mentioned *in vivo* and *ex vivo* DED models, over a period of 21 days and 18 hours, respectively. In the *in vivo* model, the treatment with the artificial tear promoted significant improvements in the cornea and the conjunctiva at 7 days after continued treatment (significantly reduced fluorescein and cytological scores compared with baseline). In the *ex vivo* model, the formulation improved the ocular surface with two instillations after 18 hours, though the small sample size did not allow statistical analysis. Objective-4: Assessment of the effects of the novel artificial tear formulations on NET formation as a proof of concept. Excessive amounts and impaired clearance of NETs have been observed on the ocular surface of DED patients and linked with the pathogenesis of the disease.[5,6] Effects of the novel artificial tear formulations on NET formation (NETosis) were assessed in neutrophils. The formulations did not increase NET formation in unstimulated neutrophils, demonstrating an optimal tolerance. Furthermore, in neutrophils stimulated with a NETosis inducing agent, all of the formulations reduced NET formation significantly.

Conclusions

1. All of the novel artificial tear formulations developed in this thesis, containing different combinations of liposomes (made up of phosphatidylcholine and cholesterol), a bioadhesive polymer (hydroxypropyl methylcellulose), an *in situ* gelling polymer (gellan gum), osmoprotectants (levocarnitine and trehalose), antioxidants (vitamins A, C, and E), and other formulation excipients (fructose, electrolytes, and borates) demonstrate suitable physicochemical properties for topical ophthalmic administration and are well tolerated in rabbits.
2. Incorporation of gellan gum at 0.25% with electrolytes created artificial tears with *in situ* gelling properties, capable of producing viscoelastic gels that suggest an extended ocular residence time of the ophthalmic formulations.
3. Twice-daily topical ophthalmic administration of the preservative benzalkonium chloride at 0.1% for 15 days induces significant alterations on the ocular surface of rabbits that are indicative of dry eye disease. The experimental dry eye model can be used as a platform to evaluate the efficacy of artificial tears.
4. According to the preliminary efficacy studies performed in the *in vivo* dry eye model, the novel artificial tear containing the *in situ* gelling polymer gellan gum (0.25%), the bioadhesive polymer hydroxypropyl methylcellulose (0.12%), the osmoprotectants levocarnitine (0.4%) and trehalose (1.68%), the antioxidant vitamin C (0.01%), borates (boric acid 0.84% and sodium tetraborate anhydrous 0.08%), and electrolytes (sodium chloride 0.05% and potassium chloride 0.05%) may be beneficial in DED treatment.

Key words: dry eye, artificial tear, *in situ* gelling, bioadhesion, nanotechnology

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RESUMEN

Polímeros bioadhesivos y liposomas para el tratamiento de la enfermedad de ojo seco

Duygu Acar, 2019

Introducción

La enfermedad de ojo seco (EOS) se considera una patología multifactorial que cursa con sequedad o quemazón de la superficie ocular con una prevalencia comprendida entre el 5 y el 50% de la población mundial.[1–3] La hiperosmolaridad y la inestabilidad de la película lagrimal constituyen los mecanismos principales de la fisiopatología del EOS.[4] Recientemente se ha postulado que las trampas extracelulares de neutrófilos (NET) están involucrados en la patogénesis de la enfermedad.[5,6] Las lágrimas artificiales se utilizan con frecuencia como primera línea de tratamiento del EOS.[7] Uno de los inconvenientes de estas formulaciones es el corto tiempo de residencia en la superficie ocular.[8]

Objetivos y Resultados

Objetivo-1: Desarrollo de formulaciones de lágrimas artificiales con componentes lipídicos y acuosos, que presenten propiedades fisicoquímicas adecuadas, tolerancia óptima y un tiempo de residencia prolonga do en la superficie ocular. Se han desarrollado nuevas formulaciones con componentes acuosos y lipídicos. Se formularon liposomas (compuestos por fosfatidilcolina y colesterol) con el objeto de reponer los lípidos de la película lacrimal. Se emplearon además polímeros bioadhesivos y/o gelificantes *in situ* (hidroxipropilmetilcelulosa (HPMC) y/o goma gelano respectivamente) para aumentar el tiempo de residencia ocular de las formulaciones. Además, se incluyeron antioxidantes (vitamina A, C y E), osmoprotectores (levocarnitina y trehalosa) o protectores frente a la desecación (trehalosa) por sus beneficios potenciales en el tratamiento del EOS. Las propiedades de las formulaciones: pH neutro o ligeramente alcalino (7,0–7,6); tensión superficial (44,6–48,7 mN/m), viscosidad (3,6–12,1 mPa.s), ligera hipotonicidad (222,7–272,7 mOsm/L) y distribución de tamaño (191,0–200,1 nm) resultaron adecuadas para su administración tópica oftálmica. Las formulaciones que contienen el polímero gelificante *in situ* goma gelano dieron lugar a geles viscoelásticos con propiedades pseudoplásticas y viscosidades adecuadas (2,8–3,0 mPa.s). Las formulaciones presentaron una tolerancia óptima *in vivo* (conejos). Objetivo-2: Desarrollo de nuevas herramientas para evaluar la eficacia de las formulaciones de lágrimas artificiales. Se han desarrollado dos modelos de EOS (*in vivo* y *ex vivo*). En el modelo *in vivo*, se instiló cloruro de benzalconio (BAK) al 0,1% durante 15 días. Al final del estudio se observaron signos de sequedad ocular (daño en la córnea, pérdida de células caliciformes y alteraciones en el epitelio conjuntival). El modelo *ex vivo* se optimizó en colaboración con investigadores de

la Universidad de Aston, mediante el ajuste de la frecuencia de irrigación de los ojos con una lágrima artificial. Objetivo-3. Evaluación de la eficacia de las nuevas formulaciones de lágrimas artificiales. Estudios preliminares. La formulación conteniendo un polímero gelificante *in situ* (goma gelano 0,25%), un polímero bioadhesivo (HPMC 0,12%), osmoprotectores (levocarnitina 0,4% y trehalosa 1,68%), un antioxidante (vitamina C 0,01%), electrolitos (cloruro de sodio 0,05% y cloruro de potasio 0,05%) y boratos (ácido bórico 0,84% y tetraborato de sodio anhidro 0,08%) se ensayó en los modelos de ojo seco *in vivo* y *ex vivo* anteriormente mencionados, durante 21 días y 18 horas, respectivamente. En el modelo *in vivo*, el tratamiento con la lágrima artificial generó una mejora significativa en la córnea y la conjuntiva tras 7 días de tratamiento. En el modelo *ex vivo*, el tratamiento mejoró la superficie ocular tras 18 horas, aunque el tamaño reducido de la muestra no permitió el análisis estadístico. Objetivo-4: Evaluación de los efectos de las nuevas formulaciones de lágrimas artificiales en la formación de NET como prueba de concepto. Los efectos de las nuevas formulaciones en la formación de NET (NETosis) se evaluaron en neutrófilos. Las lágrimas artificiales ensayadas no generaron un aumento en la formación de NET en neutrófilos no estimulados, demostrando así una tolerancia óptima. Además, en neutrófilos estimulados con un agente inductor de NETosis, todas las formulaciones redujeron, de forma significativa, la formación de NET en comparación con los neutrófilos estimulados que no recibieron ningún tratamiento.

Conclusiones

1. Las cinco formulaciones de lágrimas artificiales desarrolladas conteniendo liposomas (compuestos por fosfatidilcolina y colesterol), un polímero bioadhesivo (HPMC), un polímero gelificante *in situ* (goma gelano), agentes osmoprotectores (levocarnitina y trehalosa), agentes antioxidantes (vitaminas A, C y E) y otros excipientes como fructosa, electrolitos y boratos presentaron unas propiedades fisicoquímicas que resultan adecuadas para la administración tópica oftálmica. Además dichas formulaciones presentaron una tolerancia óptima *in vivo* (conejos).
2. La incorporación de goma gelano (0,25%) generó la formación de lágrimas artificiales con propiedades gelificantes *in situ,* capaces de producir geles viscoelásticos que sugieren un tiempo de residencia ocular prolongado.
3. La administración de BAK (0,1%) dos veces al día durante 15 días produjo alteraciones en la superficie ocular de conejos que son indicativas de EOS (daño corneal, reducción de la densidad de células caliciformes y alteraciones en el epitelio conjuntival). El modelo experimental de ojo seco desarrollado puede ser empleado como plataforma para evaluar la eficacia de lágrimas artificiales.
4. De acuerdo con los estudios preliminares de eficacia en el modelo animal de EOS, la nueva lágrima artificial conteniendo goma gelano (0,25%), HPMC (0,12%), osmoprotectores (levocarnitina 0,4% y trehalosa 1,68%), vitamina C (0,01%), boratos (ácido bórico 0,84% y tetraborato de sodio anhidro 0,08%) y electrolitos (cloruro sódico 0,05% y cloruro potásico 0,05%), podría resultar beneficiosa en el tratamiento de EOS.

Palabras clave: ojo seco, lágrimas artificiales, gelificación *in situ*, bioadhesión, nanotecnología

To my family

Science is the most reliable guide for civilization, for life, for success in the world.

- Mustafa Kemal Atatürk

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LIST OF ABBREVIATIONS

ADDE	Aqueous deficient dry eye
AF	Airflow
ARVO	Association for Research in Vision and Ophthalmology
BAK	Benzalkonium chloride
BCLA	British Contact Lens Association
Chol	Cholesterol
CIC	Conjunctival impression cytology
CL	Contact lens
CMC	Carboxymethyl cellulose
CMPH	Committee for Medicinal Products for Human Use
CR	Controlled rate
CS	Controlled stress
DED	Dry eye disease
DEWS	Dry Eye WorkShop
DLS	Dynamic light scattering
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EAOO	European Academy of Optometry and Optics
EDE	Evaporative dry eye
EDEN	European Dry Eye Network
eDNA	Extracellular DNA
EFA	Essential fatty acid
EMA	European Medicines Agency
EOS	Enfermedad de ojo seco
EU	European Union
HA	Hyaluronic acid
HPMC	Hydroxypropyl methylcellulose

L-carnitine	Levocarnitine
LHS	Life & Health Sciences
LVR	Linear viscoelastic region
MGD	Meibomian gland dysfunction
MUC	Mucin
NET	Neutrophil extracellular trap
NIBUT	Non-invasive tear break-up time
OAHFA	(O-acyl)-ω-hydroxy-fatty acid
OSD	Ocular surface disease
PAA	Polyacrylic acid
PAS	Periodic acid-Schiff
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PEG	Polyethylene glycol
PLL	Poly-L-lysine
PMA	Phorbol 12-myristate 13-acetate
PTFE	Polytetrafluoroethylene
PVA	Polyvinyl alcohol
RH	Relative humidity
ROS	Reactive oxygen species
rpm	Rate per minute
RPMI	Roswell Park Memorial Institute
SC	Subcutaneous
SD	Standard deviation
SH	Sodium hyaluronate
STF	Simulated tear fluid
TBUT	Tear break-up time
TFOS	Tear Film & Ocular Surface
TSP	Tamarind seed polysaccharide
UK	United Kingdom

USA	United States of America
Vit	Vitamin

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1. INTRODUCTION

1.1. Tear film structure and function

The lacrimal functional unit, composed of the ocular surface (cornea and conjunctiva), lacrimal glands, meibomian glands, and the connecting nerves, regulates tear homeostasis.[9]

The tear film, overlying the cornea and the conjunctiva, is a dynamic structure that plays a crucial role in maintaining a healthy ocular surface. Conventionally, it has been considered to be composed of three separate layers: the outermost lipid layer, the middle aqueous layer, and the innermost mucus layer. The more recently proposed tear film structure suggests a different architecture, with an outer lipid layer and an underlying aqueous-mucin gel with a decreasing mucin gradient from epithelium to the outer surface. The two proposed structures of the tear film are shown in Figure 1.1.

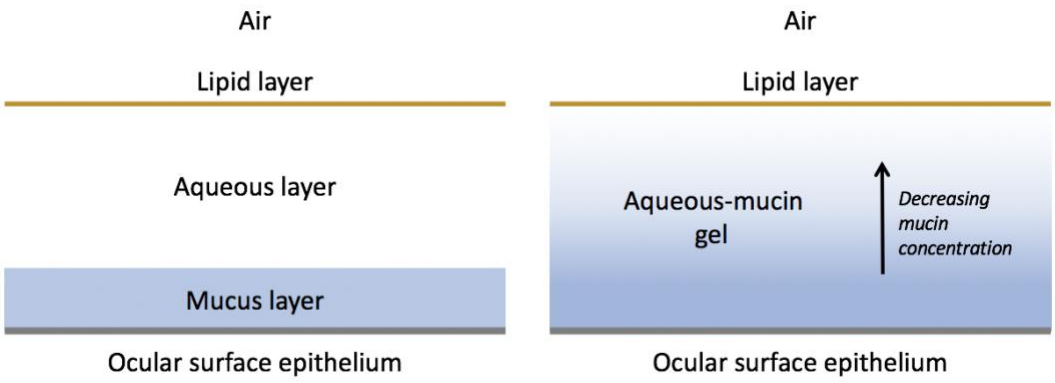

Figure 1.1. *Schematic representations of the tear film structure. Left: The classical three-layered structure. Right: The more recently proposed tear film structure, with an outer lipid layer and an underlying aqueous gel with mucin content. Adapted from Rolando et al.*[10]

Meibomian glands, located in the lid margins, secrete a lipid mixture called meibum. The meibum is spread over the ocular surface with blinking, providing the lipids of the tear film. The tear film lipid layer consists of polar and non-polar

lipids, including wax and cholesterol esters, triacylglycerol, free fatty acids, free cholesterol, and phospholipids.[11,12] Butovich et al.[12] identified also very long chain (O-acyl)-ω-hydroxy-fatty acids (OAHFAs) in human meibum. The authors suggested that OAHFAs, owing to their amphiphilic anionogenic nature, may be responsible for stabilization of the tear film lipid layer by creating an interface between the outermost non-polar lipids and the underlying aqueous phase. Figure 1.2 illustrates a proposed model of the tear film, incorporating also intercalated proteins in the lipid layer. The primary functions of the lipid layer are reducing the evaporation of the underlying phase, maintaining tear film stability and a clear optical surface, and protecting the eye from microbes and foreign bodies by constituting a barrier.[10,11]

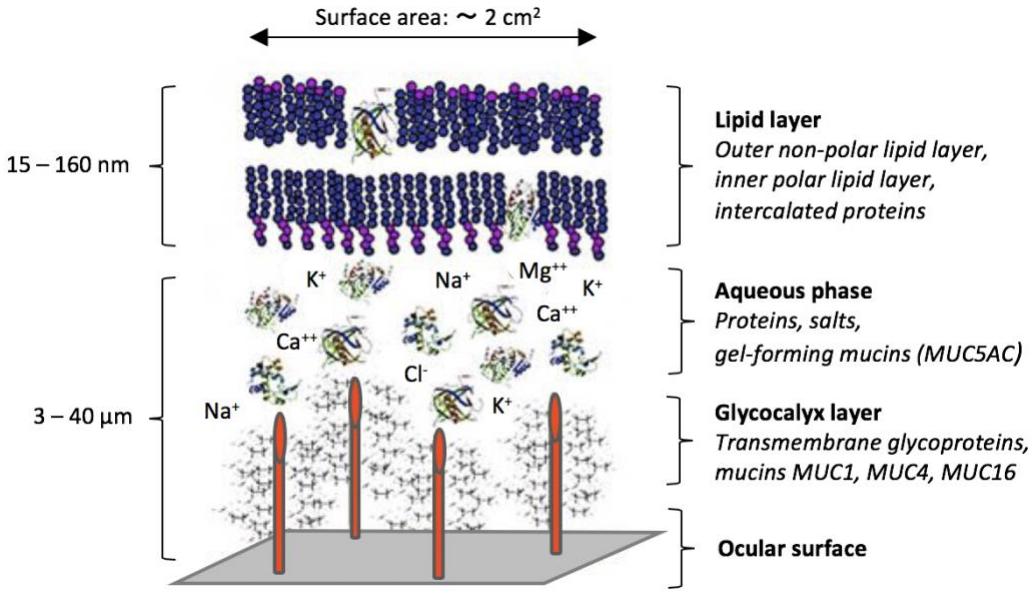

Figure 1.2. *A proposed model of the tear film. Adapted from Nichols et al.*[11]

The aqueous-mucin gel underneath the lipid layer of the tear film contains various components such as electrolytes, proteins, and mucins. The aqueous portion is secreted mainly by the lacrimal glands. Mucins lubricate the corneal and

conjunctival epithelial surfaces, stabilize the tear film by preventing the desiccation of the underlying epithelium, and provide an important barrier against pathogen penetrance.[13–15] They provide a scaffolding and hold several anti-microbial proteins such as immunoglobulins, lysozyme, transferrin, defensins, and members of the trefoil factor family at the epithelial surface.[16] Mucins contribute to rheology, having low viscosity in blinking, where high shear rates are present, and higher viscosity in the open eye, due to their shear-thinning properties.[17,18] They are of particular importance because of their ability of forming gels[19] and providing bioadhesion upon interacting with several polymers.

Mucins can be divided into two classes: membrane-associated mucins, produced by corneal and conjunctival epithelia, and secreted mucins, secreted by goblet cells in the conjunctiva.[13,14] Secreted mucins have two types: low molecular weight soluble mucins and high molecular weight gel-forming mucins.[13,16] Soluble mucins are small monomers. Gel-forming mucins, able to form oligomers and generate macromolecules with a molecular weight up to 40 MDa, are involved in rheology and gel formation.[16] Distribution of some of the membrane-associated mucins (MUC1, MUC4, MUC16) and the secreted mucin MUC5AC on the cornea and conjunctiva is illustrated in Figure 1.3.

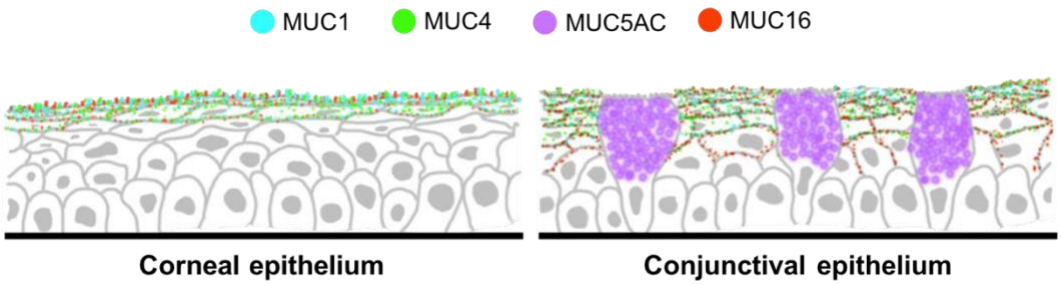

Figure 1.3. *Illustration of the corneal and conjunctival epithelium demonstrating the distribution of the mucins. Membrane-associated mucins MUC1, MUC4, and MUC16 are located within apical cell membranes, and the secreted mucin MUC5AC within mucin packets in goblet cells. Adapted from Gipson.[13]*

Tears can be classified as basal, reflex, emotional, and closed-eye tears.[9] Tear drainage occurs through the nasolacrimal drainage system, via the flow of tears through upper and lower punctum, canaliculi, lacrimal sac, and nasolacrimal duct (Figure 1.4).

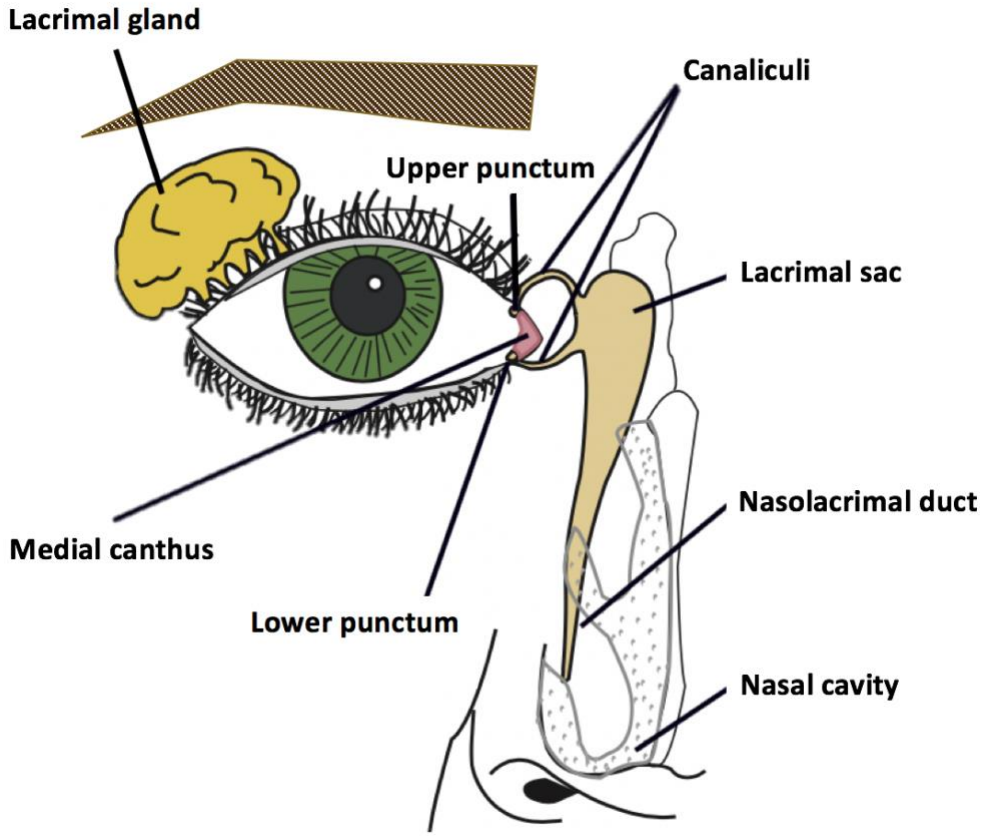

Figure 1.4. *Nasolacrimal drainage system. Adapted from Perry.*[20]

1.2. Dry Eye Disease

1.2.1. Definition and classification

Dry eye disease (DED) is a highly prevalent health problem, affecting millions of people worldwide.[21] It was defined by the National Eye Institute in 1995 as "a disorder of the tear film due to tear deficiency or excessive evaporation which causes damage to the interpalpebral ocular surface and is associated with symptoms of ocular discomfort".[22] As the understanding of the pathophysiology and mechanisms of DED advanced, the definition of the disease has evolved. The current internationally accepted definition of DED is the one produced by the Definition and Classification Subcommittee of the Tear Film & Ocular Surface (TFOS) Dry Eye WorkShop II (DEWS II) in 2017, which defines DED as "a multifactorial disease of the ocular surface characterized by a loss of homeostasis of the tear film, and accompanied by ocular symptoms, in which tear film instability and hyperosmolarity, ocular surface inflammation and damage, and neurosensory abnormalities play etiological roles".[2] The nomenclature for DED includes dry eye, dry eye syndrome, dry eye disorder, dysfunctional tear syndrome, and keratoconjunctivitis sicca, among others.

Dry eye can be divided into two main categories: aqueous deficient dry eye (ADDE) and evaporative dry eye (EDE). Aqueous deficient dry eye arises from a reduced lacrimal tear secretion and can be further categorized into Sjogren syndrome dry eye and non-Sjogren syndrome dry eye. Sjogren syndrome is an autoimmune disease affecting lacrimal and salivary glands and resulting in dry eye and dry mouth. Evaporative dry eye, on the other hand, is due to excessive tear film evaporation, which can have intrinsic or extrinsic causes that may be lid-related or ocular surface-related.[2,23] ADDE and EDE can also occur at the same time. The current classification of DED, produced by TFOS DEWS II in 2017, is illustrated in Figure 1.5.

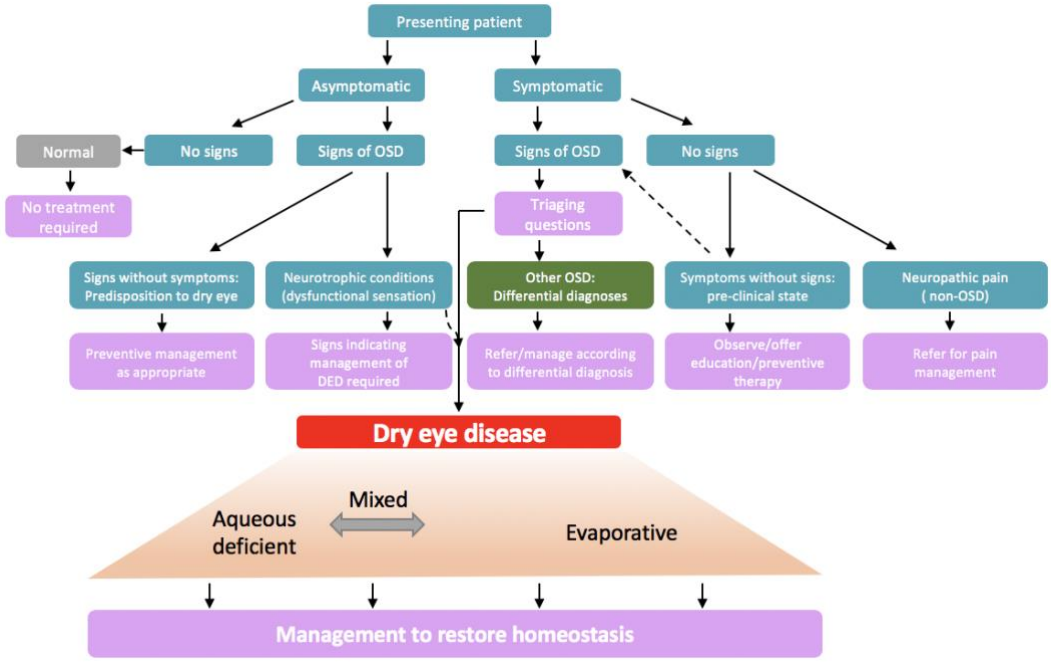

Figure 1.5. *Classification of DED according to the 2017 TFOS DEWS II Report. Adapted from Craig et al.*[2] *OSD: Ocular surface disease.*

Symptoms of DED may include dryness, burning, itching, discomfort, pain, redness, excessive tearing, gritty feeling, foreign body sensation, and eye fatigue.[3] DED is associated with a significant adverse impact on vision-related quality of life, causing difficulties in daily activities such as reading, driving, and computer use, as well as negatively affecting the patient's psychological health.[24,25] The impact of DED on quality of life has been reported to be comparable to the impact of moderate to severe angina.[26]

The lack of a universally agreed standard diagnostic criteria for dry eye complicates the prevalence studies and the identification of risk factors. Patients' symptoms evaluated subjectively via questionnaires and clinical signs assessed by diagnostic tests together may be used in the diagnosis of dry eye. Prevalence studies of dry eye present a range of 5-50%;[1] with higher occurrence in the elderly, women, and Asian people. Increased age, female sex, Asian race, meibomian gland dysfunction, Sjogren syndrome, hormone replacement therapy, contact lens

wear, computer use, environmental factors (e.g. low humidity, pollution), and the use of certain medications (e.g. antidepressants, antihistamines) are identified as risk factors for DED.[27]

1.2.2. Pathophysiology

Regardless of the type and the etiologic cause of DED, two main mechanisms play an important role in the pathophysiology of the disease: tear hyperosmolarity and tear film instability. Tear hyperosmolarity may arise as a result of reduced aqueous flow (in ADDE) or increased tear film evaporation (in EDE). Tear hyperosmolarity triggers an inflammatory cascade by activating several signaling pathways in epithelial cells that result in the release of inflammatory mediators and proteases into tears. The release of these substances causes loss of epithelial and goblet cells and damage to epithelial glycocalyx, increasing tear film instability. Furthermore, tear film instability gives rise to tear film break-up and increased evaporation, which augments tear hyperosmolarity in turn, completing what is referred to as the “Vicious Circle” of DED and damaging the ocular surface (Figure 1.6).

Tear film instability can develop secondary to tear hyperosmolarity (as a result of the events in the vicious circle) or due to several factors including meibomian gland dysfunction, topical preservative use, ocular allergy, and contact lens wear.[23] Over the years, the understanding of the complex pathogenesis of DED has advanced and many etiological causes, that can enter the circle at different points, have been identified.

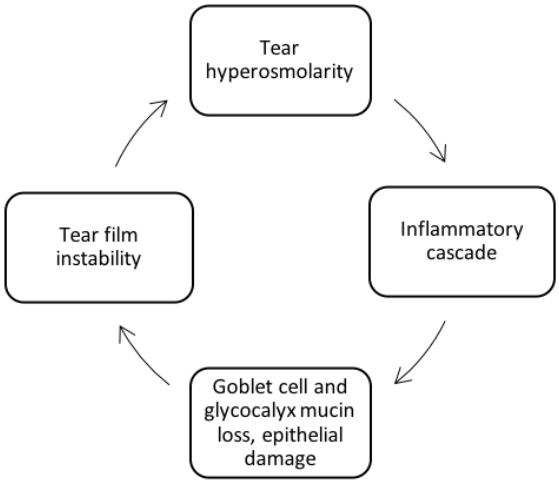

Figure 1.6. *The core mechanisms in the vicious circle of DED. Adapted from Bron et al.*[4]

Elevated levels of extracellular DNA (eDNA) and neutrophil extracellular traps (NETs) in tears have recently been explored as contributing factors to the development of ocular surface damage and pathogenesis of DED. The epithelial cells of the ocular surface, as a part of their turnover, shed into the tear film and release eDNA. Neutrophils, as part of the immune defense, release nuclear contents into the extracellular space and form NETs for defense against microbes. Tear nucleases are responsible for the clearance of eDNA and NETs in tears. However, in the tear fluid of dry eye patients, increased levels of eDNA and NETs as well as a decreased nuclease activity were identified.[5] Also, it has been shown that the formation of NETs is induced by hyperosmolarity.[28]

1.3. Management and therapy of dry eye disease

Due to the complex multifactorial nature and different severity levels of DED, several approaches have been developed for its management and therapy.

Current therapeutic approaches for DED include environmental and dietary and modifications, tear supplementation, tear conservation, tear stimulation, anti-inflammatory therapy, treatment of lid-related conditions, and surgical approaches.[29–35]

1.3.1. Environmental modifications

"Environmental" factors of a patient such as the use of medications (preservative-containing topical ophthalmic medications such as anti-glaucoma formulations, and systemic medications such as antidepressants, antihistamines, diuretics, beta-blockers, and estrogen therapy), environmental conditions (e.g. low humidity, pollution, air conditioning), contact lens wear, and digital screen use are involved in DED and have been identified as risk factors.[27] Modification of these environmental factors, or elimination when possible, is considered to be of importance in the management of DED.[36]

1.3.2. Dietary modifications

Essential fatty acids (EFAs) and antioxidants have been investigated for their potential beneficial effects in the management of DED.[37,38]

EFAs are nutrients that cannot be synthetized in human body and must be obtained from the diet. They are required for several biological processes in the body and play an important role in proper physiological functions and good health. The EFAs can be grouped as omega-3 and omega-6 fatty acids; both groups are involved in the inflammatory processes. Omega-3 fatty acids play a role in the suppression of inflammatory processes, whereas omega-6 fatty acids generate certain proinflammatory mediators.[36] Considering the role of inflammation in the pathogenesis of DED, increasing omega-3 intake, from diet and/or with oral

supplements, may be beneficial in the treatment of the disease.[34] Sources of omega-3 fatty acids include fish oil, flaxseed oil, and soybean oil, among others.

Oxidative stress, caused by an imbalance between the generation of reactive oxygen species (ROS) and the body's antioxidant defense protecting against the harmful effects of ROS, can lead to tissue damage including ocular surface damage associated with DED.[39] Therefore, dietary supplementation with antioxidants such as vitamin C, vitamin E, and alpha-lipoic acid may be beneficial in DED and are being investigated.[40]

1.3.3. Tear supplementation

Artificial tears and biological tear substitutes may be used to supplement the tear film. Artificial tears are considered the mainstay in the management of mild to moderate dry eye.[7] They are usually available as over-the-counter eye drops or sprays. Artificial tear formulations can be water-based, which typically lubricate the eye and provide symptomatic relief, and more recently they can incorporate both aqueous and lipid components to replenish all layers of the tear film. Artificial tears are discussed in detail in Section 1.4.

Autologous serum and allogeneic serum can be considered for the treatment of severe cases of dry eye. Serum is the liquid component of blood, free of blood cells and clotting factors. It is referred to as "autologous" when obtained from the patient's own blood, and "allogeneic" when obtained from a donor's blood. Recently, the use of autologous serum in dry eye has gained interest due to its advantage of containing substances found in tears that are important for the ocular surface, including vitamins, growth factors, immunoglobulins, and fibronectin, among other proteins and nutrients.[41–43] A number of studies have investigated the clinical efficacy of autologous serum in DED patients and obtained positive results, among which are improvements in symptoms, tear break-up time (TBUT), Schirmer scores, and fluorescein staining, as well as increased goblet cell

density.[44–52] Drawbacks of autologous serum include regulatory issues regarding its production, stability issues over storage, and high cost. In patients with certain health conditions such as systemic inflammation or chronic anemia, in the elderly, or when autologous serum is not available for any other reason, the use of allogeneic serum may be an option; however, a potential risk of antigenicity exists.[36,53]

1.3.4. Tear conservation

Tear retention on the ocular surface may be beneficial in dry eye, particularly in aqueous deficient dry eye. Tear conservation approaches include punctal occlusion, use of moisture chamber spectacles, and therapeutic contact lens wear.

Punctal occlusion involves blocking the lacrimal drainage by occluding either one or both of the puncta to increase tear retention on the ocular surface (Figure 1.7). Punctal occlusion can be temporary, by the insertion of absorbable punctal plugs (e.g. collagen-based); semi-permanent, by the insertion of non-absorbable plugs (e.g. silicone-based); and permanent, by surgical procedures. Collagen and silicon based punctal plugs have been shown to improve signs and symptoms of DED in clinical studies,[54–60] though the evidence is limited and further studies are needed. It is important to mention that punctal plugs may have complications and cause adverse effects, including spontaneous plug loss or plus migration, epiphora (tear overflow), eye irritation, and foreign body sensation.[61–64]

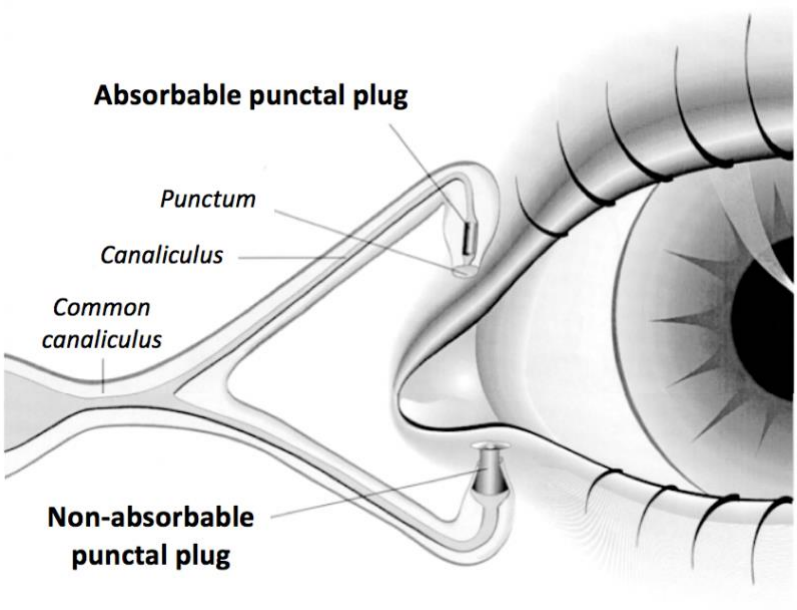

***Figure 1.7**. Punctal occlusion with absorbable and non-absorbable plugs. Adapted from Calonge.*[30]

Moisture chamber spectacles are special glasses enclosing the eyes in order to shield them against the desiccating conditions of the environment such as wind, air conditioning, and dryness. By enclosing the ocular area, they also create a humid environment. As a result, moisture chamber spectacles provide retention of tears on the ocular surface and reduce tear evaporation.[30] Therapeutic contact lenses, also referred to as bandage contact lenses, are also being explored for their potential use for tear retention.

1.3.5. Tear stimulation

Therapeutic agents called secretagogues may induce mucous and/or aqueous secretion and may be beneficial in DED. Diquafosol tetrasodium and rebamipide are examples of topically administered secretagogues. Both active substances are approved in Japan for the treatment of DED. Diquafasol tetrasodium induces aqueous and mucin secretion[65,66] and rebamipide stimulates mucin secretion.[67]

The cholinergic agonists pilocarpine and cevimeline are orally administered secretagogues available for DED associated with Sjogren syndrome.[36]

1.3.6. Anti-inflammatory therapy

As mentioned in the pathophysiology of DED, inflammation plays an important role in the pathogenesis of both aqueous deficient and evaporative dry eye and may lead to ocular surface damage. Anti-inflammatory drug substances, such as cyclosporine, corticosteroids, and tetracyclines, can be considered for the therapy and management of the disease.

Cyclosporine A, an immunosuppressant drug having anti-inflammatory effects, has gained significant interest in the treatment of DED. Topical cyclosporine A has been shown to improve dry eye signs (including tear film break-up time, Schirmer score, and corneal staining) and symptoms in DED patients and was evaluated as safe.[68–70] Cyclosporine A has been commercialized as a topical ophthalmic emulsion at the concentration of 0.05% (Restasis®; approved in 2003 by the Food and Drug Administration in the United States) and more recently as an unpreserved novel cationic nanoemulsion at the concentration of 0.1% (Ikervis®; approved in 2015 by the European Medicines Agency in Europe).

1.3.7. Treatment of lid-related conditions

Meibomian gland dysfunction (MGD) is one of the major causes of evaporative dry eye that may present with insufficient lipid production and lipid supplement to the tear film.[11] The condition results in reduced thickness and impairment of the tear film lipid layer, with a consequent tear film instability and elevated tear evaporation.[71] The approaches for the treatment of MGD associated dry eye include replenishing the tear film lipids with the use of lipid-containing artificial tears and lubricants, heat therapy for the liquefaction of thick meibomian gland

secretions clogging the glands and for enhancing the flow of lipids to the ocular surface (e.g. warm compression devices and eye masks), and physical treatments (e.g. applying force, thermal pulsation treatment such as LipiFlow, intense pulsed light therapy).[11,72,73]

Blepharitis is the inflammation of the eyelids that may arise as a result of bacterial overgrowth or mite infestation (e.g. Demodex) and can be associated with MGD.[74] Management and treatment approaches include maintaining an optimum lid hygiene (by lid wipes, scrubs, solutions), the use of topical antibiotics against bacterial overgrowth and tea tree oil against Demodex infestation, and in-office treatments.[75,76] Among in-office treatments are electromechanical lid margin debridement to remove bacteria, mites, and biofilm from the eyelids (e.g. BlephEx treatment), thermal pulsation treatment, and intense pulsed light therapy.[77]

1.3.8. Surgical approaches

In cases of severe DED not responsive to other treatment approaches, surgical procedures can be considered. These procedures include tarsorraphy (partial or total closure of eyelids with sutures), salivary gland transplantation, amniotic membrane grafts, and lid corrections for patients with eyelid abnormalities, among others.[36]

1.3.9. Choice of the treatment

The type and the severity of DED should be considered when choosing the therapeutic approach for the disease. The Management and Therapy Subcommittee of 2007 TFOS DEWS[29] reviewed the Dry Eye Preferred Practice Patterns of the American Academy of Ophthalmology and the International Task Force Delphi Panel on DED treatment and recommended different treatments based on the severity level of the disease. 2017 TFOS DEWS II Management and

Therapy Subcommittee proposed a staged management algorithm for DED (Figure 1.8) and an updated version of treatment recommendations for different steps of the disease.[36] The proposed treatment recommendations for each step, based on the staged management algorithm, are indicated in Table 1.1.[36]

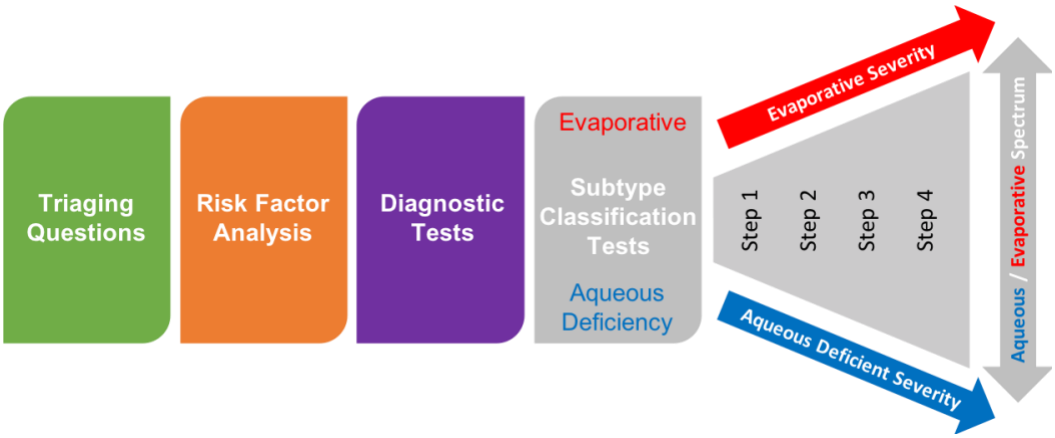

***Figure 1.8**. Staged management algorithm for DED proposed by 2017 TFOS DEWS II. Adapted from Jones et al.[36]*

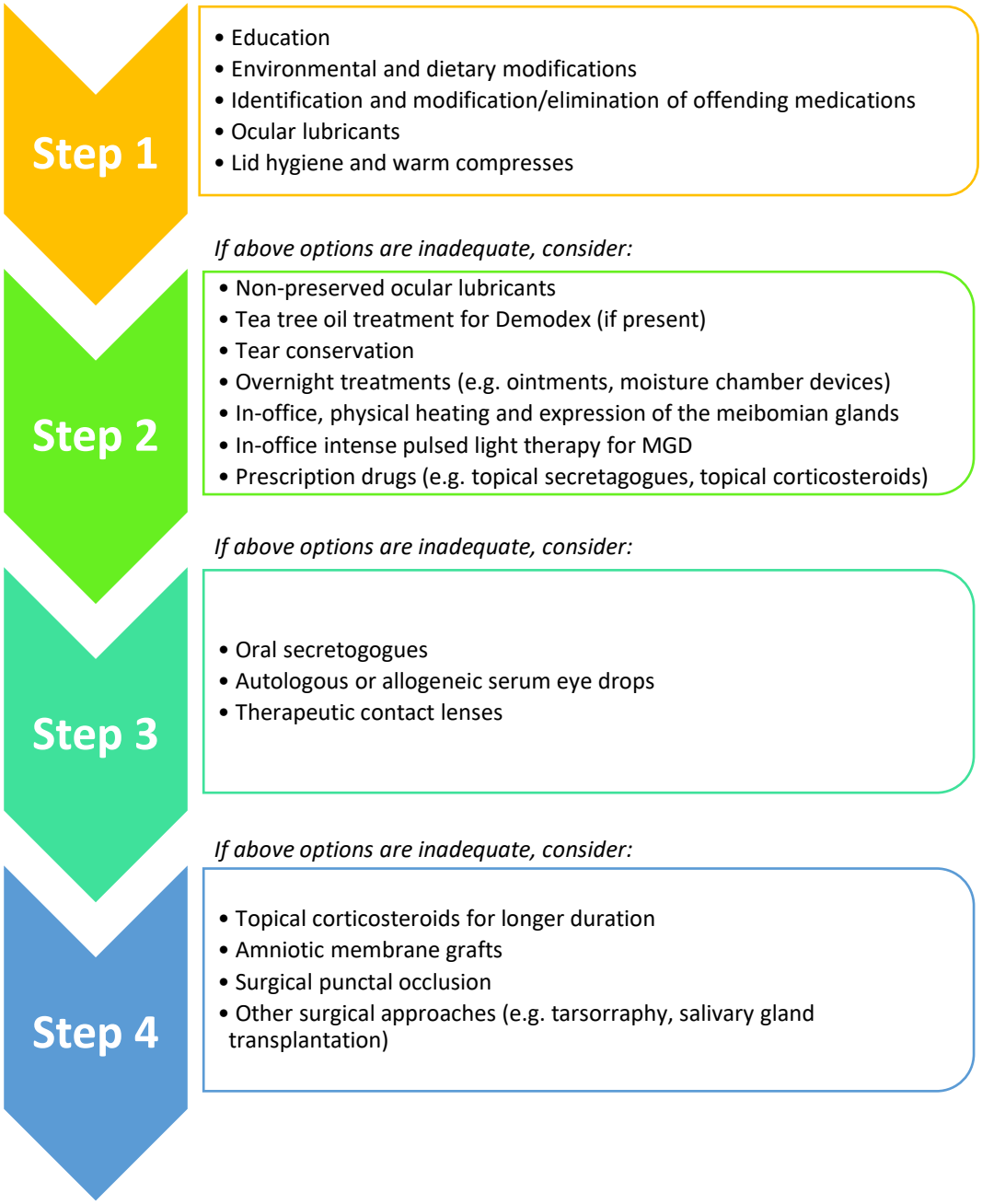

Table 1.1. *Treatment recommendations for DED proposed by the Management and Therapy Subcommittee of 2017 TFOS DEWS II. Adapted from Jones et al.*[36] *MGD: Meibomian gland dysfunction.*

1.4. Artificial tears

Artificial tears may be formulated with a broad range of components. While the conventional formulations usually employ polymers with or without electrolytes and buffering agents, novel components and strategies are being developed to enhance the therapeutic efficacy of artificial tears. The key physicochemical aspects of artificial tears, main components of the conventional formulations as well as novel components and recent approaches, are discussed below.

1.4.1. Physicochemical properties of artificial tears

1.4.1.1. pH

Tear pH plays an essential role in the normal cellular function on the ocular surface. Although a relatively broad human tear pH range, spanning from 5.2 to 8.6, was historically reported in literature,[78] more recent studies with more advanced measuring techniques have narrowed this range and reported a neutral to slightly alkaline human tear pH. Abelson et al.[79] used a direct measurement technique, employing a microcombination glass pH probe to measure the pH of the tear fluid in the inferior cul-de-sac of humans, and established a pH range of 6.5–7.6, with a mean pH value of 7.0. In another study, Yamada et al.[80] measured the pH of the tear film of humans *in vivo,* using a pH-sensitive fluorescent dye, and reported a mean pH value of 7.5.

Tear pH varies among individuals, depending on factors such as age and sex.[81] It may also vary for a given individual throughout the day. During the day, a rise in tear pH, called alkaline shift, is observed.[78,81] Nevertheless, the pH of the tear film and the ocular surface is maintained relatively stable under normal conditions. This is attributed to the well known chemical buffering capacity of tears, due to

the bicarbonate system, phosphates, ammonium, and proteins in their composition,[82,83] as well as the tear turnover.[84]

The overall buffering action of tears is of particular importance in neutralizing topical ophthalmic formulations and preventing them from causing discomfort upon instillation.[84] Ophthalmic solutions with a pH out of the range 6.6-9.0 are likely to cause ocular discomfort and damage.[84] Consequently, when formulating an artificial tear, the pH and the buffer systems to be used should receive special attention. An ideal artificial tear formulation should have neutral to slightly alkaline pH.

1.4.1.2. Surface tension

Surface tension of tears plays a very important role in the proper formation and stability of the tear film.[85] Tear components such as lipids and proteins contribute to decrease surface tension, which is reported to be in the range of 40-46 milliNewtons/meter (mN/m) in healthy state.[86,87] Tear film stability has been demonstrated to decrease as the surface tension of the tear fluid increases,[88] emphasizing the importance of maintaining the physiological range.

The surface tension of a topical ophthalmic formulation affects its interaction with the tear film and spreading ability on the ocular surface.[89] Poor spreading may cause tear film instability and ocular discomfort. It may also induce blinking and lacrimation,[90] causing formulations to wash out faster. Ophthalmic formulations with relatively low surface tension are expected to show better wetting properties. However, it was demonstrated that the instillation of an eye drop with a very low surface tension value (23 mN/m) resulted in the formation of a non-homogenous film over the ocular surface.[91] It was shown in another study that an artificial tear formulation with a surface tension value within the physiological range of tears (44 mN/m) presented better spreading ability and extended ocular residence time when compared with other two eye drops with higher surface tension values.[92]

Despite the advantages of having a surface tension value close to the values presented in tears, this is not the case for many commercialized formulations. Han et al.[89] measured the surface tension of 7 ophthalmic formulations indicated for dry eye and reported that all of them had surface tension values that are higher than the surface tension of tears. In another study, Grgurević et al.[90] determined the surface tension of 18 commercially available eye drops to evaluate their compatibility with the tear film in terms of surface tension. The authors reported that only 17% of the tested eye drops had a surface tension value within the range of 40–46 mN/m. The authors studied also the individual surface activity of some of the most commonly used ophthalmic excipients, including a number of thickening agents, solubilizing agents, and preservatives. They concluded that each of the ingredients of an ophthalmic formulation can influence its surface tension. Among the tested thickening agents, hydroxypropyl methylcellulose was the most effective in lowering the surface tension.

In light of the afore-mentioned studies, it can be concluded that in the design of artificial tear formulations, it is important to take into consideration the surface active properties of the ingredients and prepare an artificial tear with a surface tension value within or near the physiological range of natural tears.

1.4.1.3. Viscosity

Natural tears show non-Newtonian shear-thinning behavior, with low viscosity at high shear rates to prevent damage to epithelial surfaces during blinking, and a higher viscosity in the open eye, to resist drainage and tear film break-up.[17] The physiological range of the viscosity of natural tears is reported to be 1.0-8.3 millipascal seconds (mPa.s).[17,93]

It has been demonstrated that the eye drops that incorporate viscosity agents have prolonged residence time on the ocular surface.[8,94] Polymeric systems are employed in artificial tears as viscosity enhancers. Ideally, the viscosity of an artificial tear should be within or near the physiological range for those that follow

Newtonian behavior. For artificial tears with non-Newtonian behavior, on the other hand, a dynamic viscosity within or near the aforementioned range would be desired. If the viscosity of an artificial tear is very high, it may cause blurred vision and irritation when instilled.

1.4.1.4. Osmolarity

Tear hyperosmolarity occurs both in aqueous deficient and evaporative dry eye, due to low aqueous tear flow and excessive tear film evaporation, respectively. Tear hyperosmolarity activates an inflammatory cascade and causes epithelial damage, leading to tear film instability.[23]

In a multicenter study involving 10 centers and 314 subjects, the average tear osmolarity for normal subjects was found to be 300.8 milliosmoles/liter (mOsm/L), whereas subjects with mild to moderate dry eye and severe dry eye had tear osmolarity values of 315.5 mOsm/L and 336.7 mOsm/L, respectively.[95] The authors found the most sensitive threshold between healthy and mild to moderate dry eye subjects to be 308 mOsm/L. In another study, Jacobi et al.[96] obtained consistent findings with Lemp et al.[95] and suggested that a cutoff value of 308 mOsm/L would better identify the whole dry eye population, in comparison with the higher cutoff values previously described in the literature.

Considering the increased tear osmolarity in DED patients, artificial tears should ideally be slightly hypotonic or isotonic, and incorporate compatible solutes called osmoprotectants.

1.4.2. Conventional artificial tears: Main components

Conventional artificial tears are typically water-based topical ophthalmic formulations incorporating one or a combination of polymers, electrolytes,

buffering agents, and preservatives. These formulations typically supplement the aqueous layer of the tear film and provide symptomatic relief.

1.4.2.1. Polymers

Polymers are widely used in artificial tears as viscosity agents and lubricants. They can be of natural, synthetic, or semi-synthetic origin. Among the most commonly used polymers in artificial tears are hyaluronic acid, cellulose derivatives, and synthetic polymers.[97,98]

1.4.2.1.1. Hyaluronic acid

Hyaluronic acid (HA; also referred to as hyaluronan) is a widely investigated polymer that has found applications in many topical dry eye formulations in the form of sodium hyaluronate. It is a naturally occurring linear polysaccharide with repeating disaccharide units composed of D-glucuronic acid and N-acetyl-D-glucosamine. The polymer is an essential component of the extracellular matrix and found in all connective tissues (synovial fluids, vitreous humor, cartilage), where it displays a structural and rheological function, maintaining the viscoelasticity of liquid connective tissues.[99]

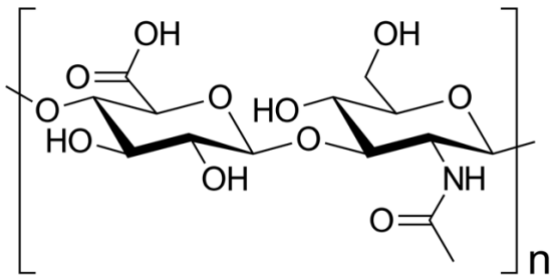

***Figure 1.9**. Chemical structure of hyaluronic acid.*

HA has several benefits which make it a desired polymer for topical dry eye formulations; it is biocompatible, biodegradable, non-toxic, and has a remarkable water-binding capacity.[100] The polymer has shear-thinning properties; its viscosity changes with changing shear conditions.[101] When the eye is open, it is highly coiled and viscous, therefore resists drainage, protects and hydrates the epithelium. During blinking, it uncoils due to lid friction and pressure and has reduced viscosity, providing smooth blinking.

1.4.2.1.2. Cellulose derivatives

Cellulose is a naturally occurring linear polysaccharide with a repeat unit of two D-glucose molecules covalently linked via β-(1→4) glycosidic bond. Cellulose derivatives carboxymethyl cellulose (CMC) and hydroxypropyl methylcellulose (HPMC) have found many applications in pharmaceutical industry including artificial tear formulations.

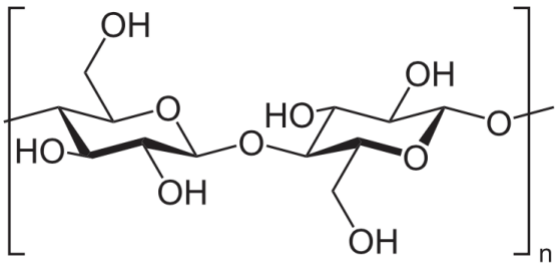

***Figure 1.10**. Chemical structure of cellulose.*

CMC, also referred to as carmellose, is a polyanionic polysaccharide usually employed in the form of sodium CMC. HPMC, also referred to as hypromellose, is a nonionic viscoelastic polymer. Both macromolecules have been evaluated as having bioadhesive properties.[102] Bioadhesion indicates the attachment of a substance to a specific biological site. When the biological site is covered by mucus

and an interaction occurs, it can be referred to as mucoadhesion.[102] Bioadhesive, or mucoadhesive polymers, interact with the mucins of the tear film and provide a prolonged residence time of the formulation on the ocular surface. HPMC was also reported to possess antioxidant properties, protecting the ocular surface.[103]

1.4.2.1.3. Synthetic polymers

Synthetic polymers pose certain advantages over the natural ones such as very high reproducibility and the possibility of tailoring to obtain a range of desired properties.[104] Among the most commonly used synthetic polymers in artificial tears are the acrylic polymer polyacrylic acid (PAA), the vinyl derivative polyvinyl alcohol (PVA), the polyol compound propylene glycol, and the polyether compound polyethylene glycol (PEG). Table 1.2 lists some of the most commonly used polymers in artificial tears.

Origin	Polymer	Group
Natural	HA	Polysaccharide
Semisynthetic	HPMC	Cellulose derivative (polysaccharide derivative)
	CMC	Cellulose derivative (polysaccharide derivative)
Synthetic	PAA	Acrylic polymer
	PVA	Vinyl derivative
	Propylene glycol	Polyol compound
	PEG	Polyether compound

Table 1.2. *Commonly used polymers in artificial tears. HA: hyaluronic acid, HPMC: hydroxypropyl methylcellulose, CMC: carboxymethyl cellulose, PAA: polyacrylic acid, PVA: polyvinyl alcohol, PEG: polyethylene glycol.*

1.4.2.2. Electrolytes

Tear film contains several electrolytes including sodium, potassium, calcium, magnesium, chloride, bicarbonate, and phosphate.[9] The electrolytes contribute to the osmolarity of the tear film and are involved in maintaining the homeostasis of the ocular surface. Some of the commercially available artificial tears (e.g. Artelac Rebalance, Bion Tears, Murine Tears, TheraTears) offer electrolytes in their formulations. The electrolyte salts incorporated into such formulations include sodium chloride, calcium chloride, magnesium chloride, potassium chloride, zinc chloride, sodium bicarbonate, and sodium phosphate, among others.

1.4.2.3. Buffering agents

Several buffer systems are employed in artificial tears as excipients to adjust and maintain the pH, with the aim of preventing irritation upon topical ophthalmic administration. Such buffering agents include boric acid, acetic acid, borates, phosphates, citrate, and sodium bicarbonate, among others.[105]

In 2012, upon encountering cases of corneal calcification in patients using phosphate-containing eye drops, the European Medicines Agency (EMA) evaluated the utilization of phosphate as buffer in medicinal eye drops.[106] The Committee for Medicinal Products for Human Use (CMPH) of the EMA stated that corneal calcification may arise from the use of phosphate-containing eye drops in patients with significant corneal damage in very rare cases, identifing the risk as very low. The Committee suggested that it should be included among the possible side effects in the product information.

1.4.2.4. Preservatives

Preservatives have been employed in artificial tear formulations in order to prevent microbial growth in multi-dose containers. Quaternary ammoniums (e.g. benzalkonium chloride, cetrimide), alcohols (e.g. chlorobutanol), mercurial

derivatives (e.g. thiomersal), amidines (e.g. polyhexamethylene biguanide), and parabens (e.g. methylparaben) are among the traditionally used preservatives in topical ophthalmic formulations.

Frequent and long-term exposure to preservatives can result in toxicity and damage to ocular surface. The most commonly used preservative in artificial tears is the quaternary ammonium compound benzalkonium chloride (BAK), which has long been associated with tear film instability and ocular cell damage. *In vitro* studies have shown that BAK can be cytotoxic for corneal and conjunctival epithelial cells at concentrations used in artificial tears (0.01%–0.02%).[107,108]

While the preservative-free unit dose artificial tears remain an alternative to the preserved multi-dose artificial tears, they generally have a higher cost and they may not be available for every brand. Consequently, several strategies have been developed to overcome the toxicity issue due to the use of preservatives in multi-dose artificial tears. These strategies include the use of newer preservatives with reduced harmful effects on the ocular surface (e.g. Polyquad®), the use of "disappearing" preservatives (also referred to as "vanishing" or "soft" preservatives), the use of ionic buffers as preservative (e.g. SofZia®; contains borate, sorbitol, propylene glycol, and zinc), and the application of special antimicrobial dispenser systems in product packaging.

The disappearing preservatives are converted to non-toxic compounds upon administration. The disappearing preservatives include sodium perborate (GenAqua®), stabilized oxychloro complex (Purite®, OcuPure®), and stabilized chlorite peroxide (Oxyd®). The disappearing preservatives and their mode of disappearance are collected in Table 1.3.

Preservative	Commercial name(s)	Mode of disappearance
Sodium perborate	GenAqua®	Converted to water, oxygen, and hydrogen peroxide upon contact with the tear film
Stabilized oxychloro complex	Purite®, OcuPure®	Degraded to sodium and chloride ions, water, and oxygen upon exposure to light
Stabilized chlorite peroxide	Oxyd®	Dissipates to water, oxygen and sodium chloride on the ocular surface

Table 1.3. *Disappearing preservatives used in artificial tears.*

Another alternative for non-preserved formulations in multi-dose containers is the use of special antimicrobial systems in packaging, such as the ABAK® system and the COMOD® system. The ABAK® system has a dispenser with an antibacterial filter, while the COMOD® system is an airless system that eliminates the contact of bacteria with the solution inside the bottle.

1.4.3. Novel artificial tears: Novel components, technologies, and approaches

1.4.3.1. Combination of lipid and aqueous components

As previously described, tear film is composed of a lipid layer and an underlying aqueous-mucin gel (Section 1.1). The thickness of the lipid layer, as well as the quality of lipids, is very important in maintaining optimal properties of the lipophilic structure, which in turn reduces the evaporation of the underlying aqueous phase and plays a very crucial role in maintaining the tear film stability. In dry eye, particularly in evaporative dry eye associated with MGD, the lipid layer of the tear film is compromised, resulting in increased tear evaporation leading to

hyperosmolarity and tear film instability. Therefore, it is of great importance to replenish both the aqueous-mucin and lipid layers of the tear film. However, Conventional water-based artificial tears replace only the aqueous layer of the tear film and not the lipid layer. Lipid-only ocular lubricants for dry eye are available in the form of ointments or gels; however, due to the fact that they are very viscous and may cause blurred vision, they have found application mainly as night time dry eye products.

Over the past years, combination of lipid and aqueous components with the aim of obtaining artificial tear formulations that more closely resemble natural tears and replenish both the lipid and the aqueous-mucin layer of the tear film has gained a lot of interest. A number of lipid-containing artificial tears are commercially available and such formulations are increasing in number. The majority of lipid-containing artificial tears are formulated in the form of emulsions, incorporating lipids such as mineral oil, castor oil, triglycerides, and phospholipids.[109,110] Recently, lipid sprays have also gained interest.[111–113] Several clinical studies evaluated the effects of lipid-containing artificial tears. Among the observed outcomes were increased lipid layer thickness, enhanced tear film stability, improved lipid layer structure, reduced tear evaporation, improvement in dry eye symptoms, and improvement in ocular surface healing and meibomian gland functionality.[114–123]

1.4.3.2. Antioxidants

An imbalance between the production and elimination of ROS can lead to elevated ROS levels and oxidative stress, which may have damaging effects.[39] In dry eye patients, elevated levels of ROS in the tear fluid, increased expression and activity of ROS generating enzymes, and reduced levels of protective antioxidant enzymes on the ocular surface were observed.[124–126] Such findings indicating the involvement of oxidative stress in dry eye have led to the consideration of employing components with antioxidant properties to target the oxidative

mechanisms on the ocular surface for the management and therapy of DED.[127–129] Vitamin A, vitamin C, and vitamin E are among the substances that possess antioxidant properties. Other components such as acetylcysteine, selenoprotein P, quercetin, gallic acid, epigallocatechin gallate, and n-propyl gallate are also being studied for their antioxidant properties and potential use in dry eye formulations.[36,130]

Among the antioxidants that can be considered for topical ophthalmic dry eye formulations, vitamin A is of particular importance for the ocular surface. It can exist in several forms such as retinol, retinal, and retinoic acid, and is present in human tear fluid as retinol.[131,132] On the ocular surface, vitamin A is involved in the correct differentiation of epithelial cells, re-epithelization of the cornea, and corneal wound healing.[133] It plays an important role in reducing conjunctival keratinization, reversing squamous metaplasia, and increasing goblet cell number.[133] Vitamin A containing ophthalmic formulations were evaluated in an experimental dry eye model[134] as well as in DED patients, and were found to be effective in the treatment of the disease.[135–137] Tseng et al.[137] evaluated the clinical efficacy of 0.01% and 0.1% (weight/weight) topical all-trans retinoic acid ointment in treating DED and reported that all patients in the study showed improvements in symptoms, visual acuity, rose bengal staining, or Schirmer test after the use of the ointment. The authors stated that the topical vitamin A treatment resulted in the reversal of squamous metaplasia as evidenced by impression cytology. In another clinical study, treatment of DED patients with vitamin A eye drops (0.05% retinyl palmitate) resulted in a significant improvement in blurred vision, tear film break-up time, Schirmer scores, and findings of impression cytology.[136]

1.4.3.3. Osmoprotectants

Hyperosmotic stress is involved in inflammation and the pathogenesis of dry eye disease. The role of several substances such as levocarnitine (L-carnitine), betaine, and erythritol as osmoprotectants has been demonstrated in several *in vitro*

studies,[138–140] as well as in an experimental dry eye.[141] While the *in vitro* studies reported suppressive effects of the three osmoprotectants on the expression or activation of proinflammatory mediators, the experimental dry eye study demonstrated also that L-carnitine restored the loss of goblet cells caused by dry eye conditions. In a study published in 2011, Evangelista et al.[142] compared three eye drops with different osmolarities (180 mOsm/L, 300 mOsm/L, 328 mOsm/L) and compositions, and found the low-osmolarity eye drop (180 mOsm/L) containing 1% L-carnitine to be the most effective in improving the quality and stability of the tear film in moderate dry eye patients.

Another osmoprotectant substance is trehalose, a natural disaccharide that plays a key role in anhydrobiosis (capability of certain organisms of surviving complete dehydration).[143] It has been demonstrated that trehalose is able to protect cells from desiccation and protect cell membranes and proteins from deactivation or denaturation.[144,145] This unique characteristic of trehalose makes it attractive for DED treatment. In fact, a trehalose-based ophthalmic solution, when compared with six other commercially available eye drops indicated for DED, showed the highest efficiency in preventing cell death during desiccation in cultured human corneal epithelial cells, as well as in preserving cellular functionality.[146] In an initial clinical study, Matsuo et al.[147] found 100 and 200 mM trehalose eye drops to be safe and effective in the treatment of moderate-to-severe DED. In another study, a 100 mM trehalose eye drop was compared with two commercially available eye drops, containing hyaluronan or cellulose, and was reported to be the most effective in the treatment of moderate to severe DED.[148]

1.4.3.4. Novel components and technologies

1.4.3.4.1. Liposomes

A novel approach for formulating lipid-based ophthalmic preparations for DED treatment is the use of liposomes. Liposomes are spherical vesicles comprised of one or more lipid bilayers surrounding an aqueous core (Figure 1.11).[149] They have

been widely investigated as a drug delivery system and employed for the delivery of various therapeutic agents.[150–154] Lipophilic drugs are mostly entrapped in the lipid bilayer of the liposomes, whereas hydrophilic drugs are entrapped within the aqueous core.

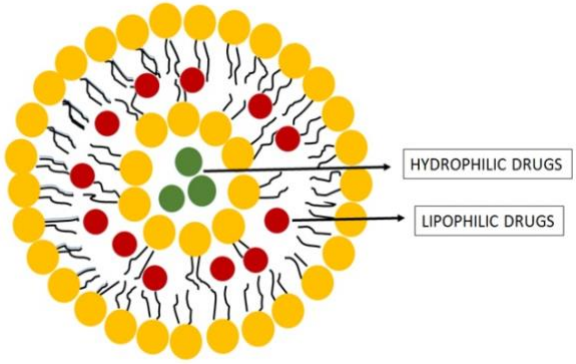

Figure 1.11. *Liposome structure.*

Liposomes are usually composed of phospholipids and cholesterol (both components are present in the tear film). In the case of artificial tears, liposomes prepared with the last mentioned components may be beneficial in replenishing the tear film lipids and in restoring the tear film lipid layer. Indeed, phosphatidylcholine was shown to increase tear film stability in a model eye.[155] Another advantage of the use of liposomes in artificial tears is that they can carry lipophilic and hydrophilic substances that may be beneficial in the treatment of DED. Currently, a small number of liposomal formulations for DED treatment are available in the market in the form of sprays. These sprays are applied onto the closed eyelid and are thought to move across the eyelid margins and reach the tear film upon blinking. There is a limited number of published studies on the liposomal sprays for DED treatment (Table 1.4).

Authors	Year	Treatment groups
Dieter et al.[111]	2006	- Liposomal spray Tears Again (Optrex ActiMist) - Triglyceride-containing gel Liposic
Craig et al.[112]	2010	- Liposomal spray Tears Again (Optrex ActiMist) - Control: Saline spray
Pult et al.[113]	2012	- Liposomal spray Optrex ActiMist - Liposomal spray DryEyesMist - Liposomal spray TearMist

Table 1.4. *Published studies on the liposomal sprays for DED treatment.*

The liposomal spray Tears Again (Optrex ActiMist) showed statistically significant clinical advantages over a triglyceride-containing gel (Liposic) in a randomized, controlled, cross-over study involving 74 patients suffering DED caused by a deficiency of the tear film lipid layer.[111] The same liposomal spray was shown to significantly increase the thickness of the tear film lipid layer in normal eyes for up to 60 minutes after the application.[112] More recently, Pult et al.[113] compared this liposomal spray (Optrex ActiMist; Tears Again) with two other commercial liposomal sprays (Table 1.5) and found that only the previously mentioned spray (Optrex ActiMist), among the three, improved ocular comfort and the tear film stability significantly (10 minutes after the application).

	Optrex ActiMist	DryEyesMist	TearMist
Other brand names	Tears Again, Liponit	Hylite	Eyerain
Ingredients	Soy lecithin, sodium chloride, ethanol, phenoxyethanol, vitamin A palmitate, vitamin E	Liposomate isoflavonoids, sodium hyaluronate, camomile extract, N-hydroxymethylglycinate, isotonic buffer solution	Liposomate isoflavonoids, sodium hyaluronate, N-hydroxymethylglycinate

Table 1.5. *Compared liposomal sprays for DED treatment. Adapted from Pult et al.[113]*

1.4.3.4.2 *In situ* gelling systems

In situ gelling systems are polymeric solutions that are able to form a gel when in contact with various body fluids. This phenomenon is known as sol-to-gel transition and occurs as a result of changes in parameters such as pH, temperature, and ions. *In situ* gelling systems can be used as drug delivery systems to extend the contact time of the formulation at the site of administration and provide a sustained release of the drug component. They have been employed in ocular, oral, rectal, and vaginal delivery, and as injectable systems to a specific site (e.g. tumor site).[156] Among the investigated polymers as *in situ* gelling agents are chitosan, alginates, gellan gum, xyloglucans (e.g. tamarind seed polysaccharide; TSP), Carbopol, pluronics, and cellulose derivatives.[157]

On the ocular surface, *in situ* gelling systems undergo gelation upon contact with the tear fluid and resist nasolacrimal drainage. Consequently, they extend the ocular residence time of topical ophthalmic formulations and increase bioavailability.[158] Gellan gum, chitosan, alginate, TSP, and pluronics are among the polymers that have been investigated for use in topical ophthalmic formulations as *in situ* gelling agents.

Gellan gum has been employed in a number of applications in the pharmaceutical industry.[159,160] It has been incorporated into ophthalmic formulations to provide extended release and increased bioavailability of anti-glaucoma and anti-inflammatory drugs.[160] Gellan gum is a high molecular weight microbial polysaccharide. The polymer exists as a random coil in solution at high temperature and undergoes a conformational change to adopt a double helical structure with decreasing temperature. Subsequent aggregation of double helices leads to a three-dimensional network formation (Figure 1.12), producing a gel.[161,162] The random coil-double helix transition temperatures of 1% high-acyl and 1% low-acyl gellan gum solutions were found to be 76°C and 25°C, respectively.[162] Coil to double helix transition and the subsequent aggregation of the helices are influenced by the presence and type of cations, including the

cations of the tear film.[163,164] The biocompatibility and unique gelling properties make gellan gum an interesting polymer for topical ophthalmic formulations, though it has not yet been employed in commercially available artificial tear formulations.

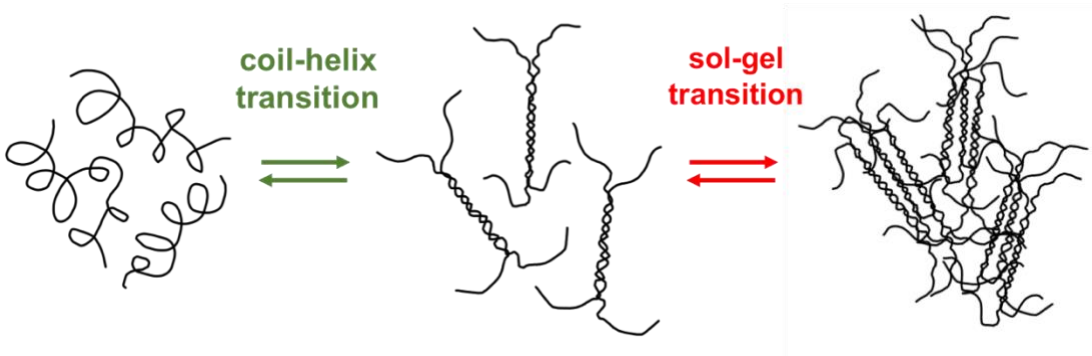

Figure 1.12. *Coil-helix and sol-gel transitions of gellan gum. Adapted from Miyoshi et al.*[164]

Polymers such as chitosan, alginate, TSP, and pluronics, have also been investigated for their gelling properties and for potential use as ocular *in situ* gelling systems. A number of studies showed that these polymers, used alone or in combination as *in situ* gelling systems in topical ophthalmic formulations, provided sustained release of the drug substances they were formulated with.[165–168] Table 1.6 summarizes some of these studies. Among these polymers, TSP, a natural polysaccharide obtained from the seeds of Tamarind (Tamarindus indica) tree, has been employed in an artificial tear formulation (HydraMed Lubricating Eye Drops) at a concentration of 0.2% in combination with sodium hyaluronate 0.2%.

Gelling agent	*In situ* sol-to-gel transition trigger	Active ingredient	Reference
TSP (xyloglucan with a galactose removal of 44%) 1.0%, 1.5%, and 2.0%	• temperature: 22–27°C	Pilocarpine hydrochloride	Miyazaki et al.[165] (2001)
Chitosan 0.25% Pluronic F-127 9%	• pH: 6.9–7.0 • temperature: 35–37°C	Timolol maleate	Gupta et al.[166] (2007)
Chitosan 0.25% Gellan gum 0.5%	• pH: 6.5-7.0 • ion	Timolol maleate	Gupta et al.[167] (2010)
Sodium alginate 0.8% TSP 0.8%	• ion	Ketorolac tromethamine	Gupta et al.[168] (2014)

Table 1.6. *In situ gelling systems used in topical ophthalmic formulations. TSP: tamarind seed polysaccharide.*

1.4.3.4.3. Cationorm®: A novel cationic nanoemulsion

Cationorm® is an unpreserved cationic oil-in-water nanoemulsion (Figure 1.13) indicated for dry eye. It contains light and heavy mineral oil, cetalkonium chloride (cationic agent), tyloxapol and poloxamer 188 (non-ionic surfactants), glycerol (osmotic agent), tromethamine and tris hydrochloride (buffer), and purified water. The emulsion is obtained using the cationic nanoemulsion technology Novasorb® developed by Novagali Pharma (Santen SAS).[169]

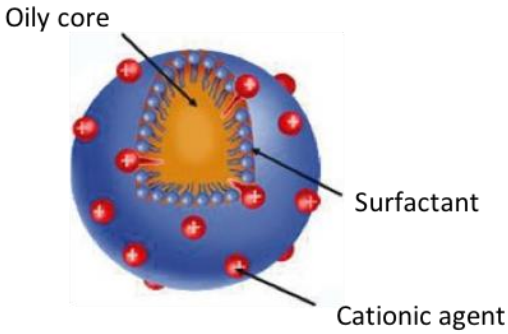

Figure 1.13. *A lipid nanodroplet of the cationic oil-in-water emulsion. Adapted from Daull et al.*[170]

Electrostatic interaction between the positively charged nanodroplets of the emulsion and the negatively charged ocular surface leads to good spreading of the formulation over the ocular surface[169] (Figure 1.14) and extends the ocular residence time of the formulation. The product is claimed to supplement all the layers of the tear film: the lipid content of the emulsion replenishes the lipid layer and reduces tear evaporation, whereas its osmotic agent is protective of the aqueous layer.

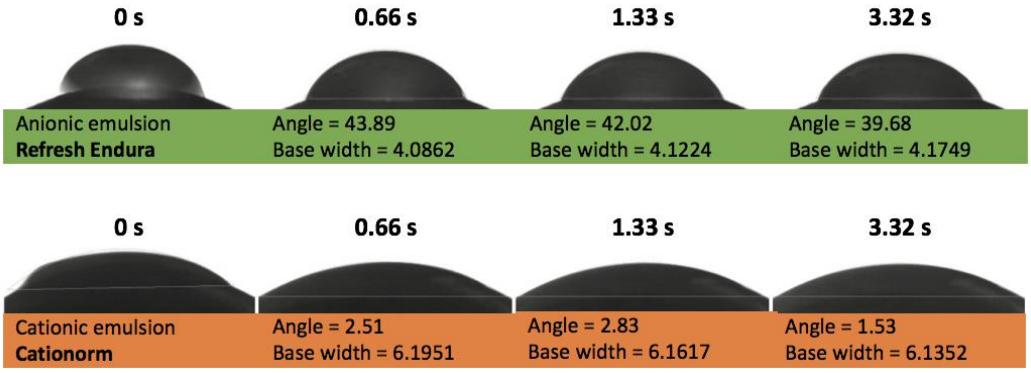

Figure 1.14. *Comparison of the dynamic contact angle and the base width of the cationic emulsion Cationorm with an anionic emulsion (Refresh Endura) instilled on rabbit eyes. Adapted from Lallemand et al.*[169]

The interactions of Cationorm® with human meibium and the tear film lipid layer were investigated *in vitro* and *in vivo*.[171] *In vitro* studies were performed by forming pseudo-binary films of the nanoemulsion with the meibium collected from the lid margins of healthy volunteers, through Langmuir surface balance experiments, and showed that the nanoemulsion enhanced the thickness and elasticity of the meibium films. *In vivo* interactions of the formulation with the tear film lipid layer, observed by specular microscopy in a case study, resulted in increased thickness of the tear film lipid layer and supported the *in vitro* findings. Clinical studies demonstrated that the treatment with Cationorm® resulted in the improvement of dry eye signs and symptoms, including improved TBUT, lissamine green staining and corneal fluorescein staining results.[172–174] The formulation showed good tolerability and safety in *in vitro* and clinical studies.[173–175] Cationic nanoemulsions of cetalkonium chloride showed also anti-inflammatory properties *in vitro* in stimulated human cells and in a rat model of corneal scraping.[176,177]

The Novasorb® technology has also been used to develop cationic nanodroplets as a drug vehicle for both dry eye and non-dry eye ophthalmic formulations. A list of the products developed with the Novasorb® technology[169,178] can be seen in Table 1.7.

Product	Active ingredient	Indication
Cationorm	–	Dry eye
Ikervis	Cyclosporin A	Severe dry eye
Vekacia	Cyclosporin A	Vernal keratoconjunctivitis
Catioprost	Latanoprost	Glaucoma associated with ocular surface disease

Table 1.7. *Products developed with the Novasorb® technology.*

1.4.3.4.4. PLL-g-PEG: A novel polymer system

Poly(L-lysine)–graft–poly(ethylene glycol) (PLL-g-PEG) is a water-soluble, polycationic copolymer with a polypeptide backbone and PEG side chains. The copolymer has been shown to attach to negatively charged surfaces through electrostatic interaction due to the multiple positive charges on the PLL backbone.[179,180] *In vitro* studies demonstrated that PLL-g-PEG reduces the frictional forces and enhance lubrication in aqueous environments.[181,182]

This graft copolymer has been incorporated into an artificial tear formulation containing the demulcent glycerin (1%). A significant increase in tear film break-up time in dry eye patients, compared with a commercially available propylene glycol and PEG containing artificial tear, was observed.[183] The authors of the study attributed this effect to the interactions between the negatively charged epithelial cells and membrane-bound mucins of the ocular surface, the copolymer, and glycerin, resulting in extended ocular residence time and improved tear film stability. The formulation was evaluated as safe.

1.4.3.4.5. Manuka honey

Honey has been known to possess antimicrobial, anti-inflammatory and wound healing properties due to its acidic pH, low water content, high sugar content, and the glucose degradation product hydrogen peroxide.[184–186] It has found medicinal applications, especially in wound healing. Manuka (Leptospermum species) honey, produced by the honey bees from the Manuka bush native to Australia and New Zealand, has recently gained interest for its stronger antimicrobial properties, attributed mainly to its high content of the antibacterial component methylglyoxal.[187,188] Manuka honey has been investigated for ocular surface conditions such as blepharitis and post-operative corneal edema.[76,189–191]

In a prospective pilot study, Albietz et al.[192] investigated the ocular flora of DED patients and the effect of an antibacterial honey (Medihoney™ Antibacterial

Honey; containing a mix of honeys including Manuka honey) on the flora. The authors found a significant difference in the count of bacterial colony forming units isolated from eyelid margins and conjunctiva of dry eye and non-dry eye groups. After a three times daily topical administration of the antimicrobial honey for a period of 3 months, a significant decrease in the bacterial count was observed in DED patients. The authors stated that the antibacterial properties of the honey could provide potential benefits in DED patients.

There are currently two Manuka honey containing topical products with regulatory approval in Australia, New Zealand, and Europe, indicated for use in dry eye: an eye drop and a gel preparation. The eye drop preparation (Optimel Antibacterial Manuka+ Eye Drops) contains 16% Manuka honey in a saline solution with the preservative benzoic acid, whereas the gel preparation (Optimel Antibacterial Manuka+ Eye Gel) has a Manuka honey concentration of 98% and is unpreserved. The effects and safety of the 16% Manuka honey eye drop were evaluated in patients with contact lens (CL) related dry eye.[193] A significant improvement in dry eye symptoms in patients with CL related dry eye was observed and the product was evaluated as safe in CL wearers. In another study, the efficacy of the Manuka honey containing eye drop and the gel preparation was assessed in patients with evaporative dry eye due to MGD, as an adjunctive therapy to conventional treatments (warm compresses, lid massage and preservative-free lubricant).[194] Both the 16% Manuka honey eye drop and the 98% Manuka honey gel treatments resulted in a significant increase in meibum quality and gland expressibility, while significantly reducing lid margin isolated Staphylococcus spp. count and the need for the lubricant use in DED patients after an 8-week treatment, compared with the conventional therapy-only control group. The 16% drops also reduced the epithelial damage on the ocular surface. However, transient redness and stinging were reported as adverse effects in both studies.

1.5. Animal models of dry eye as a tool to assess the efficacy of artificial tears

Animal models of dry eye are valuable tools for studying the pathophysiologic mechanisms of the disease and identifying disease biomarkers, as well as for the evaluation and comparison of the therapeutic efficacy of dry eye treatments. Though animal models do not always correlate well with the clinical setting, preclinical studies are an important step that may give insights regarding the safety and efficacy of therapeutic agents before clinical studies.

Several aspects of the ocular surface and the tear film, such as ocular surface damage, tear production, tear osmolarity, and tear film stability, have been evaluated in animal models of dry eye.[195] Examples of the tests that may be performed for such evaluations are listed in Table 1.8. Dogs, rabbits, mice, rats, pigs, and monkeys are among the animals that have been used in dry eye research.[196–198]

Aspect to be evaluated	Tests
Ocular surface damage	○ Ocular surface staining: - Fluorescein staining - Lissamine green staining - Rose bengal staining ○ Impression cytology
Tear production	○ Schirmer test ○ Cotton thread test
Tear osmolarity	○ Osmolarity measurement with an osmometer
Tear film stability	○ Tear break-up time

Table 1.8. *Examples of the tests for the evaluation of the ocular surface and the tear film in animal models of dry eye.*

Several *in vivo* models of dry eye have been developed, using different types of animals and approaches to induce the disease, depending on the focus of the investigation. *In vivo* models developed for studying the pathophysiologic mechanisms and causative factors of dry eye may employ genetic alterations of animals or other strategies such as closure of meibomian orifices by cauterization and surgical approaches (e.g. removal of lacrimal glands).[199] Some of the most commonly employed methods to induce dry eye for efficacy assessment of DED treatments are administration of pharmaceutical agents, exposure to desiccating environmental stress, and mechanical approaches.

Lacrimal gland injection of botulinum toxin B[200] and topical ophthalmic administration of the antimuscarinic agent atropine[201] have been employed to induce dry eye in animals. Another antimuscarinic agent scopolamine has been used in combination with desiccating environmental stress conditions to develop animal models of dry eye.[202–206] Parameters such as humidity, air flow, and temperature can be adjusted to achieve desiccating environmental stress in the room where animals are housed or in controlled environmental chambers.[202–208] Furthermore, mechanical approaches such as holding the eye open with a speculum to prevent blinking have also been used to induce dry eye.[209] Some of the dry eye treatments assessed using such dry eye models are listed in Table 1.9.

DED inducing conditions	Model animal	Evaluated dry eye treatments	Reference
Pharmaceutical agent administration			
Botulinum toxin B (injection to the lacrimal gland)	Mouse	The topical steroid fluorometholone and the non-steroidal anti-inflammatory drugs nepafenac and ketorolac	Zhu et al.[200] (2012)
Atropine sulfate 1% (topical ophthalmic administration)	Rabbit	Cyclosporine A-encapsulated liposomes vs. commercially available cyclosporine A emulsion (Restasis®)	Karn et al.[201] (2014)
Desiccating environmental stress			
Dessicating environmental stress (RH: 25%±2%, AF: 2-4 m/s, T: 23±2°C) & placement of the animal on a jogging board (7.5 h/day)	Rat	D-β-hydroxybutyrate	Nakamura et al.[207] (2005)
Intelligently controlled environmental system (RH: 15.3% ±3.0%, AF: 2.1± 0.2 m/s, T: 21°C–23°C)	Mouse	CMC+HA artificial tears (Optive Fusion®) vs. CMC-only (Refresh Tears®) vs.HA-only (Hycosan®) artificial tears	She et al.[208] (2015)
Desiccating environmental stress + scopolamine administration			
Controlled environment chamber (RH: 30%, AF: 15 L/min, T: 21-23°C) & SC scopolamine injection	Mouse	Topical omega-3 and omega-6 fatty acids	Rashid et al.[202] (2008)
Controlled environment chamber (RH:20%, AF: 20 L/min, T:21–22°C) & scopolamine transdermal patch	Mouse	Oil-in-water emulsion containing natural triglycerides, a mixture of phospholipids, and glycerol (Emustil); alone or in combination with a commercially available SH 0.2% formulation	Scifo et al.[203] (2010)
Exposure to an air draft and 30% ambient humidity & SC scopolamine hydrobromide injection	Mouse	Topical omega-3 fatty acids and hyaluronic acid mixtures	Li et al.[204] (2014)
Desiccating environmental stress (Placement of animals between 2 fans for a continuous AF of 15 L/min in a 25°C room with an ambient humidity of 30%) & SC scopolamine injection	Mouse	SH 0.18% artificial tears	Oh et al.[205] (2014)
Controlled environment chamber (RH: <25%, AF: 15 L/min, T: 20-22°C) & transdermal scopolamine patch	Mouse	Topical cationic emulsion of cyclosporine A (Ikervis®) and its vehicle	Daull et al.[206] (2016)
Mechanical approaches for desiccation			
Holding the eye open with a speculum	Rabbit	Ocular lubricants containing SH, CMC, or HPMC	Zheng et al.[209] (2014)

Table 1.9. *Examples of dry eye treatments evaluated in different animal models of dry eye. RH: relative humidity, AF: airflow, CMC: carboxymethyl cellulose, HA: hyaluronic acid, SC: subcutaneous, SH: sodium hyaluronate, HPMC: hydroxypropyl methylcellulose.*

BAK, a preservative widely used in ophthalmic formulations, has been shown to possess cytotoxic effects that may cause ocular surface damage and dry eye, as mentioned in Section 1.4.2.4. These effects of BAK have led to its exploration as a potential approach to induce dry eye in animal models. Topical administration of BAK has been employed to induce dry eye in mice and rabbits (Table 1.10).[210–214] As rabbit eyes are more similar to human eyes in terms of their size and have a larger exposed ocular surface that facilitates testing,[199] rabbits may be preferable to mice.

BAK treatment	Main outcomes	Model stability after the cessation of the BAK treatment	Reference
Mouse models			
0.2% twice daily; for 1 week	↓ tear volume ↓ tear break-up time ↑ corneal fluorescein scores ↑ rose bengal scores ↑ inflammatory index Ultrastructural changes in the corneal epithelium	Not observed	Lin et al.[210] (2011)
Rabbit models			
0.1% twice daily; for 2 weeks	↓ tear secretion ↑ fluorescein scores ↑ rose bengal scores ↓ goblet cell density ↓ MUC5AC Histopathological and ultrastructural disorders of the cornea and the conjunctiva	Not observed	Xiong et al.[211] (2008)
0.1% twice daily; for 2, 3, 4, or 5 weeks (groups 2W, 3W, 4W, and 5W, respectively)	↓ tear secretion ↑ fluorescein scores ↓ goblet cell density ↓ MUC5AC Histopathological and ultrastructural disorders of the cornea and the conjunctiva	Group 5W the most stable; dry eye signs sustained for 2-3 weeks	Li et al.[212] (2012)

Table 1.10. *Animal models of dry eye developed with topical administration of BAK.*

In vivo animal models have some disadvantages and limitations such as the feasibility and reproducibility issues and ethical considerations. *In vitro* and *ex vivo* animal models of dry eye are emerging as alternative tools to the *in vivo* models to investigate the mechanisms of dry eye and to evaluate the therapeutic efficacy of dry eye formulations. A number of two dimensional and three dimensional *in vitro* models using corneal or conjunctival cells have employed different methods to mimic dry eye conditions, including exposure to desiccating environmental conditions,[215–217] osmotic stress,[218] and inflammation-inducing agents.[219] An interesting *ex vivo* dry eye model example is the porcine dry eye model developed by Choy et al.,[220,221] using the whole porcine eyeball with conjunctiva tissue, lacrimal gland and the nictitating membrane in the system, and simulating dry eye by adjusting the lacrimation/blink interval in the model. Such models, once proven reliable and standardized, may be useful in dry eye research due to several potential advantages such as the opportunity of adjusting several parameters (e.g. blinking rate, temperature, humidity) as desired, increased practicality and reproducibility, and eliminating the use of laboratory animals along with the ethical considerations.

2. OBJECTIVES AND APPROACH

2.1. Main objective

Dry eye disease (DED) is associated with impairments of the tear film and the ocular surface.[2] Replenishing the tear film and restoring the ocular surface are crucial for the treatment of the disease. One of the most commonly employed therapeutic approaches in DED is the use of artificial tears. A large number of artificial tear formulations are commercially available. However, most of these formulations are water-based polymeric solutions, supplementing the aqueous phase of the tear film and providing only a temporary relief of the symptoms. Short ocular residence time, requiring frequent administration, is another drawback of many of these formulations.[8] Current research in DED treatment falls short of addressing these issues.

The main objective of this doctoral thesis was the design and development of novel artificial tear formulations for the treatment of dry eye disease. In order to accomplish this objective, specific objectives were set as indicated below.

2.2. Specific objectives

Objective 1: Development of artificial tear formulations containing novel components that replenish the tear film, with suitable physicochemical properties, optimum tolerance, and extended ocular residence time

In order to achieve this objective, the following approach was taken:

- Literature review on tear film to identify the key characteristics to mimic and the constituents that might achieve this, as well as on advances in DED treatments

- Design and preparation of five novel artificial tear formulations containing liposomes and/or bioadhesive polymers and/or *in situ* gelling polymers

- Physicochemical characterization of the novel artificial tear formulations in terms of liposome size, pH, surface tension, viscosity, and osmolarity

- Rheological evaluation of the artificial tears containing a gelling agent, upon simulating *in situ* gelling on the ocular surface

- Assessment of the *in vivo* tolerance of the formulations in rabbits

The results obtained are presented in Chapter 3 entitled "DEVELOPMENT AND CHARACTERIZATION OF NOVEL ARTIFICIAL TEAR FORMULATIONS".

Objective 2: Development of novel tools to evaluate the efficacy of the artificial tear formulations

In order to achieve this objective, the following approach was taken:

- Development of an *in vivo* dry eye model: Dry eye was induced in rabbits by twice a day topical ophthalmic administration of the preservative benzalkonium chloride (0.1%) for 15 days. The experimental dry eye model was characterized by assessing the ocular surface with corneal fluorescein staining and conjunctival impression cytology.

  The results obtained are presented in Chapter 3 entitled "DEVELOPMENT AND CHARACTERIZATION OF NOVEL ARTIFICIAL TEAR FORMULATIONS".

- Optimization of an *ex vivo* dry eye model: A porcine *ex vivo* anterior eye model developed at Aston University (UK) was optimized as a dry eye model, in collaboration with researchers from Aston University in the framework of the EDEN Project.

The results obtained are presented in Chapter 4 entitled “PRELIMINARY EFFICACY STUDIES ON DRY EYE UTILIZING THE ASTON BIOLOGICAL ANTERIOR EYE MODEL”.

Objective 3: Evaluation of the efficacy of the novel artificial tear formulations with preliminary studies

In order to achieve this objective, preliminary efficacy studies were performed in the previously mentioned *in vivo* and *ex vivo* dry eye models:

- Preliminary efficacy studies in the *in vivo* dry eye model over a 21-day treatment period

The results obtained are presented in Chapter 3 entitled “DEVELOPMENT AND CHARACTERIZATION OF NOVEL ARTIFICIAL TEAR FORMULATIONS”.

- Preliminary efficacy studies in the *ex vivo* dry eye model over an 18-hour treatment period

The results obtained are presented in Chapter 4 entitled “PRELIMINARY EFFICACY STUDIES ON DRY EYE UTILIZING THE ASTON BIOLOGICAL ANTERIOR EYE MODEL”.

Objective 4: Assessment of the effects of the novel artificial tear formulations on neutrophil extracellular trap (NET) formation as a proof of concept

NETs are extracellular fibers of nuclear and granule components, produced by neutrophils through a process termed NETosis.[222] Elevated levels of NETs on the ocular surface have been associated with DED pathogenesis.[5,6]

In order to achieve this objective, the author collaborated with researchers from the University of Birmingham (UK) that work on NETosis. The following approach was taken:

- Isolation of neutrophils from the blood of a healthy donor
- Incubation of the formulations with unstimulated and stimulated neutrophils and subsequent NET visualization

The results obtained are presented in Chapter 5 entitled “NETOSIS STUDIES”.

3. DEVELOPMENT AND CHARACTERIZATION OF NOVEL ARTIFICIAL TEAR FORMULATIONS

3.1. Introduction

Artificial tears are considered the mainstay in the management of dry eye disease (DED).[97] Numerous artificial tear formulations with a diverse range of components are currently available in the market. However, most of these formulations do not quite resemble natural tears in composition and do not aim to restore the tear film.[29] Furthermore, they have a short ocular residence time and require repeated daily administration.[8]

An ideal artificial tear formulation should contain both aqueous and lipid components to mimic natural tears, and have certain physicochemical properties such as neutral to slightly alkaline pH, suitable surface tension and viscosity.[223] A surface tension near the physiological range of natural tears (that is reported to be 40–46 mN/m)[86,87] provides a good spreading of the formulation over the ocular surface. For artificial tears that follow Newtonian behavior, the desirable viscosity would be in the physiological range of 1.0–8.3 mPa.s;[17,93] whereas for those that follow non-Newtonian behavior, a dynamic viscosity in the same range would be suitable. Additionally, an ideal artificial tear formulation should be well tolerated on the ocular surface, provide extended ocular residence time, and be preservative-free. Artificial tears can be prepared isotonic or slightly hypotonic.

This thesis aimed to develop novel artificial tear formulations that effectively replace and restore the altered, unstable tear film. With this purpose, the novel formulations incorporated both aqueous and lipid components and took into consideration parameters such as pH, surface tension, viscosity, and osmolarity. Five different artificial tear formulations were designed, developed, and characterized (Table 3.1).

Formulations 1 and 1a	Liposomal formulations containing hydroxypropyl methylcellulose, levocarnitine, vitamin A, vitamin E, fructose, and borates
Formulations 2 and 2a	Liposomal formulations containing gellan gum, hydroxypropyl methylcellulose, levocarnitine, vitamin A, vitamin E, trehalose, electrolytes, and borates
Formulation 3	Formulation containing gellan gum, hydroxypropyl methylcellulose, levocarnitine, vitamin C, trehalose, electrolytes, and borates

Table 3.1. *The novel artificial tear formulations designed and developed in this thesis.*

Liposomes, which mimic the lipids of the tear film, were employed in the formulations to restore the lipid layer of the tear film. Bioadhesive polymers and/or *in situ* gelling polymers were used to provide bioadhesion and gelation on the ocular surface, respectively, in order to extend the ocular residence time of the formulations. Antioxidants, osmoprotectants, antidessicants, and components that protect the corneal epithelium were incorporated to obtain artificial tears that do not only provide a symptomatic relief of DED, but are also potentially effective in the treatment of the disease. The rationale for the use of each component is indicated in Table 3.2.

Component	Rationale for use in the formulations
Liposomes (made up of phosphatidylcholine and cholesterol)	Resemble the lipids of the tear film, able to carry lipophilic and hydrophilic substances that may be beneficial in the treatment of DED
Hydroxypropyl methylcellulose	Bioadhesive polymer; interacts with the mucins of the tear film and extends the ocular residence time of the formulation
Gellan gum	*In situ* gelling polymer; undergoes sol-to-gel transition upon contact with the tear fluid
Levocarnitine	Osmoprotectant; potentially reduces hyperosmotic stress and the expression of proinflammatory cytokines
Trehalose	Osmoprotectant, antidessicant
Vitamin A	Antioxidant, protects corneal epithelial cells
Vitamin C	Antioxidant
Vitamin E	Antioxidant
Fructose	Possesses high water retention properties
Electrolytes (NaCl and KCl)	Enhance the gelation of the formulations containing gellan gum upon contact with the tear fluid
Borates	Buffering agent

Table 3.2. *Formulation components and the rationale for their use. DED: dry eye disease.*

For the formulations incorporating the *in situ* gelling polymer gellan gum, the *in situ* gelling was mimicked and rheological evaluation was performed. Furthermore, *in vivo* tolerance of all of the artificial tears was assessed in rabbits.

Another aim of the thesis was to develop a suitable *in vivo* platform for the efficacy studies of the artificial tears. With this purpose, an experimental model of dry eye was developed with topical ophthalmic administration of the preservative benzalkonium chloride in rabbits. Preliminary efficacy study of Formulation 3 was performed in the experimental dry eye model.

3.2. Materials and methods

3.2.1. Materials

Phosphatidylcholine obtained from soy lecithin (Phospholipon® 90 G) was purchased from Lipoid GmbH (Ludwigshafen, Germany). Cholesterol, retinyl acetate (vitamin A), 2-phospho-L-ascorbic acid trisodium salt (vitamin C), DL-α-tocopherol acetate (vitamin E), gellan gum (Gelzan™, Gelrite®), boric acid, sodium tetraborate anhydrous, calcium chloride dihydrate, sodium bicarbonate, sodium hydroxide pellets, and benzalkonium chloride were supplied by Sigma-Aldrich Co. (St. Louis, MO, USA). Sodium chloride, potassium chloride, magnesium chloride hexahydrate, periodic acid (EMSURE®), Schiff's reagent, Papanicolaou's solution 1b Hematoxylin solution S, and the mounting medium Entellan® new were purchased from Merck KGaA (Darmstadt, Germany). Chloroform (stabilized with ethanol), ethanol 70% v/v, ethanol 96% v/v, ethanol absolute, and xylene (mixture of isomers) were supplied by Panreac Química SLU (Barcelona, Spain). Hydroxypropyl methylcellulose was purchased from Abarán Materias Primas S.L. (Madrid, Spain), levocarnitine from Fagron Ibérica SAU (Barcelona, Spain), and sodium fluorescein (Colircusí Fluoresceína®, 2%) from Alcon Cusí, S.A. (Barcelona, Spain). D-fructose and D-(+)-trehalose dihydrate were supplied by ThermoFisher (Kandel) GmbH (Karlsruhe, Germany), membrane disc filters (Supor® 200) by Pall Corporation (Michigan, USA), and 24-well cell culture plates (Cellstar®) by Greiner Bio-One (Kremsmünster, Austria).

3.2.2. Animals

A total of fourteen male New Zealand white rabbits (purchased from San Bernardo Farm, Navarra, Spain) were used: six rabbits (3.0–4.0 kg) for *in vivo* tolerance

studies and eight rabbits (1.5–2.2 kg) for the development of an animal model of dry eye. The animals were housed individually with free access to food and drinking water, in a room with an alternating 12-hour light/dark cycle. The room was maintained at 22°C with 50% relative humidity. Animal experiments were performed in accordance with the EU Directive 2010/63/EU for animal experiments, UK Animals (Scientific Procedures) Act 1986, and ARVO Statement for the Use of Animals in Ophthalmic and Visual Research. The study protocols were approved by the Complutense University of Madrid (Comité de Experimentación Animal & Comité de Ética de Experimentación Animal) and by Director General de Agricultura y Ganadería, Comunidad de Madrid (approval references: PROEX 011/16 and PROEX 316/16).

3.2.3. Preparation of the artificial tear formulations

3.2.3.1. Preparation of Formulations 1 and 1a

Liposomes were prepared by the thin-film hydration method described by Bangham et al.[224] and modified by Vicario-de-la-Torre et al.[225] under aseptic conditions. The lipid phase of the liposomes consisted of phosphatidylcholine (PC), cholesterol (Chol), vitamin A (vit A), and vitamin E (vit E). Briefly, 400 mg of PC, 50 mg of Chol, 4 mg of vit E, and 400 ng of vit A were dissolved in 25 mL of the organic solvent chloroform. The solvent was then evaporated using a rotary evaporator (Büchi Rotavapor R-205 coupled to B-490 Heating Bath, Vac® V-500 Vacuum Pump, and V-800 Vacuum Controller, Massó Analítica S.A., Spain) under following conditions:

i) at 33°C, 200 mbar, and 150 rpm; for 30 minutes
ii) at 33°C, 100 mbar, and 150 rpm; for 30 minutes

Compressed nitrogen (Alphagaz™, AL Air Liquide España S.A., Madrid, Spain) was applied for 5 minutes to remove any residual solvent. The resulting dry lipid film

was hydrated with 10 mL of an aqueous solution (1.8% fructose, 0.84% boric acid (H_3BO_3), and 0.08% sodium tetraborate anhydrous ($Na_2B_4O_7$), with a pH of 7.0). For an optimal hydration of the lipid film upon the addition of the aqueous medium, the flask was first vigorously hand-shaken and subsequently placed on the rotary evaporator without vacuum (at 33°C and 200 rpm for 20 minutes).

After hydration of the lipid film, the liposomal suspension was collected from the evaporating flask and maintained in the dark at room temperature (25°C) for 2 hours to allow vesicle swelling. With the aim of obtaining a population of lipid vesicles that are homogenous in terms of size and structure, sonication and subsequent liposome extrusion were performed. Sonication was carried out in an ultrasonic bath (Ultrasons-H, J.P. Selecta S.A., Barcelona, Spain) at 5-10°C for 15 minutes. Liposome extrusion was performed with an extruder (Lipex® Extruder, Transferra Nanosciences Inc., Burnaby, BC, Canada), forcing the suspension through Nuclepore™ polycarbonate filters (Whatman International Ltd., Kent, UK) (pore size of 0.8 µm and 0.2 µm; 10 times each). After the extrusion process, the liposomal suspension was maintained at 2-8°C for 24 hours.

The final formulations were obtained by diluting the liposomal suspension 1:2 with an aqueous solution containing 1% hydroxypropyl methylcellulose (HPMC), 0.2% or 0.8% levocarnitine (for Formulation 1 and Formulation 1a, respectively), 1.8% fructose, 0.84% H_3BO_3, and 0.08% $Na_2B_4O_7$. Compositions of the formulations are indicated in Table 3.3.

	FORMULATION 1 Concentration (w:v)	FORMULATION 1A Concentration (w:v)
PC (liposomes)	2%	2%
HPMC	0.5%	0.5%
Levocarnitine	0.1%	0.4%
Vitamin E	0.02%	0.02%
Vitamin A	0.002%	0.002%
Fructose	1.8%	1.8%
H_3BO_3	0.84%	0.84%
$Na_2B_4O_7$	0.08%	0.08%

Table 3.3. *Compositions of Formulations 1 and 1a. Concentrations are expressed in percentage (weight/volume). PC: phosphatidylcholine, HPMC: hydroxypropyl methylcellulose, H_3BO_3: boric acid, $Na_2B_4O_7$: sodium tetraborate anhydrous.*

3.2.3.2. Preparation of Formulations 2 and 2a

Liposomes were prepared as described in Section 3.2.3.1, using an aqueous solution composed of 1.68% trehalose, 0.84% H_3BO_3, and 0.08% $Na_2B_4O_7$. Formulations 2 and 2a (Table 3.4) were obtained by mixing the liposomal suspension with the following solutions that were prepared separately in the above-mentioned aqueous solution of trehalose and borates:

- gellan gum 0.5%
- HPMC 0.6%
- levocarnitine 2% or 8% (for Formulation 2 and Formulation 2a, respectively)
- NaCl 0.4% and KCl 0.4%

	FORMULATION 2 Concentration (w:v)	FORMULATION 2A Concentration (w:v)
PC (liposomes)	0.5%	0.5%
Gellan gum	0.25%	0.25%
HPMC	0.12%	0.12%
Levocarnitine	0.1%	0.4%
Vitamin E	0.005%	0.005%
Vitamin A	0.0005%	0.0005%
NaCl	0.05%	0.05%
KCl	0.05%	0.05%
Trehalose	1.68%	1.68%
H_3BO_3	0.84%	0.84%
$Na_2B_4O_7$	0.08%	0.08%

Table 3.4. *Compositions of Formulations 2 and 2a. Concentrations are expressed in percentage (weight/volume). PC: phosphatidylcholine, HPMC: hydroxypropyl methylcellulose, NaCl: sodium chloride, KCl: potassium chloride, H_3BO_3: boric acid, $Na_2B_4O_7$: sodium tetraborate anhydrous.*

3.2.3.3. Preparation of Formulation 3

The following solutions were prepared separately, in an aqueous solution of 1.68% trehalose, 0.84% H_3BO_3, and 0.08% $Na_2B_4O_7$:

- gellan gum 0.5%
- HPMC 0.6%
- levocarnitine 8%
- Vitamin C 0.08%
- NaCl 0.4% and KCl 0.4%

These solutions were mixed to obtain Formulation 3 with the composition indicated in Table 3.5.

	FORMULATION 3 Concentration (w:v)
Gellan gum	0.25%
HPMC	0.12%
Levocarnitine	0.4%
Vitamin C	0.01%
NaCl	0.05%
KCl	0.05%
Trehalose	1.68%
H_3BO_3	0.84%
$Na_2B_4O_7$	0.08%

Table 3.5. *Composition of Formulation 3. Concentrations are expressed in percentage (weight/volume). HPMC: hydroxypropyl methylcellulose, NaCl: sodium chloride, KCl: potassium chloride, H_3BO_3: boric acid, $Na_2B_4O_7$: sodium tetraborate anhydrous.*

Compositions of the five artificial formulations developed in this thesis are indicated in Table 3.6. In all cases, solutions were sterilized either by steam sterilization (121°C, 20 minutes) or by filtration (pore size: 0.2 µm).

	Formulation 1	Formulation 1a	Formulation 2	Formulation 2a	Formulation 3
PC (liposomes)	2%	2%	0.5%	0.5%	-
Gellan gum	-	-	0.25%	0.25%	0.25%
HPMC	0.5%	0.5%	0.12%	0.12%	0.12%
Levocarnitine	0.1%	0.4%	0.1%	0.4%	0.4%
Vitamin E	0.02%	0.02%	0.005%	0.005%	-
Vitamin A	0.002%	0.002%	0.0005%	0.0005%	-
Vitamin C	-	-	-	-	0.01%
NaCl	-	-	0.05%	0.05%	0.05%
KCl	-	-	0.05%	0.05%	0.05%
Fructose	1.8%	1.8%	-	-	-
Trehalose	-	-	1.68%	1.68%	1.68%
H_3BO_3	0.84%	0.84%	0.84%	0.84%	0.84%
$Na_2B_4O_7$	0.08%	0.08%	0.08%	0.08%	0.08%

Table 3.6. *Compositions of the five artificial tear formulations developed in this thesis. Concentrations are expressed in percentage (weight/volume). PC: phosphatidylcholine, HPMC: hydroxypropyl methylcellulose, NaCl: sodium chloride, KCl: potassium chloride, H_3BO_3: boric acid, $Na_2B_4O_7$: sodium tetraborate anhydrous.*

3.2.4. Physicochemical characterization of the artificial tear formulations

The artificial tear formulations were characterized in terms of liposome size, pH, surface tension, viscosity, and osmolarity.

3.2.4.1. Liposome size

The particle size and the size distribution profile of liposomal vesicles were determined by dynamic light scattering (DLS, also referred to as photon correlation spectroscopy or quasi-elastic light scattering). In this technique, a monochromatic light beam, usually coming from a laser, is shot through the sample and the fluctuations of scattered light intensity are measured. Measurements were performed at room temperature and in triplicate, using the equipment Microtrac® Zetatrac™ Particle Size & Zeta Potential Analyzer (Microtrac Europe GmbH, Meerbusch, Germany), operated through Microtrac FLEX software (Version 10.5.3). Results were expressed as mean ± standard deviation (SD) in nanometers (nm).

3.2.4.2. pH

The pH was determined at room temperature with a pH meter (GLP 22, Crison Instruments S.A., Barcelona, Spain). Measurements were performed in triplicate and results were expressed as mean ± SD. The pH meter was calibrated with standard buffer solutions of pH 4.0 and 7.0 before the measurements.

3.2.4.3. Surface tension

The surface tension of the formulations was determined with a tensiometer (Krüss K-11 Tensiometer, Krüss GmbH, Hamburg, Germany) employing the Wilhelmy plate method. In this method, the sample liquid is brought into contact with an optimally wettable plate that is suspended from a precision balance and perpendicularly oriented to the interface. The force exerted on the plate due to the wetting is measured and the surface tension is calculated by the equipment. The plate used in the analyses was made of platinum. Measurements were performed at 33°C (temperature of the ocular surface) and in triplicate. Results were expressed as mean ± SD in milliNewtons/meter (mN/m).

3.2.4.4. Viscosity

The viscosity of the artificial tear formulations was determined with a rheometer (HAAKE RheoStress 1, ThermoFisher Scientific) using the parallel plate system (plate diameter=60 mm). For each formulation, the shear rate was increased stepwise in 20 steps from 0 to 1000 s^{-1} and each data point was measured at its equilibrium (steady state measurement). Measurements were performed at 33°C and in triplicate. The rheometer was operated through HAAKE RheoWin Measuring and Evaluation Software (Version: 4.30.0025). Results were expressed as mean ± SD in millipascal seconds (mPa.s).

3.2.4.5. Osmolarity

The osmolarity of the artificial tears was determined using a freezing-point osmometer (Gonotec Osmomat 3000). Measurements were performed in triplicate and results were expressed as mean ± SD in milliosmoles/liter (mOsm/L).

3.2.5. Rheological studies

To mimic the *in situ* gelling of the artificial tears on the ocular surface, the formulations were mixed with simulated tear fluid (STF)[226] at a proportion of 25:7 v/v (25 μL of the formulation and 7 μL of STF) and left for 150 seconds to allow gel formation. STF contained the electrolytes of tears that are involved in gel formation and consisted of 0.577% NaCl, 0.211% KCl, 0.013% $CaCl_2$, 0.0076% $MgCl_2$, and 0.218% $NaHCO_3$. The rheological properties of the resultant gels were evaluated with a rheometer (HAAKE RheoStress 1, ThermoFisher Scientific; plate diameter=60 mm) at 33°C, employing rotational and oscillatory measurements.

The flow curves were obtained both in the controlled rate (CR) and the controlled stress (CS) modes. In the CR mode, the shear rate was increased from 0-1000 s^{-1} in 20 steps, and subsequently decreased from 1000-0 s^{-1} in 20 steps. In the CS mode,

the shear stress was set to increase in 20 steps from 0-5 Pa (Formulation 3) or 0-5.1 Pa (Formulations 2 and 2a) and decrease to 0 Pa in 20 steps upon reaching the maximum set value. Ascending and descending flow curves were obtained by plotting shear stress versus shear rate in both the CR and the CS modes. The rheological behavior of the samples was observed and the dynamic viscosity at elevated shear rates was calculated in both the CR and the CS modes. The stress value at which the material yields and starts to flow, called the yield stress, was calculated in the CS mode.

The linear viscoelastic region (LVR) of the samples, in which the material maintains its structure, was determined by an oscillatory stress sweep at the fixed frequency of 0.5 Hz. A stress value from within the LVR was chosen and used for a subsequent frequency sweep over the range of 0.1-100 Hz for further characterization of the samples. The elastic (storage) modulus G' and the viscous (loss) modulus G" were evaluated.

3.2.6. *In vivo* tolerance studies

In vivo tolerance studies of the artificial tear formulations were performed in six male New Zealand white rabbits (each formulation was tested on a separate occasion). 30 µL of the artificial tear was administered in one eye of each animal every 30 min for 6 hours, whereas the contralateral eye received the same volume of non-preserved isotonic saline solution and served as control. Macroscopic evaluation of the ocular surface and the clinical signs was performed before the first instillation, and at 3, 6, and 24 hours after the first instillation, in compliance with the protocol previously described by Enríquez de Salamanca et al.[227] Clinical signs (corneal opacity, conjunctival alterations, eyelid swelling, discharge, and indicators of animal discomfort) were assessed and graded from 0 to 2 according to the grading system used by the authors (Table 3.7).

	Grade 0	Grade 1	Grade 2
Cornea	No alterations	Mild opacity	Intense opacity
Conjunctiva	No alterations	Mild hyperemia/ mild edema	Intense hyperemia/ intense edema/ hemorrhage
Eyelids	No swelling	Mild swelling	Obvious swelling
Discharge	No discharge	Mild discharge without moistened hair	Intense discharge with moistened hair
Discomfort	No reaction	Blinking	Enhanced blinking/ intense tearing/ vocalizations

Table 3.7. *Grading system used in the in vivo tolerance studies. Adapted from Enríquez de Salamanca et al.*[227]

3.2.7. *In vivo* efficacy studies

3.2.7.1. Development of an animal model of dry eye

Study design

Dry eye was induced in rabbits (n=8) by topical ophthalmic administration of the preservative benzalkonium chloride (BAK), following the protocols described by Xiong et al. and Li et al.[211,212] To this, one eye of each animal was treated with 30 µL of isotonic 0.1% BAK solution twice a day (8 A.M. and 7 P.M.) for 15 days. The contralateral eye received no treatment and served as a control. The ocular surface of the animals was evaluated prior to the BAK treatment (day 0), during the BAK treatment (day 7), and at the end of the BAK treatment (day 15) with two assays: corneal fluorescein staining and conjunctival impression cytology (CIC). Due to the irritated state of the animals and the slightly invasive nature of the

assay, in the control group on days 7 and 15, CIC samples were taken from four animals (n=4).

i. Corneal fluorescein staining

Corneal fluorescein staining has been used as an indicator of corneal damage. One drop of a 2% sodium fluorescein solution was instilled into the conjunctival sac. The cornea was evaluated after 2 minutes with a slit lamp (Kowa SL-15L, Kowa Company Ltd., Tokyo, Japan), using the cobalt blue filter. The cornea was divided into five zones (central, superior, inferior, nasal, and temporal; as in Figure 3.1) and each zone was assigned a grade from 0 to 4 according to the Efron Grading Scale.[228] The grades of the five zones were summed up to obtain the total fluorescein score between 0–20.

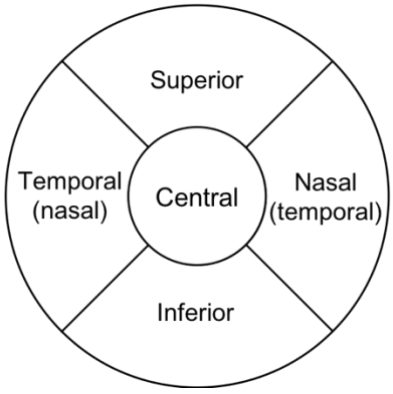

Figure 3.1. *Corneal zones used in the grading of fluorescein staining.*

ii. Conjunctival impression cytology

Samples of conjunctival cells were taken from the superior bulbar conjunctiva of the eyes by gently pressing a 5 mm x 5 mm membrane disc filter for 5 seconds with the help of a forceps. The samples with the cells obtained from the ocular surface were fixed in 96% ethanol for 30 minutes, stained with periodic acid-Schiff

(PAS) and hematoxylin, dehydrated and cleared.[229] PAS staining was carried out in 24-well culture plates with the following steps:

- Distilled water: 10 minutes
- Periodic acid (1%): 10 minutes
- Distilled water: 3 minutes
- Schiff's reagent: 3 minutes
- Distilled water: 5 minutes

Hematoxylin staining and the subsequent dehydration and clearing of the samples (with increasing grades of ethanol and xylene, respectively) were carried out in staining dishes with the following steps:

- Hematoxylin: 20 seconds
- Distilled water: 5 minutes
- Ethanol 70%: 2 minutes
- Ethanol 96%: 2 times for 2 minutes
- Ethanol 100%: 2 times for 2 minutes
- Xylene: 9 minutes

Samples were then mounted on a microscope slide with the mounting medium Entellan® and examined under microscope (Nikon Eclipse Ci, Nikon Corporation, Tokyo, Japan) after a 24-hour waiting period. Microscopic evaluation was performed at 400X magnification following the protocol described by Zuazo et al.,[230] which uses a semi-quantitative estimation of five cytological criteria chosen according to the grading system described by Nelson et al.[231] Each cytological criterion was assigned a score according to Table 3.8 and the scores of the five criteria were summed up to obtain the total cytological score. Images were captured using Nikon NIS-Elements Imaging Software (Version 4.0).

Cytological criteria	Reference range
Cellularity	**0:** abundant **1:** moderate **2:** scarce
Cell-cell contact	**0:** associated cells **1:** associated cells and isolated cells **2:** isolated cells only
Nucleus/cytoplasm ratio	**0:** 1/2 **1:** 1/3 – 1/4 **2:** >1/5
Goblet cells	**0:** abundant (≥30) **1:** moderate (<30) **2:** scarce (<10) **3:** absent (<3)
Metaplasia	**0:** absent **1:** incipient **2:** moderate **3:** advanced

Table 3.8. *Cytological criteria and the relative reference ranges used in the evaluation of the epithelial and the goblet cells. Adapted from Zuazo et al.[230] Goblet cell count was presented as cells/image field of view at 400X magnification.*

The total cytological score of the five criteria was also expressed according to Nelson grading (Table 3.9).

Nelson grade	Cytological score
0	0–3
1	4–5
2	6–9
3	≥10

Table 3.9*. Nelson grading in relation to the cytological score. Adapted from Zuazo et al.[230]*

3.2.7.2. Preliminary efficacy study

Preliminary efficacy study of Formulation 3 was performed in the experimental dry eye model described in Section 3.2.7.1, immediately after inducing dry eye in rabbits with a 15-day BAK treatment. The dry eye models (n=8) were divided into two groups: the treatment group (n=6) and the control group (n=2). The treatment group was treated with 30 µL of the test formulation three times a day (8 A.M., 1.30 P.M., and 7 P.M.) for a period of 21 days. The control group received the same volume of a non-preserved isotonic saline solution with the same frequency for 21 days. The contralateral eye of each animal, not treated with BAK in the previous study (non-dry eye; "healthy" eyes), received a non-preserved isotonic saline solution during this study and they were used as a reference to normal conditions (reference group; n=8). Efficacy of the formulation was evaluated with corneal fluorescein staining and CIC, as described in Section 3.2.7.1, on days 7, 14, and 21 of the treatment. Fluorescein scores and cytological scores obtained at the end (on day 15) of the previous experimental dry eye study were accepted as baseline scores for this study. In the reference group, on days 7, 14 and 21, CIC samples were taken from four animals (n=4).

3.2.7.3. Statistical analysis

Statistical analyses of fluorescein and cytological scores were performed with Mann-Whitney test, using the GraphPad Prism software (Version 7.0a), as the scores were assigned based on distinct grading scales. P-values were two-tailed and $P<0.05$ was considered to be statistically significant.

3.3. Results

3.3.1. Physicochemical characterization of the artificial tear formulations

The results obtained in the characterization of the physicochemical properties of the five artificial tear formulations are reported in Table 3.10. Data are expressed as mean ± SD.

	Formulation 1	Formulation 1a	Formulation 2	Formulation 2a	Formulation 3
Liposome size (nm)	194.40 ± 1.80	191.03 ± 9.72	200.07 ± 4.37	194.53 ± 0.55	–
pH	6.96 ± 0.03	6.99 ± 0.03	7.58 ± 0.03	7.41 ± 0.05	7.06 ± 0.01
Surface tension (mN/m)	48.73 ± 0.15	46.37 ± 2.75	45.57 ± 1.55	44.60 ± 0.26	46.97 ± 0.50
Viscosity* (mPa.s)	10.25 ± 0.52	12.08 ± 0.19	4.01 ± 0.06	4.64 ± 0.12	3.64 ± 0.02
Osmolarity (mOsm/L)	222.67 ± 2.08	241.33 ± 0.58	250.33 ± 0.58	265.67 ± 1.15	272.67 ± 3.06

Table 3.10. *Results obtained in the physicochemical characterization of the five artificial tear formulations. Formulation 3 did not contain liposomes. Data are expressed as mean ± SD. *Mean value (± SD) within the shear rate interval of 200-1000 s^{-1}.*

The liposomal vesicles of the liposome-containing formulations (Formulations 1, 1a, 2, and 2a) showed a unimodal size distribution (Figures 3.2 and 3.3).

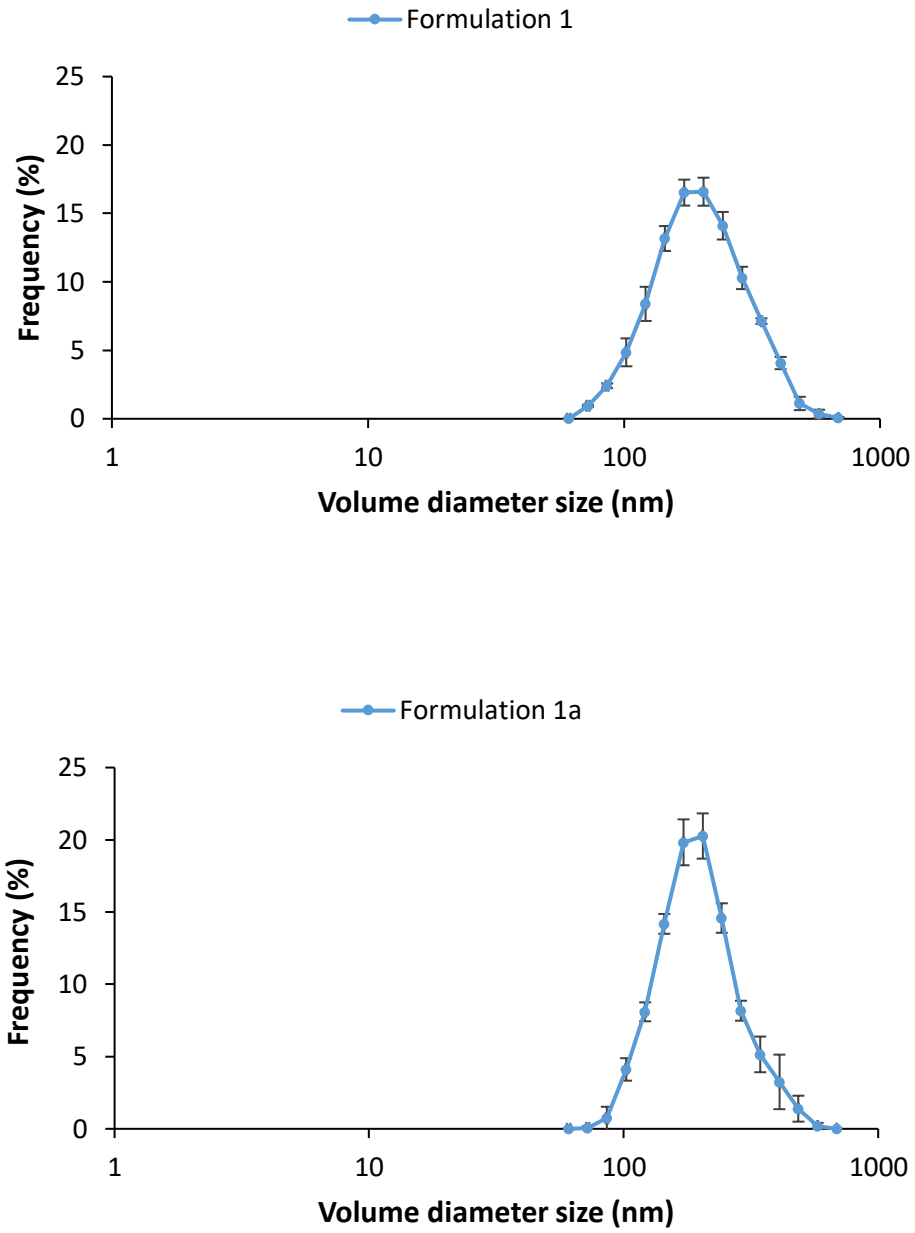

Figure 3.2. *Size distribution of the liposomal vesicles of Formulations 1 and 1a.*

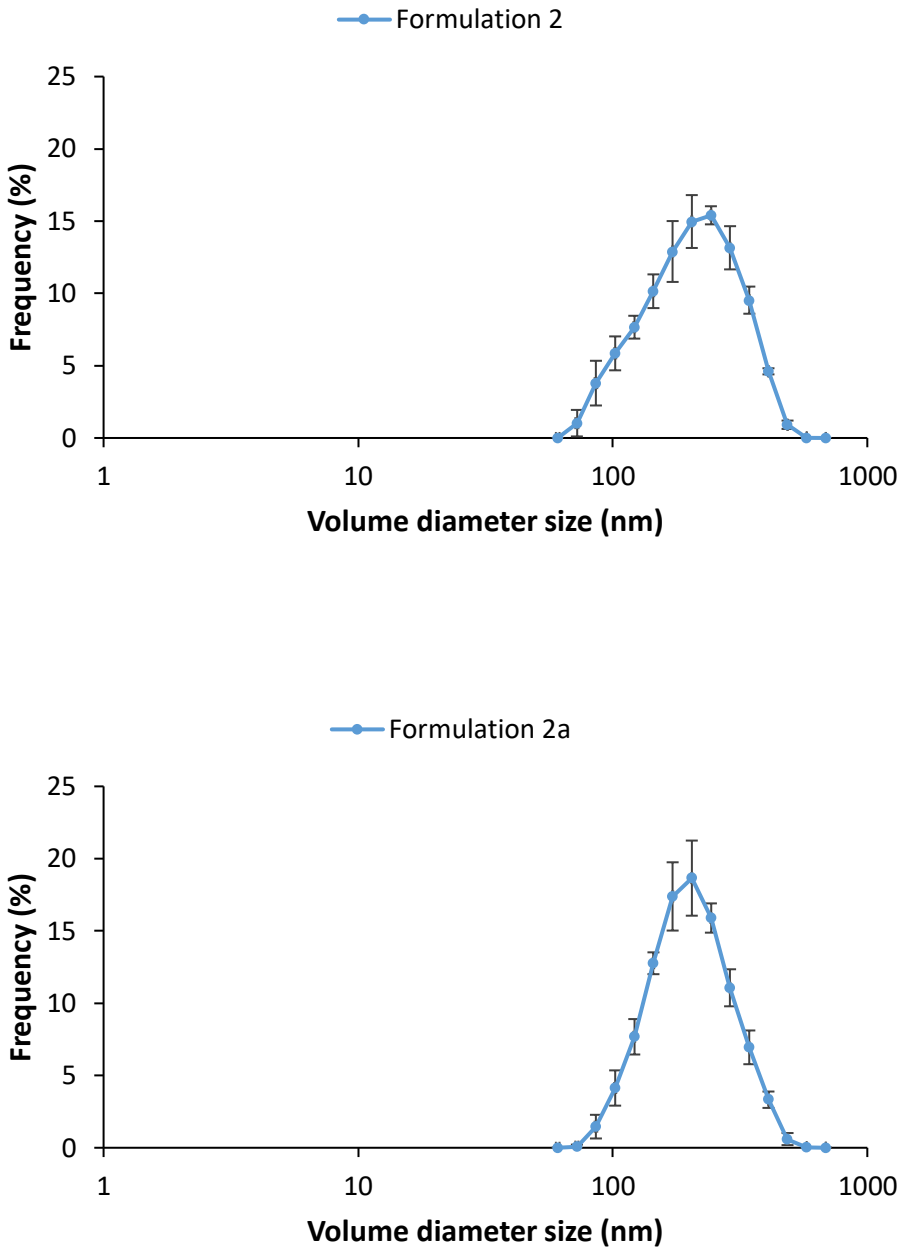

Figure 3.3. *Size distribution of the liposomal vesicles of Formulations 2 and 2a.*

The artificial tears followed Newtonian behavior as can be seen in rheograms illustrated in Figure 3.4. Formulations 1 and 1a had higher viscosities than the other formulations (within the shear rate interval of 200-1000 s^{-1}).

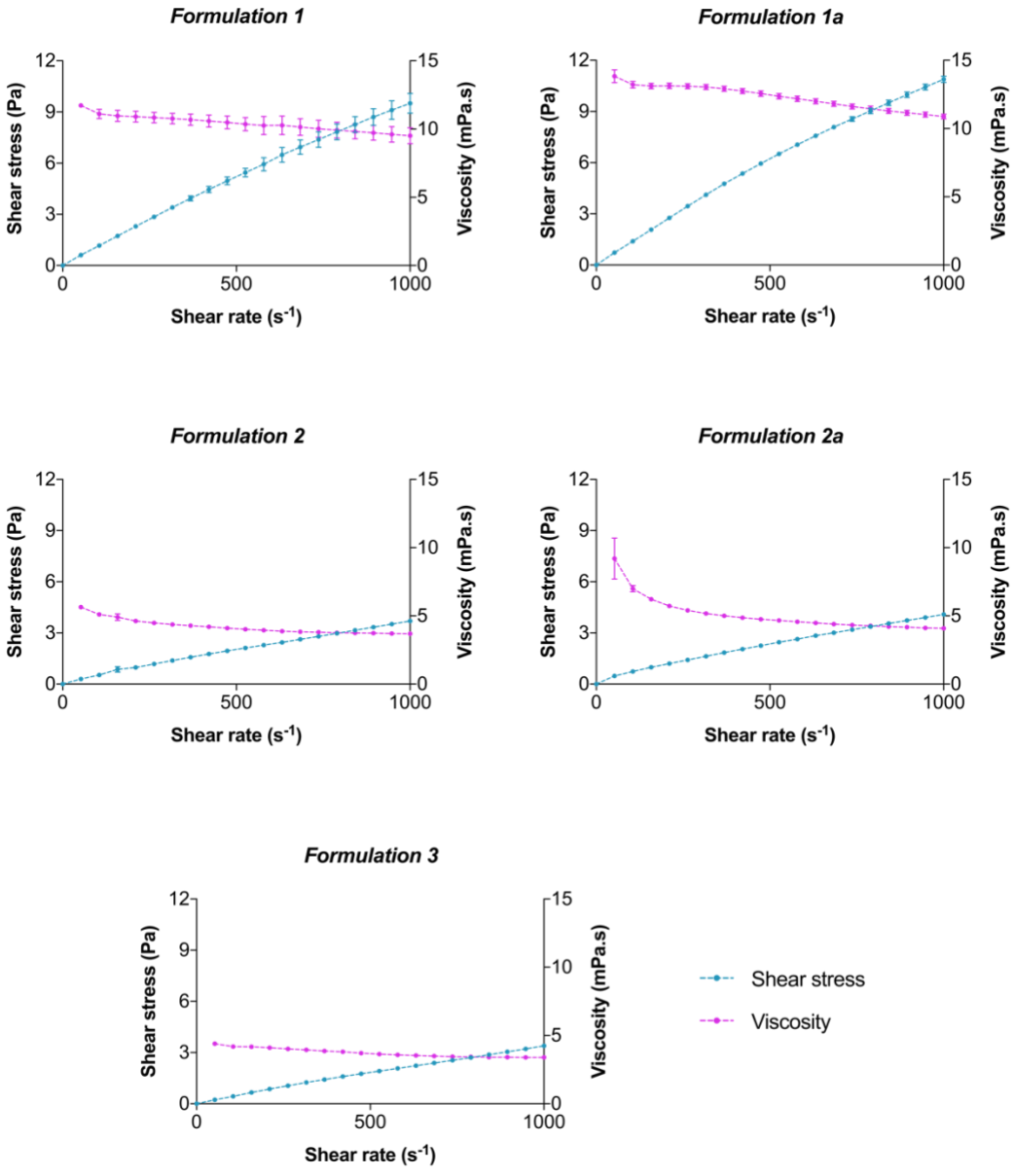

Figure 3.4. *Rheograms of the artificial tear formulations.*

The physicochemical properties of the liposome-containing artificial tear formulations were compared with their corresponding initial liposomal suspensions (Tables 3.11 and 3.12). The comparisons revealed that the liposome size, surface tension, and viscosity increased upon dilution of the liposomal suspensions with their corresponding polymeric solutions, whereas the pH remained similar.

	Liposomal suspension 1	Formulation 1	Formulation 1a
Liposome size (nm)	172.57 ± 0.47	194.40 ± 1.80	191.03 ± 9.72
pH	6.96 ± 0.02	6.96 ± 0.03	6.99 ± 0.03
Surface tension (mN/m)	25.70 ± 0.66	48.73 ± 0.15	46.37 ± 2.75
Viscosity* (mPa.s)	1.12 ± 0.04	10.25 ± 0.52	12.08 ± 0.19

Table 3.11. *Comparison of the physicochemical properties of Liposomal suspension 1, Formulation 1, and Formulation 1a. Data are expressed as mean ± SD. *Mean value (± SD) within the shear rate interval of 200-1000 s^{-1}.*

	Liposomal suspension 2	Formulation 2	Formulation 2a
Liposome size (nm)	164.40 ± 1.91	200.07 ± 4.37	194.53 ± 0.55
pH	7.45 ± 0.01	7.58 ± 0.03	7.41 ± 0.05
Surface tension (mN/m)	26.93 ± 0.42	45.57 ± 1.55	44.60 ± 0.26
Viscosity* (mPa.s)	1.12 ± 0.03	4.01 ± 0.06	4.64 ± 0.12

Table 3.12. *Comparison of the physicochemical properties of Liposomal suspension 2, Formulation 2, and Formulation 2a. Data are expressed as mean ± SD. * Mean value (± SD) within the shear rate interval of 200-1000 s^{-1}.*

3.3.2. Rheological studies

The flow curves of the gels obtained in the controlled rate (CR) mode are illustrated in Figure 3.5. Pseudoplastic behavior and a hysteresis area between the ascending and the descending flow curves are observed. Gels seem to show thixotropic behavior (the viscosity values obtained during the stepwise increase of the shear rate are greater than the ones obtained during the stepwise decrease).

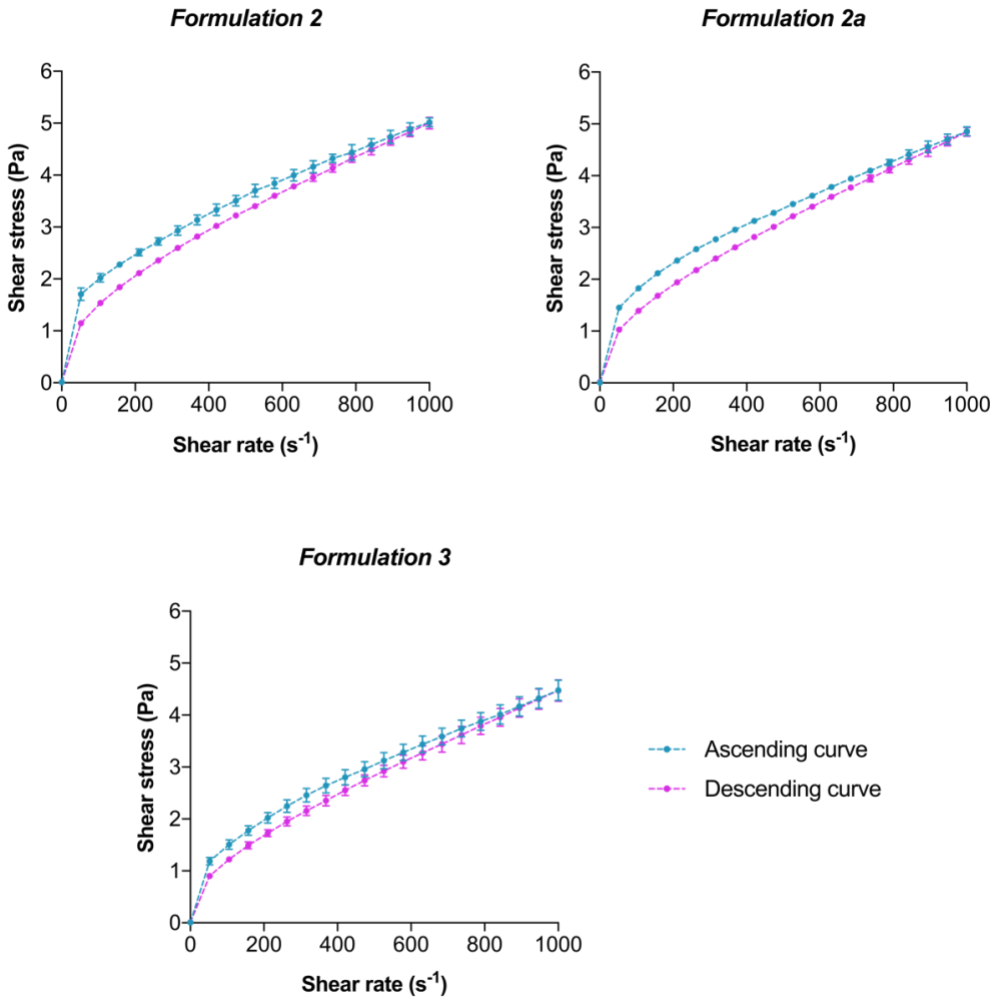

Figure 3.5. *Flow curves of the gels in the CR mode.*

The dynamic viscosity values of the gels at elevated shear rates (800-1000 s^{-1}) in the CR mode are repoted in Table 3.13.

Analyzed sample	Dynamic viscosity (mPa.s)
Formulation 2 – STF	2.77±0.15
Formulation 2a – STF	2.83±0.06
Formulation 3 – STF	2.97±0.25

Table 3.13. *Dynamic viscosities of the gels at elevated shear rates (800-1000 s^{-1}) in the CR mode.*

When the flow curves are obtained in the controlled stress (CS) mode (Figure 3.6), a yield stress can be observed in addition to the pseudoplastic behavior, reflecting a Herschel-Bulkley fluid model. The materials exhibit elastic behavior below the yield stress; whereas above the yield stress they exhibit viscous behavior and flow. The yield stress and the dynamic viscosity values (at elevated shear rates; 800-1100 s^{-1}) of the gels are repoted in Table 3.14.

Analyzed sample	Yield stress (Pa)	Dynamic viscosity (mPa.s)
Formulation 2 – STF	1.96±0.29	2.95±0.21
Formulation 2a – STF	1.98±0.04	3.00±1.17
Formulation 3 – STF	1.64±0.01	2.85±0.35

Table 3.14. *Yield stress and dynamic viscosity values of the gels (at elevated shear rates; 800-1100 s^{-1}) in the CS mode.*

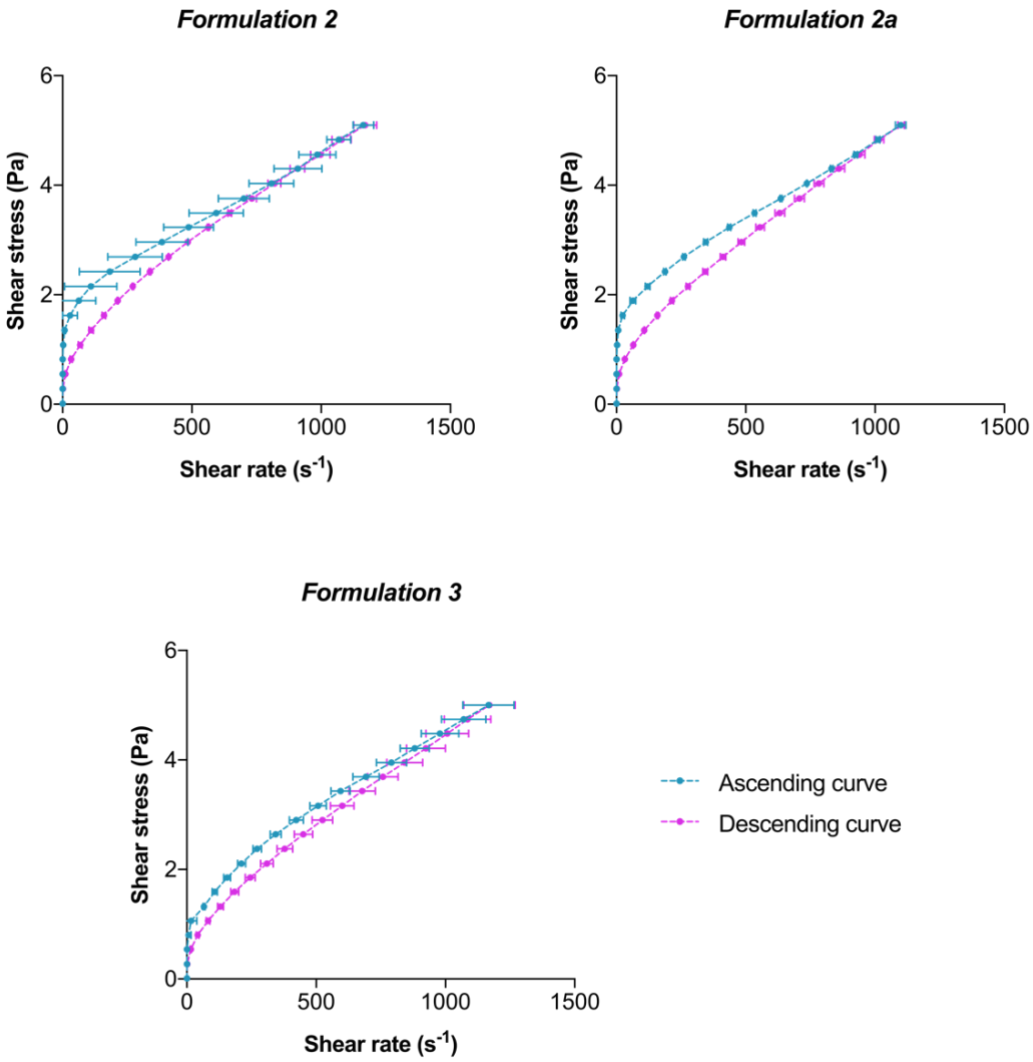

Figure 3.6. *Flow curves of the gels in the CS mode.*

The linear viscoelastic region of each gel was determined with an oscillatory stress sweep (Figure 3.7). The stress values chosen from the LVR for the subsequent frequency sweep measurements were as follows: 0.4 Pa for Formulations 2 and 2a, and 0.1 Pa for Formulation 3.

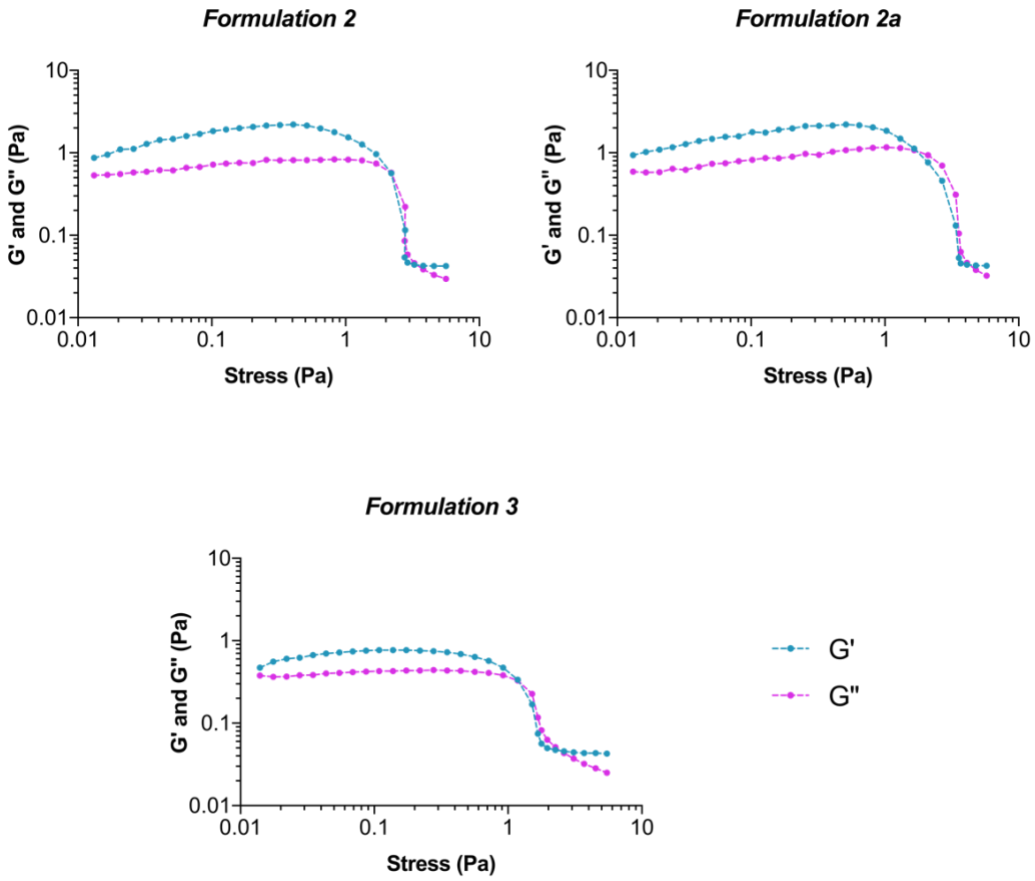

Figure 3.7. *Oscillatory stress sweep measurements of the gels showing the linear viscoelastic regions.*

The results obtained with the frequency sweep (Figure 3.8) showed that the elastic modulus G' is greater than the viscous modulus G'', indicating that the gels exhibit viscoelastic properties with elastic behavior. At intermediate oscillation frequencies (1-10 Hz), the dynamic moduli G' and G'' demonstrate low dependency on the frequency. At higher oscillation frequencies (i.e. greater than 20 Hz), the viscous modulus G'' increases more rapidly than the elastic modulus G', indicative of a strong loss in elastic response.

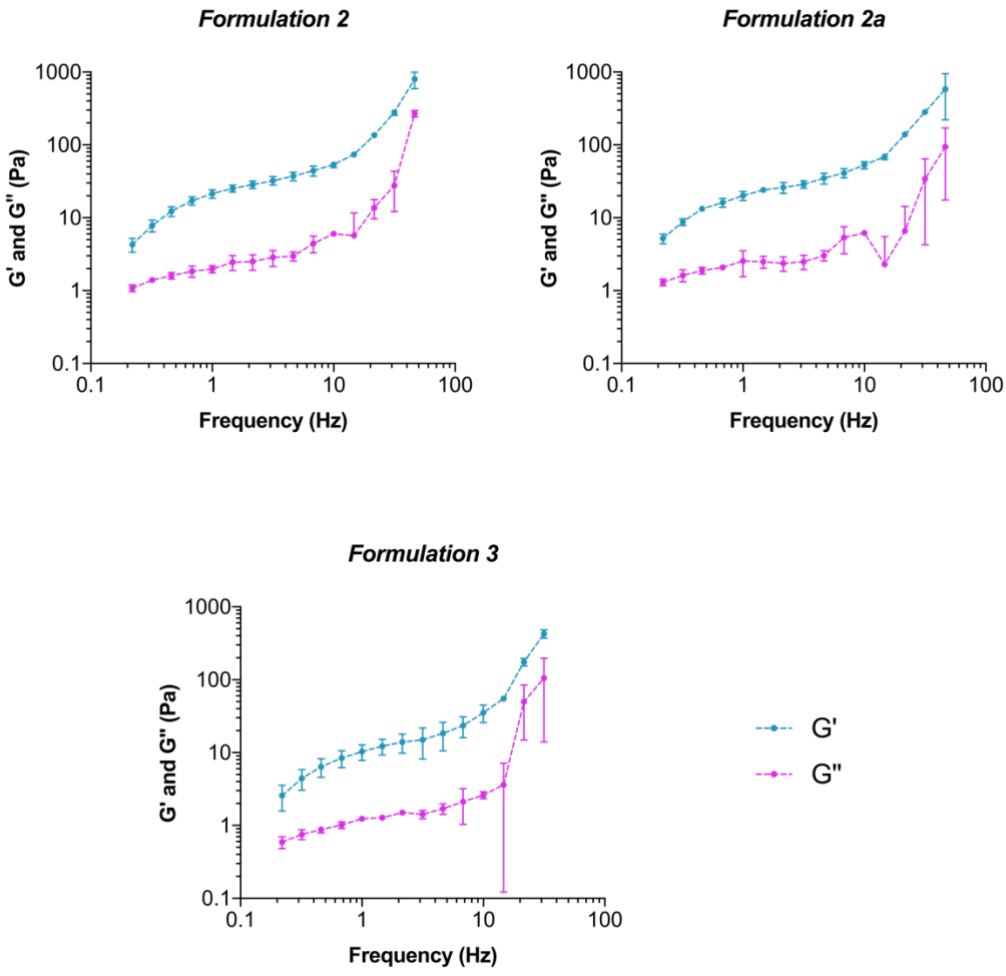

Figure 3.8. *Frequency sweep measurements of the gels.*

3.3.3. *In vivo* tolerance studies

Prior to the experiments, all of the animals had a normal ocular surface and showed no clinical signs or indicators of discomfort (grade 0). During the experiments, the animals showed neither indicators of discomfort nor clinical signs (grade 0) generated by any of the artificial tear formulations within 24 hours after the first instillation (Table 3.15).

Cornea	No alterations (grade 0)
Conjunctiva	No alterations (grade 0)
Eyelids	No swelling (grade 0)
Discharge	No discharge (grade 0)
Discomfort	No reaction (grade 0)

Table 3.15. *Results of the in vivo tolerance studies.*

3.3.4. *In vivo* efficacy studies

3.3.4.1. Development of an animal model of dry eye

Corneal fluorescein staining

Fluorescein scores of the untreated control group and the BAK group on days 0, 7, and 15 of the BAK treatment are reported in Table 3.16.

Day	Control group	BAK group
0	2.13 ± 1.73	1.75 ± 1.91
7	2.14 ± 2.41	6.86 ± 3.39
15	0.88 ± 1.73	13.75 ± 5.18

Table 3.16. *Fluorescein scores on days 0, 7, and 15 of the BAK treatment.*

No significant differences were found in fluorescein scores between the BAK group and the control group at baseline (day 0). Nevertheless, fluorescein scores increased significantly in the BAK group on day 7 *(P<0.05)* and day 15 *(P<0.001)* compared with baseline and the control group. In the control group, no significant differences from baseline were observed on days 7 and 15 (Figure 3.9).

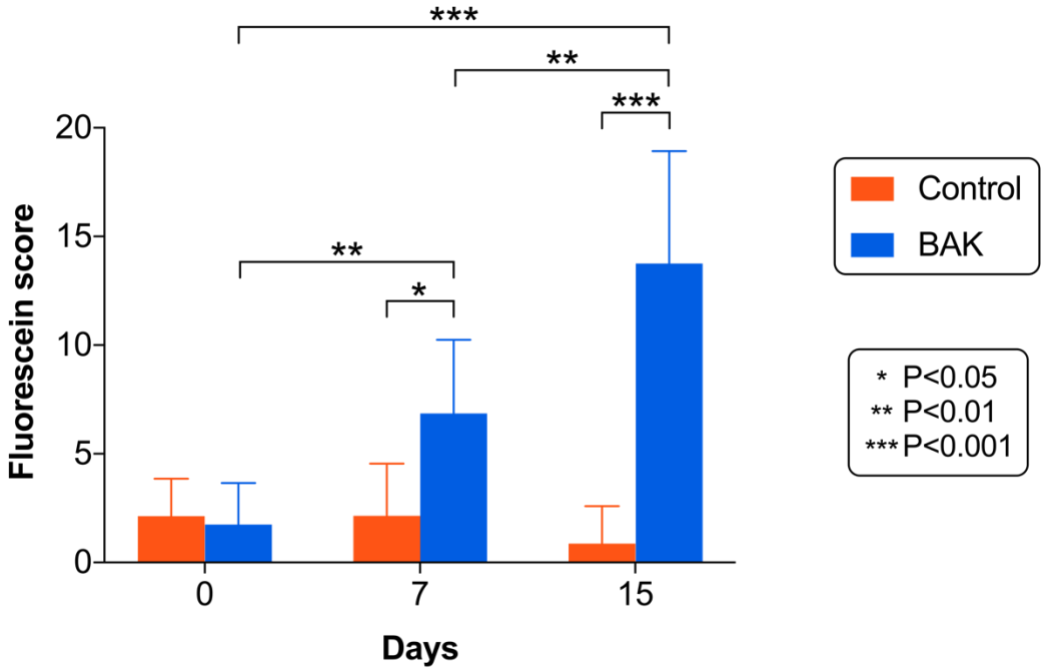

Figure 3.9. *Fluorescein scores on days 0, 7, and 15 of the BAK treatment. Sum of 0-4 scale grading for 5 corneal zones. *P<0.05, **P<0.01, ***P<0.001.*

Representative images of corneal fluorescein staining in BAK-treated eyes prior to the BAK treatment (day 0), during the BAK treatment (day 7), and at the end of the BAK treatment (day 15) are illustrated in Figure 3.10.

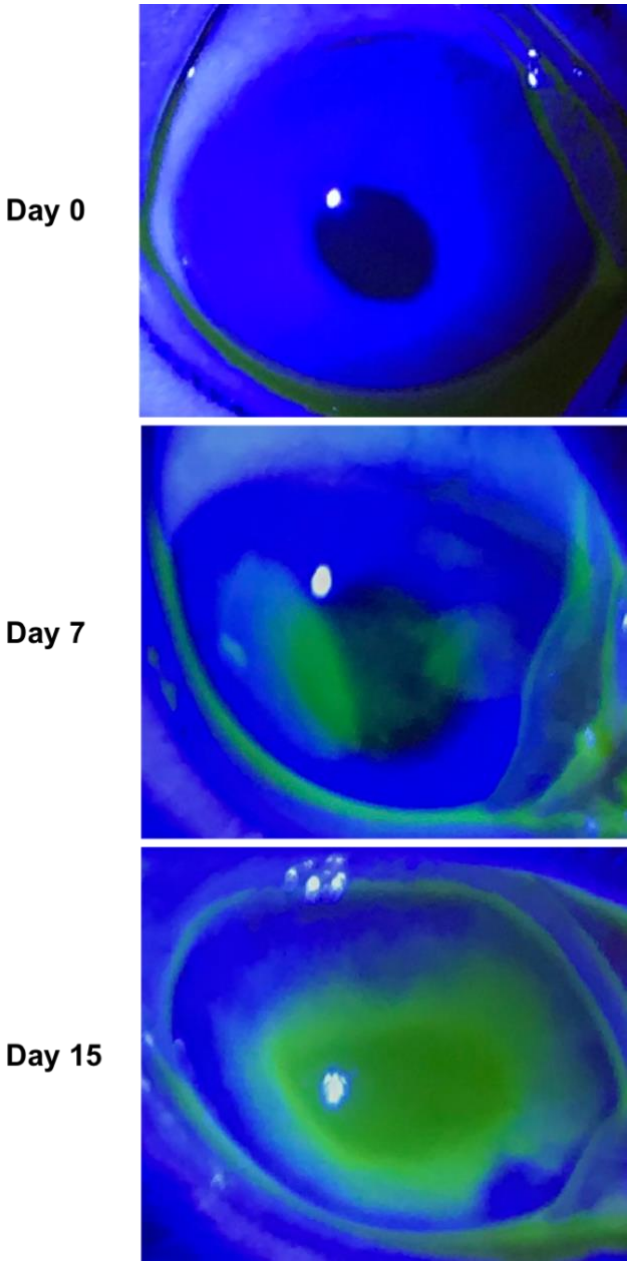

Figure 3.10. *Representative images of corneal fluorescein staining in BAK-treated eyes.* ***Day 0:*** *No obvious staining was observed prior to the BAK treatment.* ***Day 7:*** *Staining was observed.* ***Day 15:*** *Diffuse and heavier staining was observed in all five corneal zones.*

Conjunctival impression cytology

Cytological scores and corresponding Nelson grades of the untreated control group and the BAK group on days 0, 7, and 15 of the BAK treatment are reported in Table 3.17.

Day	Control group		BAK group	
	Cytological score	***Nelson grade***	***Cytological score***	***Nelson grade***
0	0.75 ± 0.46	0	0.63 ± 0.52	0
7	1.75 ± 1.26	0	6.71 ± 0.95	2
15	1.75 ± 1.26	0	8.25 ± 1.58	2

Table 3.17. *Cytological scores and corresponding Nelson grades on days 0, 7, and 15 of the BAK treatment.*

No significant differences were found in cytological scores between the BAK group and the control group at baseline (day 0). Cytological scores increased significantly in the BAK group on days 7 and 15 compared with baseline *(P<0.001)* and the control group *(P<0.01)*. In the control group, no significant differences from baseline were observed on days 7 and 15 (Figure 3.11).

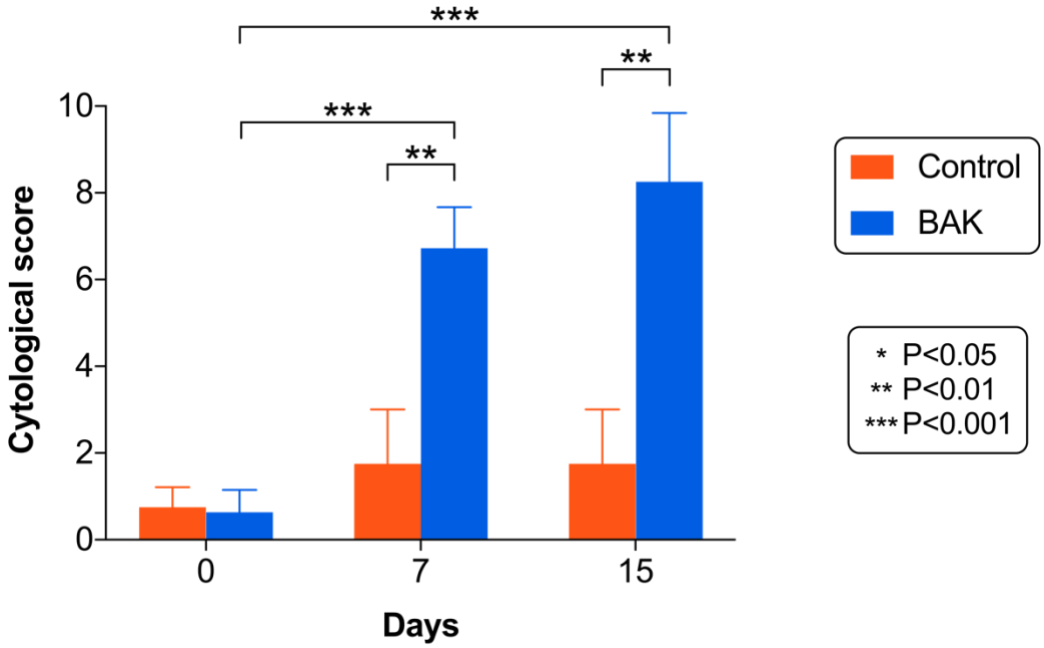

Figure 3.11. *Cytological scores on days 0, 7, and 15 of the BAK treatment. *P<0.05, **P<0.01, ***P<0.001.*

Representative images of conjunctival impression cytology in BAK-treated eyes prior to the BAK treatment (day 0), during the BAK treatment (day 7), and at the end of the BAK treatment (day 15) are illustrated in Figure 3.12.

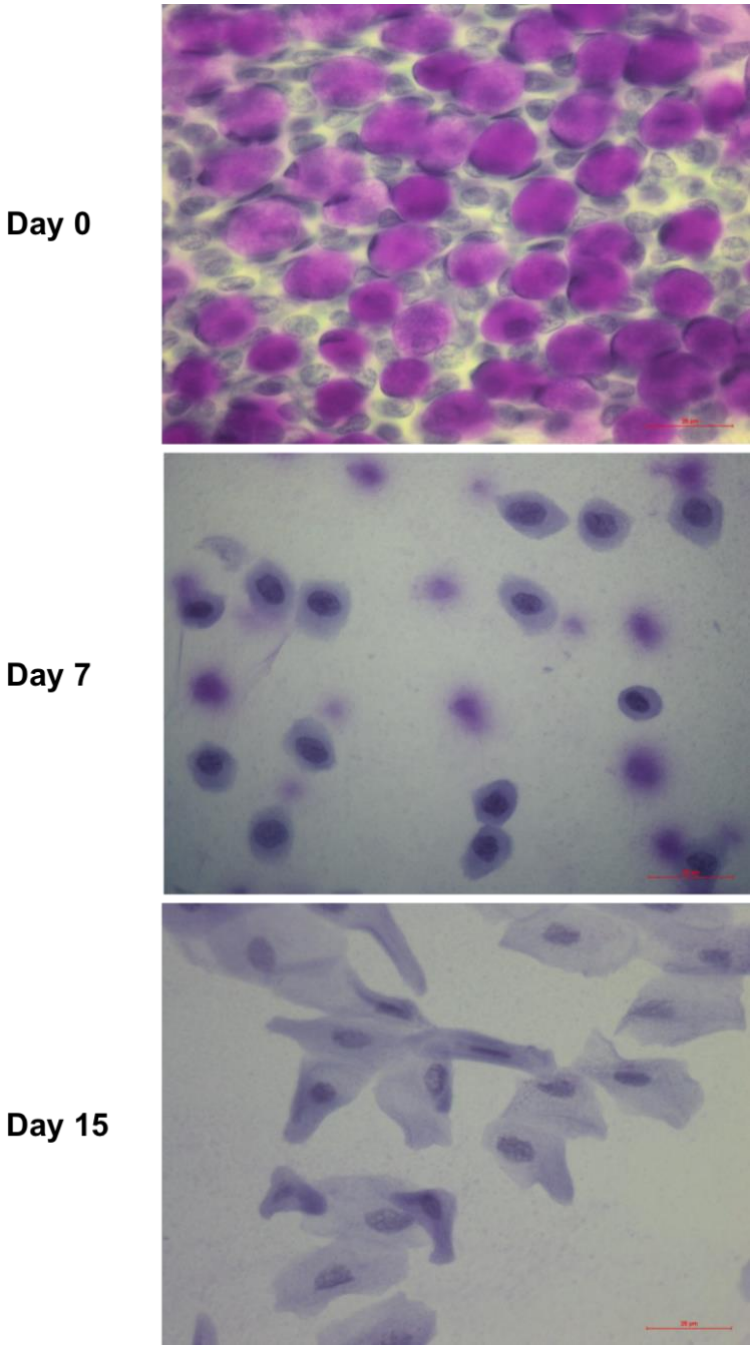

Figure 3.12. *Representative images of conjunctival impression cytology in BAK-treated eyes.* ***Day 0:*** *Baseline, prior to the BAK treatment, Nelson grade 0. Epithelial cells (violet): small and abundant. Cell-cell contact: very high, all cells are associated. Nucleus/cytoplasm ratio: 1/2. Goblet cells (purple): plump, well-defined, and abundant.* ***Day 7****: Nelson grade 2. Epithelial cells: larger and scarce. Cell-cell contact: diminished, cells are mostly isolated. Nucleus/cytoplasm ratio: 1/3–1/4. Goblet cells: smaller, poorly defined, and moderate in number.* ***Day 15****: Nelson grade 2. Epithelial cells: larger, abnormally shaped, and scarce. Cell-cell contact: low. Nucleus/cytoplasm ratio: >1/5. Goblet cells: absent. Scale bars: 25 μm.*

3.3.4.2. Preliminary efficacy study

Corneal fluorescein staining

The fluorescein score of the dry eye models obtained at the end of the previous experimental dry eye study (13.75 ± 5.18; n=8) was accepted as the baseline score (day 0) for the control and the treatment groups of this study, whereas the score of the untreated eyes from the previous study (0.88 ± 1.73; n=8) was accepted as the baseline score for the reference group. Fluorescein scores of the control group (dry eye models treated with isotonic saline solution; n=2 after baseline), the treatment group (dry eye models treated with Formulation 3; n=6 after baseline), and the reference group ("healthy" eyes treated with isotonic saline solution; n=8 after baseline) during the efficacy study are reported in Table 3.18.

Day	Control group	Treatment group	Reference group
0	13.75 ± 5.18	13.75 ± 5.18	0.88 ± 1.73
7	6.00 ± 2.83	1.50 ± 1.52	0.38 ± 0.74
14	2.50 ± 0.71	0.33 ± 0.52	0.50 ± 0.76
21	1.00 ± 0.00	0.00 ± 0.00	0.25 ± 0.46

Table 3.18. *Fluorescein scores during the preliminary efficacy study.*

In the treatment group, fluorescein scores decreased significantly *(P<0.01* compared with baseline) and appeared to return to the normal values (*P>0.05* compared with the reference group) already on day 7 (Figure 3.13). In the control group, the first statistically significant decrease from baseline was observed on day 14 *(P<0.05)*, with the values returning to normal levels on day 21 (*P>0.05* compared with the reference group). Compared with the control group, significantly lower fluorescein scores were observed in the treatment group on days 14 and 21 (*P<0.05*).

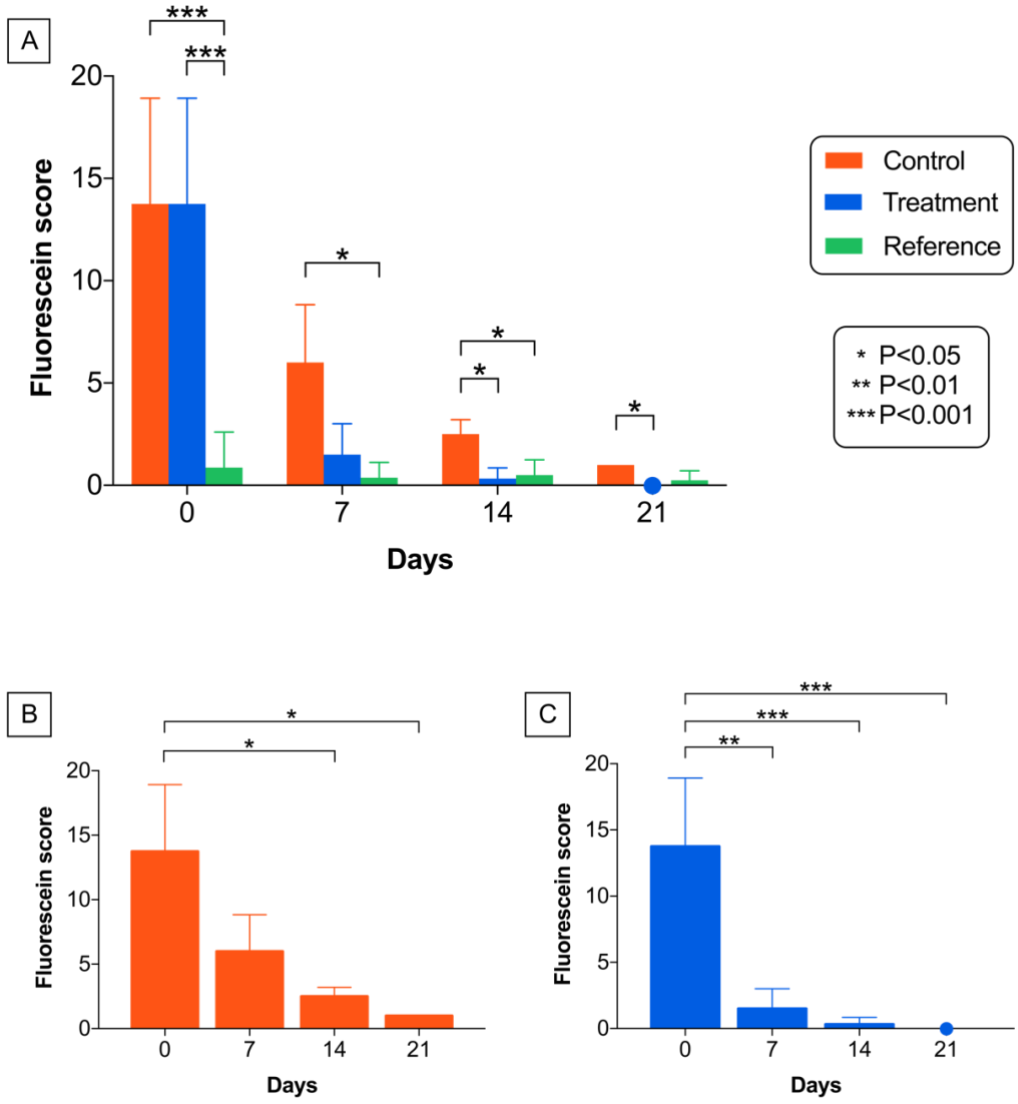

Figure 3.13. *Fluorescein scores and the statistical significance during the preliminary efficacy study A) between groups on each test day, B) between each test day inside the control group, C) between each test day inside the treatment group. The fluorescein score of the treatment group on day 21 was 0.00 ± 0.00; it is represented with a closed circle in the bar charts (A and C). The reference group had no statistically significant difference between any test day throughout the study and was not illustrated with a separate bar chart. *P<0.05, **P<0.01, ***P<0.001.*

Representative images of corneal fluorescein staining in the experimental model of dry eye prior to the treatment with the test formulation (day 0) and at days 7, 14, and 21 of the treatment are illustrated in Figure 3.14.

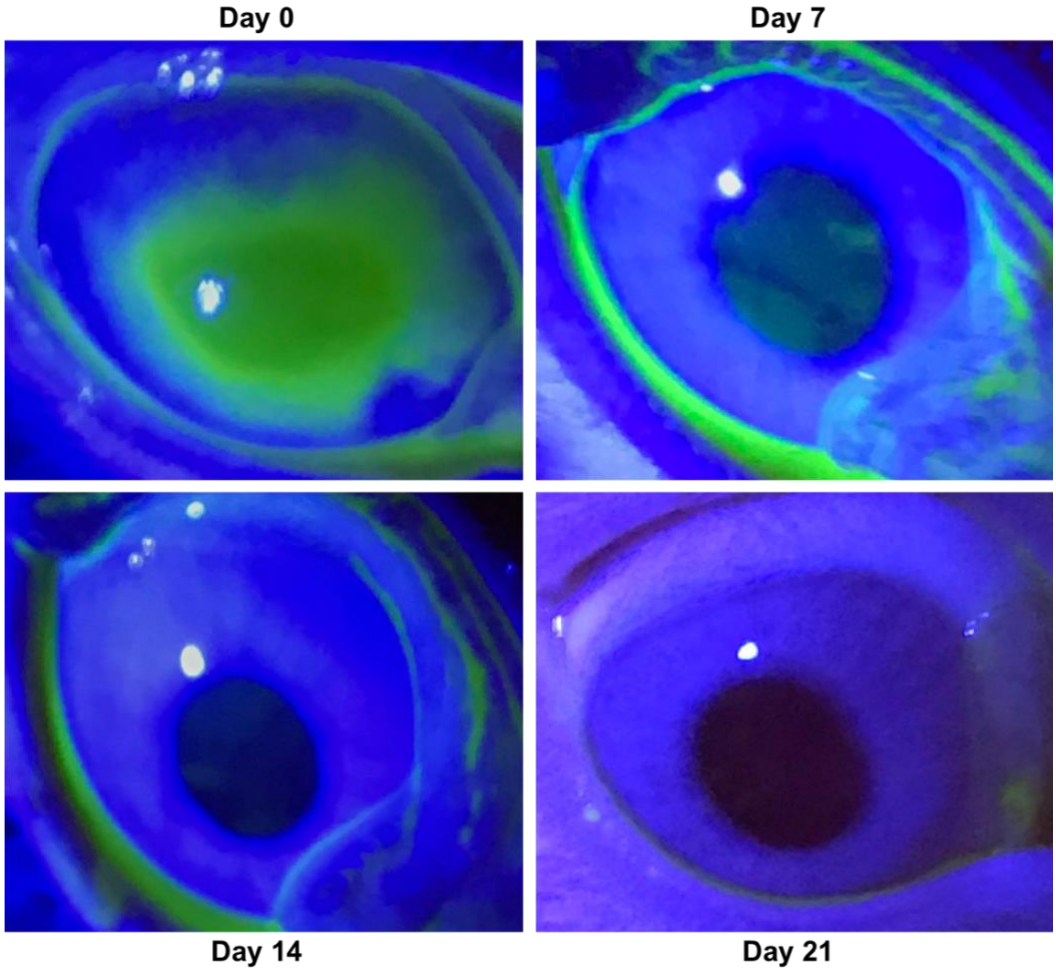

Figure 3.14. *Representative images of corneal fluorescein staining in the experimental model of dry eye treated with the test formulation.* ***Day 0****: Experimental dry eye, prior to the treatment with the test formulation. Diffuse and heavy staining was observed in all five corneal zones.* ***Day 7:*** *Staining was remarkably diminished; with a few light patches observed in the central corneal zone.* ***Days 14 and 21****: No obvious staining was observed.*

Conjunctival impression cytology

The cytological score of the dry eye models obtained at the end of the previous experimental dry eye study (8.25 ± 1.58; n=8) was accepted as the baseline score (day 0) for the control and the treatment groups of this study, whereas the score of the untreated eyes from the previous study (1.75 ± 1.26; n=4) was accepted as the baseline score for the reference group. Cytological scores and the corresponding Nelson grades obtained during the preliminary efficacy study are reported in Table 3.19 (n=2 for the control group, n=6 for the treatment group, and n=4 for the reference group after baseline).

Day	Control group		Treatment group		Reference group	
	Cytological score	*Nelson grade*	*Cytological score*	*Nelson grade*	*Cytological score*	*Nelson grade*
0	8.25 ± 1.58	2	8.25 ± 1.58	2	1.75 ± 1.26	0
7	7.50 ± 2.12	2	4.50 ± 0.84	1	1.00 ± 0.82	0
14	7.50 ± 0.71	2	3.17 ± 1.47	0	1.50 ± 1.29	0
21	6.50 ± 0.71	2	0.33 ± 0.52	0	1.00 ± 1.41	0

Table 3.19. *Cytological scores and the corresponding Nelson grades during the preliminary efficacy study.*

Compared with baseline, cytological scores decreased significantly in the treatment group at day 7 *(P<0.001)* and continued to decrease on the next test days, whereas no statistically significant difference from baseline was observed in the control group on any test day (Figure 3.15). Compared with the control group, significantly lower cytological scores were observed in the treatment group at day 21 *(P<0.05)*. In the treatment group, no more significant differences were found on day 14 compared with the reference group. Cytological scores of the control group were higher than the reference group throughout the study (days 7, 14, and 21); however, statistically significant results were not obtained, probably due to the small total sample size in these particular comparisons: n=2 for the control

group and n=4 for the reference group (the guide of the statistics software used in the analyses indicates that the Mann-Whitney test always gives a P-value greater than 0.05 when the total sample size is seven or less, no matter how much the groups differ[232]).

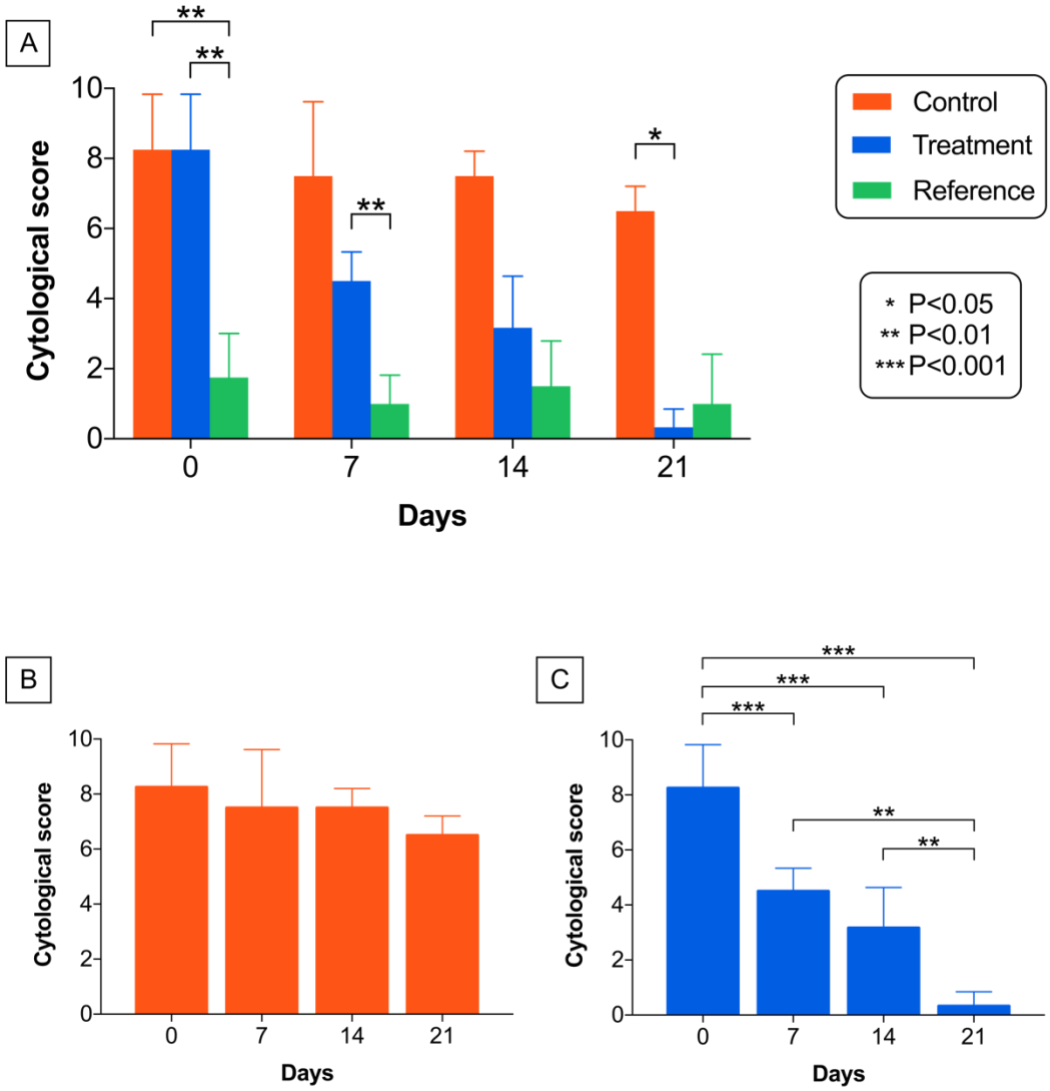

Figure 3.15. *Cytological scores and the statistical significance during the preliminary efficacy study A) between groups on each test day, B) between each test day inside the control group (no significant difference was observed between any test day), C) between each test day inside the treatment group. The reference group had no statistically significant difference between any test day throughout the study and was not illustrated with a separate bar chart. *P<0.05, **P<0.01, ***P<0.001.*

Representative images of conjunctival impression cytology in the experimental model of dry eye prior to the treatment with the test formulation (day 0) and at days 7, 14, and 21 of the treatment are illustrated in Figure 3.16.

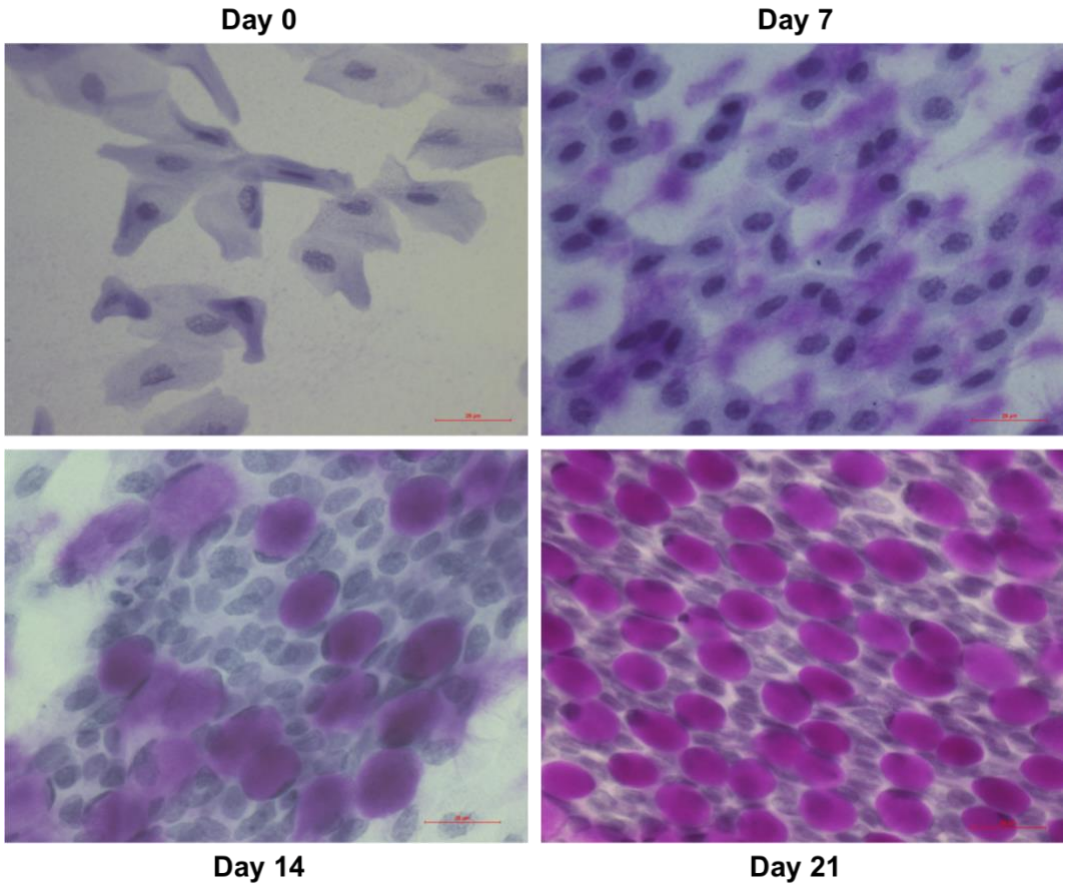

Figure 3.16. *Representative images of conjunctival impression cytology in the experimental model of dry eye treated with the test formulation.* ***Day 0****: Experimental dry eye, prior to the treatment with the test formulation, Nelson grade 2. Epithelial cells (violet): large, abnormally shaped, and scarce. Cell-cell contact: low. Nucleus/cytoplasm ratio: >1/5. Goblet cells (purple): absent.* ***Day 7****: Nelson grade 1. Epithelial cells: smaller but mostly polygonal, and moderate in number. Cell-cell contact: moderate. Nucleus/cytoplasm ratio: 1/3–1/4. Goblet cells: smaller than normal, poorly defined, and moderate in number.* ***Day 14****: Nelson grade 0. Epithelial cells: small and abundant. Cell-cell contact: high. Nucleus/cytoplasm ratio: 1/2. Goblet cells: plump, mostly well-defined, and moderate in number.* ***Day 21:*** *Nelson grade 0. Epithelial cells: small and abundant. Cell-cell contact: very high, all cells are associated. Nucleus/cytoplasm ratio: 1/2. Goblet cells: plump, well-defined, and abundant. The state and the number of the epithelial and the goblet cells have returned to normal. Scale bars: 25 μm.*

3.4. Discussion

Dry eye disease is associated with alterations and instability of the tear film; therefore, restoring the tear film is fundamental in its management. This study aimed at developing novel artificial tear formulations that resemble natural tears and restore the impaired tear film and the ocular surface in DED. Liposomes, consisting of phosphatidylcholine and cholesterol, were employed to replenish the lipids of the tear film. The *in situ* gelling polymer gellan gum was incorporated because of its unique gel-forming property when in contact with certain ions present in tears,[161–164] with the aim of resisting the lacrimal drainage and extending the ocular residence time of the formulation. HPMC was used to provide bioadhesion[102] and increase the ocular residence time. Antioxidants vitamin A, vitamin C, vitamin E, and osmoprotectants levocarnitine and trehalose were incorporated to enhance the potential efficacy of the artificial tears in the treatment of DED.

When formulating an artificial tear, the key physicochemical parameters such as pH, surface tension, viscosity, and osmolarity should be taken into consideration. Human tears have a pH of 7.0–7.5,[79,80] and a buffering capacity that prevents the ophthalmic formulations within the pH range of 6.6–9.0 from causing discomfort.[82–84] The formulations developed in this thesis had neutral to slightly alkaline pH (7.0–7.6), very similar to the pH of human tears, suggesting optimum tolerance of the formulations on the ocular surface in terms of their pH.

The surface tension of natural tears is reported to be 40–46 mN/m,[86,87] and the viscosity 1.0–8.3 mPa.s.[17,93] The surface tension of an ophthalmic formulation affects its interaction with the tear film and spreading ability over the ocular surface.[89] The viscosity, if excessively high, can cause non-uniform mixing of the formulation with natural tears, blurred vision, and discomfort. The surface tension of the formulations developed in this thesis ranged from 44.6 to 48.7 mN/m, and the viscosity from 3.6 to 12.1 mPa.s, suggesting good spreading of the

formulations over the ocular surface without causing tear film instability, blurred vision, or discomfort. Furthermore, the liposomal vesicles of the liposome-containing artificial tears had a suitable size for a topical ophthalmic formulation.

Tear hyperosmolarity is one of the main mechanisms in DED pathogenesis; it may lead to inflammation and tear film instability.[4] Therefore, restoring the physiological tear osmolarity and protecting the ocular surface from hyperosmotic stress is important in DED management. With this purpose, the formulations developed in this thesis incorporated levocarnitine for its osmoprotectant properties,[138–141] and trehalose for both osmoprotectant and antidessicant properties.[144–146] In fact, trehalose eye drops (100 mM and 200 mM) were previously found effective in the treatment of moderate to severe DED in an initial 4-week clinical study.[147] Furthermore, in a 4 week cross-over clinical study, 100 mM trehalose eye drops were found to be more effective in improving dry eye signs in moderate to severe DED patients compared with two commercially available eye drops containing either hyaluronan or hydroxyethylcellulose.[148] Considering these findings and the increased tear osmolarity in dry eye, it can be postulated that the artificial tears developed in this thesis, which are slightly hypotonic (with osmolarity values ranging from 222.7 to 272.7 mOsm/L) and incorporate components with osmoprotectant and antidessicant properties, would be suitable for DED patients with potential beneficial effects.[233,234]

Gellan gum has been employed in ophthalmic preparations as an *in situ* gelling agent in order to provide sustained release and improve bioavailability of anti-inflammatory and and anti-glaucoma agents;[159,160] however, it has not yet been utilized in commercially available artificial tears. The rheological data obtained in this study showed that the gellan gum containing formulations (Formulations 2, 2a, and 3) behaved as *in situ* gelling systems, which may be attributed to the ability of three-dimensional network formation of the gelling polymer in presence of certain ions in tears. The gelling formulations exhibited viscoelastic properties with elastic gel behavior (storage modulus>loss modulus) when mixed with STF.

The dynamic viscosities of the simulated *in situ* gels at elevated shear rates (800–1100 s^{-1}) ranged from 2.8–3.0 mPa.s. Such dynamic viscosities in the physiological range of natural tears and the pseudoplastic or "shear-thinning" properties exhibited by the tested gels could prevent discomfort after administration and avoid damage to the ocular surface during blinking where high shear rates are present (estimated to be between 4250–28500 s^{-1}).[17,235] Additionally, the *in situ* gel forming artificial tears may provide extended ocular residence time after topical ophthalmic administration in DED treatment.

As shown in the results of this work, the *in vivo* tolerance studies indicated that the formulations resulted well-tolerated in rabbit eyes. In fact, they do not generate any indicators of discomfort or undesirable alterations on the ocular surface of rabbits.

One of the aims of this thesis was to develop an *in vivo* dry eye model in rabbits as a tool to evaluate the efficacy of artificial tears. The preservative BAK has shown to be cytotoxic for corneal and conjunctival epithelial cells. BAK has been previously utilized to successfully induce dry eye in rabbits.[211,212] In these previous studies, a 2-week treatment with BAK resulted in dry eye signs including decreased tear secretion, increased fluorescein and rose bengal scores, and decreased goblet cell density. Li et al.[212] investigated the stability of the rabbit dry eye models induced with 0.1% BAK treatment for different durations (2, 3, 4, and 5 weeks). Although a gradual self-recovery was observed over time after the cessation of the BAK treatment and the 5-week BAK treated model resulted the most stable, the authors postulated that 2-4 week BAK treatment can successfully produce a satisfactory and applicable dry eye model (assessing the fluorescein scores and goblet cell density). On this basis, twice-daily topical administration of BAK (0.1%) for 15 days was used as an approach to induce dry eye in rabbits in this thesis, following the protocols previously described. The model was characterized by corneal fluorescein staining and CIC. The fluorescein dye stains the damaged epithelial cells or the disrupted intercellular junctions in the cornea.[195,236,237] CIC,

on the other hand, is a technique whereby the superficial layers of the ocular surface are removed and evaluated; histological staining of CIC samples reveals the state and the number of the conjunctival epithelial cells and the goblet cells. During this study, both fluorescein and cytological scores increased significantly on day 7 and day 15 in BAK-treated eyes. The results demonstrated that twice-daily topical administration of BAK at 0.1% for 15 days causes significant alterations on the ocular surface of rabbits that are indicative of dry eye disease, including corneal damage and alterations in the conjunctival epithelial and goblet cells. These findings are consistent with the findings obtained in the aforementioned studies using the same method and suggest that the experimental dry eye model can be used as a platform to evaluate the efficacy of artificial tears.

The results obtained in the preliminary efficacy study of Formulation 3 in the experimental dry eye demonstrated a remarkable and statistically significant improvement in the ocular surface in as early as 7 days of the treatment (fluorescein and cytological scores decreased significantly compared with baseline). Corneal fluorescein staining results returned to normal in 7 days and the CIC results in 14 days. It should be noted that an improvement was observed on the ocular surface in the untreated control group as well; however, it was much slower, in agreement with the work of other authors.[212] The scores of the two groups were significantly different on day 14 (for corneal fluorescein staining) and on day 21 (for both corneal fluorescein staining and CIC). These preliminary findings, indicative of the improvement of corneal damage and the return of the conjunctival epithelial and goblet cells to their normal state after treatment with the artificial tear formulation, suggest that the formulation may be beneficial in restoring the impaired ocular surface in DED.

A limitation of the study was the small number of animals used in the development of the experimental model of dry eye and in the subsequent preliminary efficacy study, which may have affected the power of the statistical

analyses. Also, among the artificial tears developed, only Formulation 3 was tested. More comprehensive studies with larger sample sizes would be helpful to draw more conclusive results.

In conclusion, the results obtained in this study demonstrated that the artificial tear formulations developed in this thesis present suitable properties for topical ophthalmic administration and that they are well-tolerated. With the incorporation of the *in situ* gelling polymer gellan gum, the artificial tears behave as *in situ* gelling systems, which could be beneficial in prolonging the ocular residence time of the formulations. Topical administration of BAK may be a useful method to induce dry eye in rabbits, and the experimental model of dry eye developed this way, in turn, could be a useful platform for the efficacy studies of artificial tear formulations. The preliminary efficacy study of the test formulation presents a proof of concept with promising preliminary results. Due to their potential of restoring the tear film and improving the ocular surface, the novel formulations developed in this thesis may be beneficial in the treatment of DED.

4. PRELIMINARY EFFICACY STUDIES ON DRY EYE UTILIZING THE ASTON BIOLOGICAL ANTERIOR EYE MODEL

4.1. Introduction

In dry eye research, various animal models have been developed to elucidate the complex pathophysiology of the disease and to investigate the efficacy of new dry eye therapies.[173–188] While *in vivo* models constitute valuable tools in dry eye research, certain drawbacks such as high cost and time consumption, along with ethical considerations, have led to the exploration of alternative platforms.[238,239]

In vitro and *ex vivo* ocular models, progressing with the advances in tissue engineering and biomedical technologies, have gained interest to provide reliable alternatives to the *in vivo* models.[240–243] Compared with *in vitro* cell cultures that only provide partial information, *ex vivo* models possess the advantage of more closely reflecting the physiologic conditions and maintaining natural cell response. Furthermore, ocular surface *ex vivo* models offer the opportunity of tailoring several parameters such as blinking rate, temperature, and humidity, in order to induce and study dry eye at different severity levels and to investigate the effect of new dry eye treatments.[220,221]

F. Menduni and Prof. J.S. Wolffsohn from the Ophthalmic Research Group of Aston University (Birmingham, UK) have developed a novel anterior eye model named the "Aston Biological Anterior Eye Model".[244] In the framework of the European Dry Eye Network "EDEN", an optimization of the model and preliminary studies of the efficacy of the artificial tear formulations developed in this thesis were performed. The efficacies of the formulations were evaluated in terms of restoring and maintaining a healthy ocular surface in the physiologically active porcine eye model.

4.2. The Aston Biological Anterior Eye Model

The Aston Biological Anterior Eye Model is a novel *ex vivo* complete anterior eye model that uses porcine eyes (Figure 4.1).[244] In the model, porcine anterior segments are mounted in perfusion chambers and placed in a controlled system. The environmental control system is composed of a perfusion system and an irrigation system, designed to mimic tear replenishment and reproduce physiological conditions. The perfusion system supplies the endothelial back side of the mounted anterior segments with a physiological solution at controlled temperature, pressure, and flow rate; while the irrigation system hydrates the surface of the eye model at a controlled frequency. The model is placed in a laminar flow hood to avoid contamination.

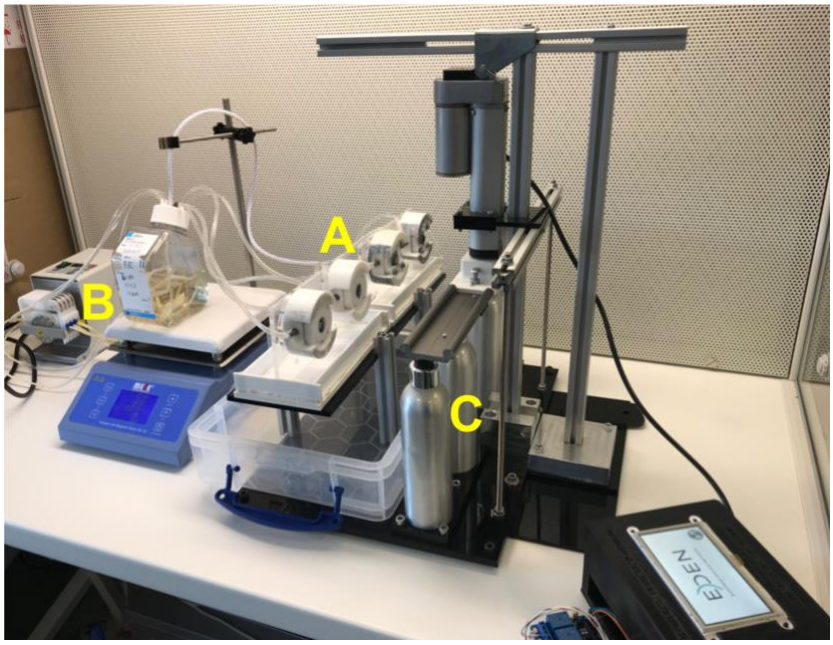

Figure 4.1. *The Aston Biological Anterior Eye Model developed by Menduni and Wolffsohn.[244] A: Perfusion chambers with mounted porcine anterior segments. B: perfusion system. C: irrigation system.*

4.2.1. Eyes

Porcine eyes were enucleated from white domestic pigs (12-25 weeks old) after the animals were killed at a local abattoir (waste material) in Birmingham, UK. The enucleated eyes were transported to the laboratory at 4°C in Dulbecco's Modified Eagle's Medium (Lonza, Berkshire, UK) supplemented with 20% w/v dextran (Sigma Aldrich, UK), 10% Foetal Bovine Serum (Sigma-Aldrich, UK), 1% v/v L-glutamine (Lonza, Berkshire, UK), 1% penicillin (10,000 units/ml; Sigma-Aldrich, UK) and streptomycin (10,000 mg/ml; Sigma-Aldrich, UK). The globes were dissected and mounted in the model immediately upon arrival.

The porcine eyes used in the initial experiments were collected from animals that underwent scalding process at the abattoir (Figure 4.2.A). For a potentially better tissue quality, collection of eyes not subjected to scalding was arranged for later experiments (Figure 4.2.B).

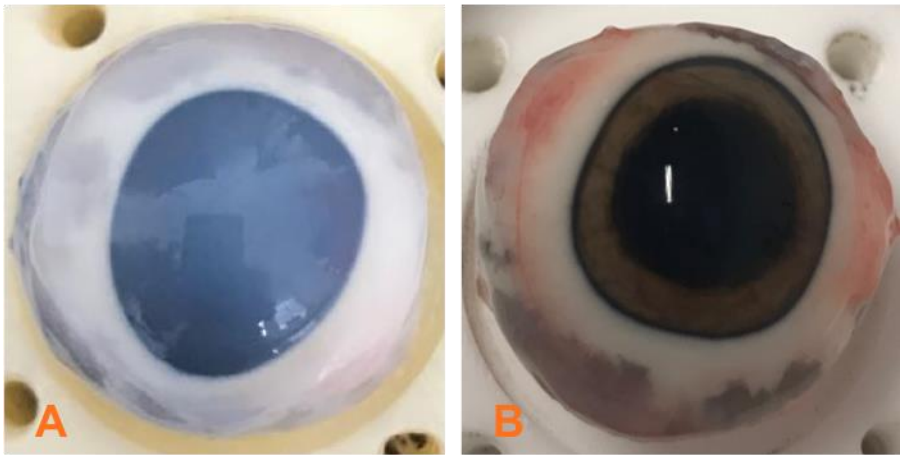

Figure 4.2. *Enucleated porcine eyes. A: subjected to scalding process. B: not subjected to scalding process.*

4.2.2. Chambers

Enucleated eyes were mounted using the suction and the perfusion chambers (Figure 4.3) made of polytetrafluoroethylene (PTFE), the biologically and chemically inert and autoclavable material.

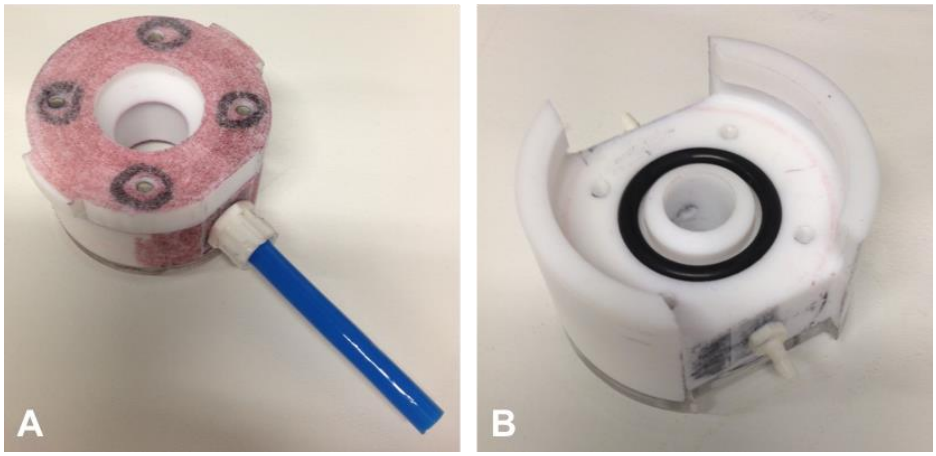

Figure 4.3. *Suction chamber (A) and perfusion chamber (B) made of PTFE.*

The surface aperture of the perfusion chamber was optimized to provide a larger area of exposure and facilitate access to conjunctiva in order to ensure optimal sampling in conjunctival impression cytology (CIC) assays (Figure 4.4).

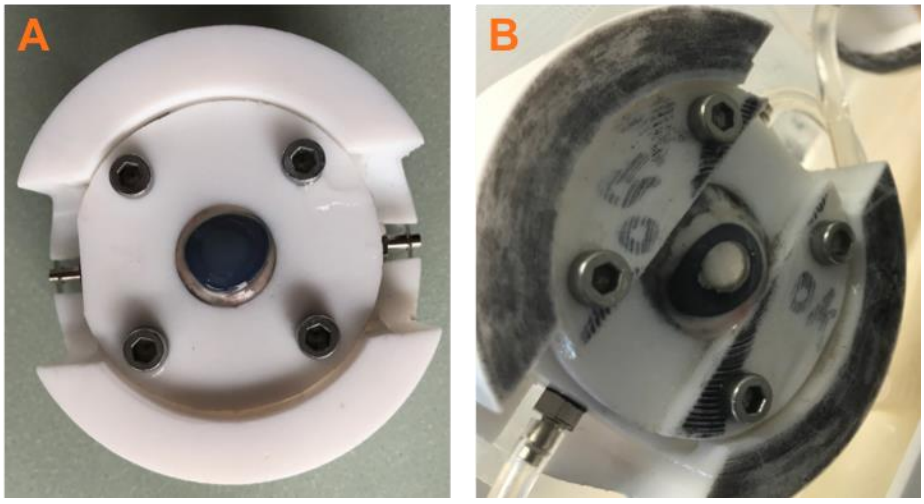

Figure 4.4. *Perfusion chamber, before (A) and after (B) surface aperture optimization.*

4.2.3. Mounting protocol

The enucleated eyeball is located in the suction chamber with the ocular surface facing down. A mild vacuum is created through suction with a syringe to ensure that the eyeball stays firmly in position during dissection. Dissection is performed using a vibrating blade (PMF 220 CE, Bosch), separating and discarding the posterior segment while keeping the anterior segment in the chamber. The perfusion chamber is clamped securely onto the suction chamber and the suction chamber is displaced, leaving the mounted anterior segment in the perfusion chamber. The mounting procedure is illustrated in Figure 4.5.

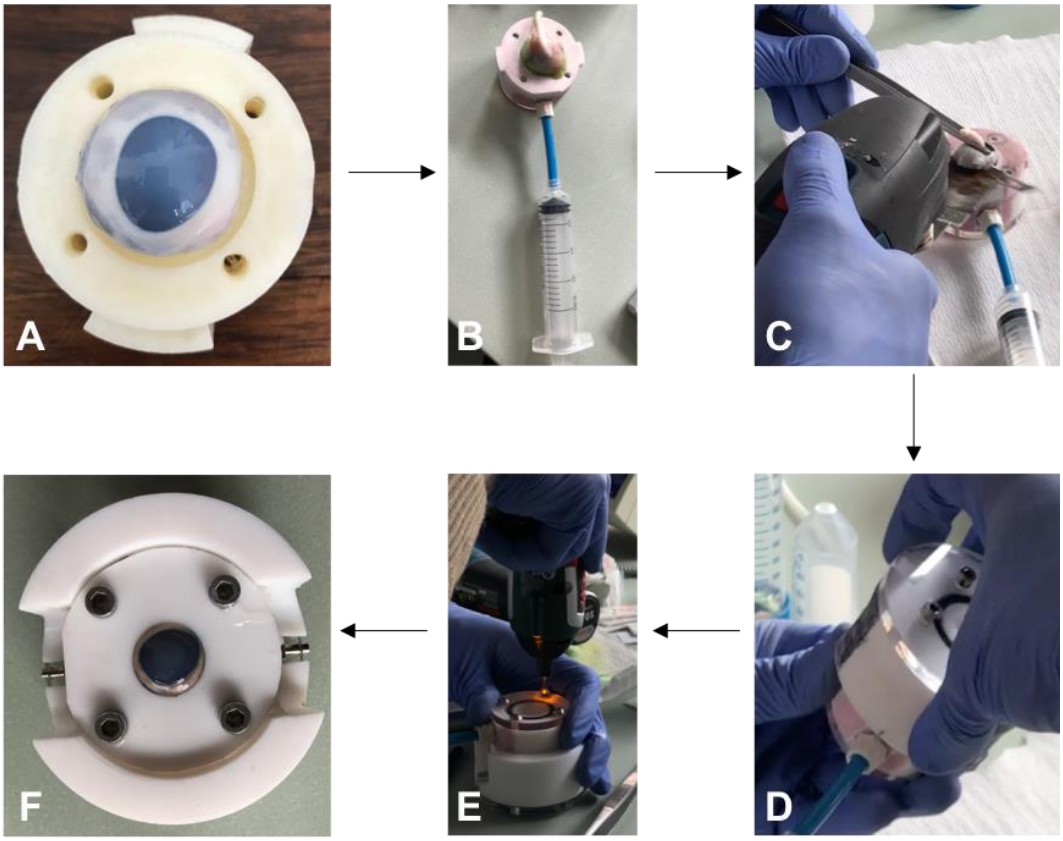

Figure 4.5. *Mounting procedure.* ***A:*** *Enucleated eye.* ***B:*** *Placement of the eyeball in the suction chamber.* ***C:*** *Dissection with a vibrating blade.* ***D:*** *Clamping of the perfusion chamber.* ***E:*** *Release of the suction chamber.* ***F:*** *Mounted anterior segment.*

4.2.4. Perfusion system

Endothelial perfusion of the mounted anterior segments was achieved by passing a culture medium, heated to 37°C with a hot plate (SH-5H, MESE Ltd., Leeds, UK), through the perfusion chambers via a multichannel peristaltic pump (ISM597, Ismatec REGLO, Wertheim, Germany). Each perfusion chamber possessed a separate inlet, through which it received the perfusion medium, and a separate outlet, from which the medium was circulated back to the reservoir (Figure 4.6). The perfusion medium was composed of Dulbecco's Modified Eagle's Medium (Lonza, Berkshire, UK) supplemeted with 10% Foetal Bovine Serum (Sigma-Aldrich, UK), 1% v/v L-glutamine (Lonza, Berkshire, UK), 1% penicillin (10,000 units/ml; Sigma-Aldrich, UK) and streptomycin (10,000 mg/ml; Sigma-Aldrich, UK). The flow rate of the perfusion medium was adjusted to 1 ml/min for continuous supply to the anterior segment. The perfusion medium was replaced with a fresh one every 24 hours when the model was run beyond 24 hours.

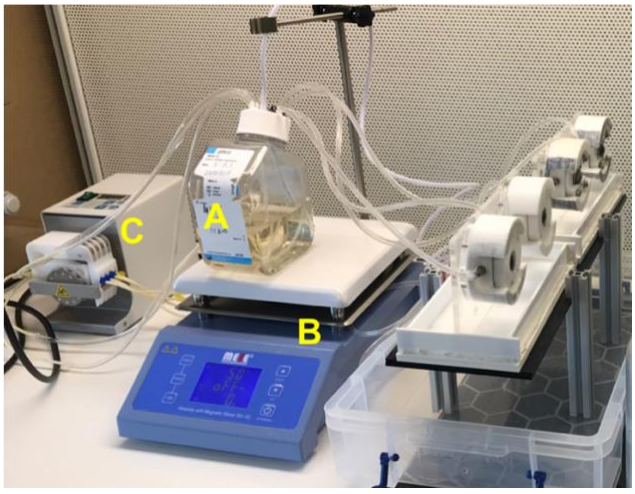

Figure 4.6. *Perfusion system. A: Perfusion medium. B: Hot plate. C: Multichannel peristaltic pump.*

4.2.5. Irrigation system

Epithelial irrigation of the mounted anterior segments was performed via an automated irrigation system, consisting of a spray unit and an electronic control unit (Figure 4.7). With the perfusion chambers placed vertically, the irrigation medium was sprayed onto the exposed surface of the eye model at set intervals in order to keep the epithelial surfaces hydrated, while simulating physiological conditions with repetitive tear replenishment and desiccation phases. The irrigation medium used in the system was the same as the perfusion medium. The system had a capacity of 4 spray bottles, allowing a maximum of 4 perfusion chambers with the mounted anterior segments to be used at once.

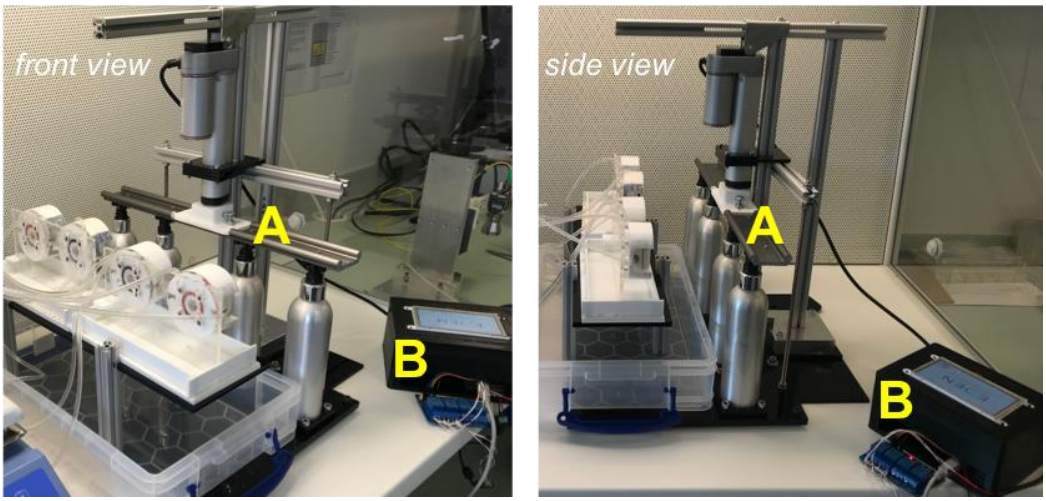

Figure 4.7. *Automated irrigation system; front and size views. A: Spray unit. B: Electronic control unit.*

4.3. Dry eye model optimization

Dry eye disease may arise due to reduced aqueous tear secretion or excessive tear evaporation, as described previously. Based on this fact, the dry eye state in the Aston Biological Anterior Eye Model was designed to be induced with a reduced irrigation frequency that simulates a decrease of the tear supplement to the surface of the eye and extend the recurring desiccation period.

The irrigation frequency for “normal” conditions and “dry eye” conditions in the porcine eyes were selected thanks to the assessment of the tear film stability by non-invasive tear break-up time (NIBUT) measurements, using a tearscope (EASYTEAR®view+ All in One, Easytear, Italy). With this method, a grid pattern was reflected on the tear film and the distortion of the reflected pattern, indicative of the formation of dry spots on the tear film, was evaluated.

Freshly enucleated porcine eyes (n=3) were mounted in the Aston Biological Anterior Eye Model and the grid pattern reflected on the corneas were videotaped after irrigation. Alterations in the reflected pattern and the time taken for their occurrence were evaluated. Representative images, extracted from the video at different time points, are illustrated in Figure 4.8.

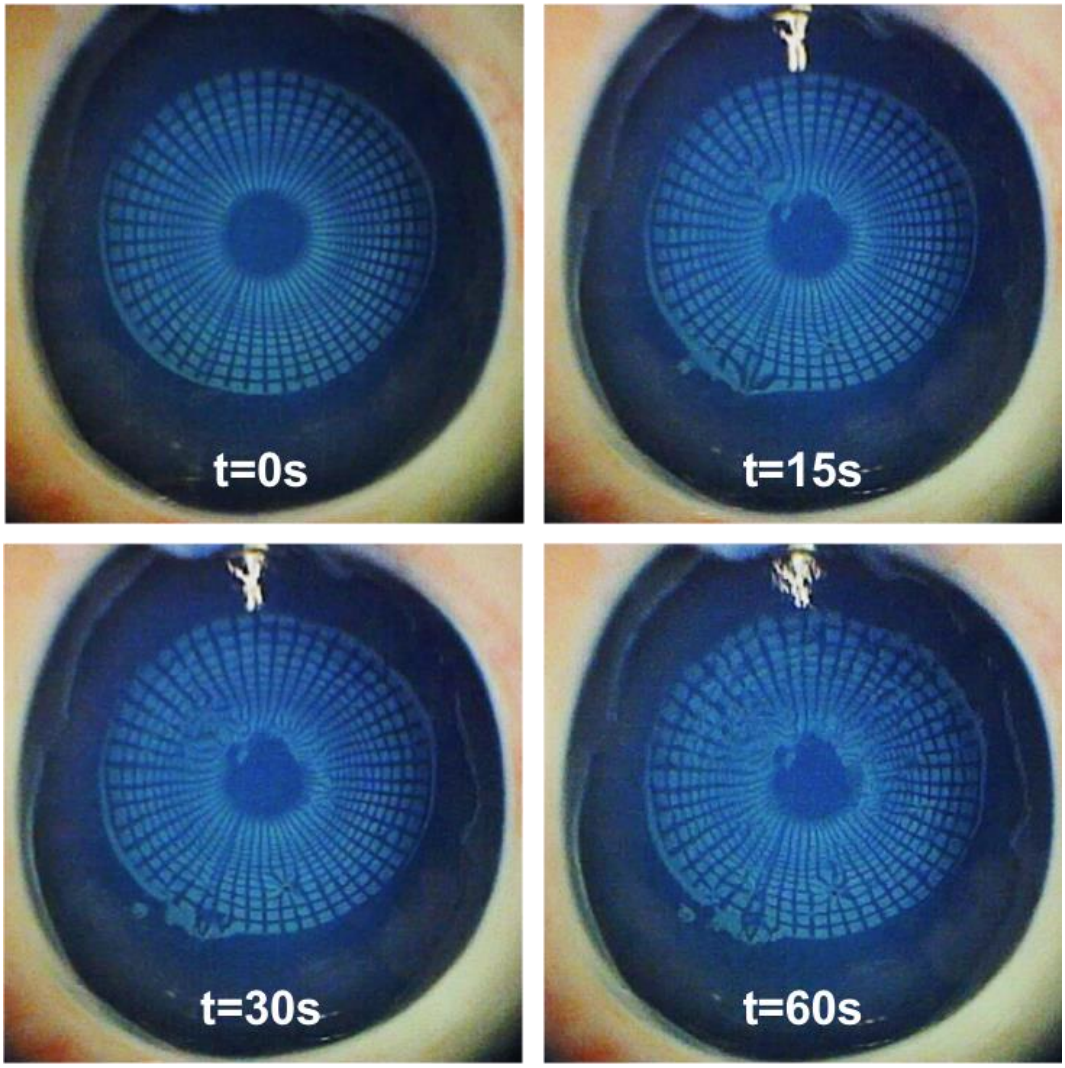

Figure 4.8. *Representative NIBUT images of a porcine eye after irrigation.*

As it can be seen from Figure 4.8, NIBUT measurements demonstrated that the tear film appears to lose its stability after 30 seconds. Based on this evaluation, an irrigation interval of 20 seconds for normal conditions and 40 seconds for dry eye conditions were selected. Also, corneal fluorescein staining and CIC were performed for the assessment of normal conditions and dry eye conditions, in eyes sprayed at relative selected frequencies.

Corneal fluorescein staining

Corneal fluorescein staining was performed in freshly enucleated porcine eyes (baseline; n=4) subjected to scalding process at the abattoir, and after a 2-hour irrigation period in the Aston Biological Anterior Eye Model with either 20-second intervals (normal state; n=2) or 40-second intervals (dry eye state; n=2). For each measurement, fluorescein stain was applied to the eyes with sterile strips (Bio Fluoro strips impregnated with 1 mg of sodium fluorescein, Biotech Vision Care Pvt Lt., India) and the corneal staining was evaluated under blue illumination with a yellow filter (Aston fluorescein enhancement filter) after 2 minutes.

No visible differences in corneal fluorescein staining were observed between the baseline, normal state, and dry eye state (Figure 4.9), which may be due to a possible tissue deterioration during the scalding process at the abattoir or during transportation/handling. These findings suggest that corneal fluorescein staining may not be a suitable test for the ocular surface assessment of the enucleated porcine eyes, collected post-scalding and mounted in the model.

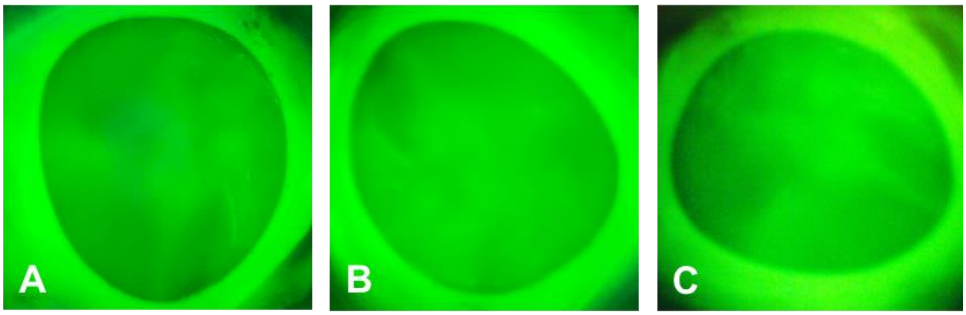

Figure 4.9. *Representative images of corneal fluorescein staining in porcine eyes.* ***A:*** *Freshly enucleated porcine eye (baseline).* ***B:*** *Porcine eye irrigated with 20-second intervals for 2 hours (normal state).* ***C:*** *Porcine eye irrigated with 40-second intervals for 2 hours (dry eye state). No visible differences were observed between the baseline, normal state, and dry eye state.*

Conjunctival impression cytology

CIC was performed in freshly enucleated porcine eyes (baseline; n=4) subjected to scalding process at the abattoir, and after a 2-hour irrigation period in the Aston Biological Anterior Eye Model with either 20-second intervals (normal state; n=2) or 40-second intervals (dry eye state; n=2). The assay and the microscopic evaluation (Leica light microscope, Leica Microsystems, Germany) of the samples were performed as described in Section 3.2.7.1. For the histological staining in the assay, periodic acid, Schiff's reagent, Papanicolaou's solution 1b Hematoxylin solution S, xylene, and the mounting medium Entellan® were purchased from Sigma-Aldrich (Dorset, UK), Supor® 200 membrane disc filters from Pall Corporation (Michigan, USA), and ethanol absolute from Fisher Scientific (Loughborough, UK).

Cytological scores and the corresponding Nelson grades of the model eyes at baseline (n=4), normal state (n=2), and dry eye state (n=2), obtained by the semi-quantitative estimation of five cytological criteria as described in Section 3.2.7.1, are reported in Table 4.1.

	Cytological score	Nelson grade
Baseline (n=4)	8.00 ± 0.82	2
2h; 20s irrigation interval (n=2)	7.50 ± 0.71	2
2h; 40s irrigation interval (n=2)	10.00 ± 2.83	3

Table 4.1. *Cytological scores and the corresponding Nelson grades obtained with different irrigation frequencies in the Aston Biological Anterior Eye Model.*

The microscopic evaluation of CIC samples revealed alterations in the number and the state of conjunctival epithelial cells; whereas no goblet cells were observed. The freshly enucleated eyes demonstrated a Nelson grade of 2 already at baseline. The eyes irrigated every 20 seconds for 2 hours, to establish normal conditions, maintained the same Nelson grade as baseline; whereas an increase in the cytological score and the Nelson grade was observed in the eyes irrigated every 40 seconds for 2 hours to establish dry eye conditions. Statistical analysis was not performed due to the small sample size. Representative CIC images are illustrated in Figure 4.10.

These findings suggest that 20-second and 40-second irrigation intervals may be suitable to establish normal and dry eye conditions (respectively) in the Aston Biological Anterior Eye Model. Furthermore, CIC may be a useful assay for the ocular surface evaluation in the model. Further studies with an increased number of eyes would be necessary to obtain conclusive results.

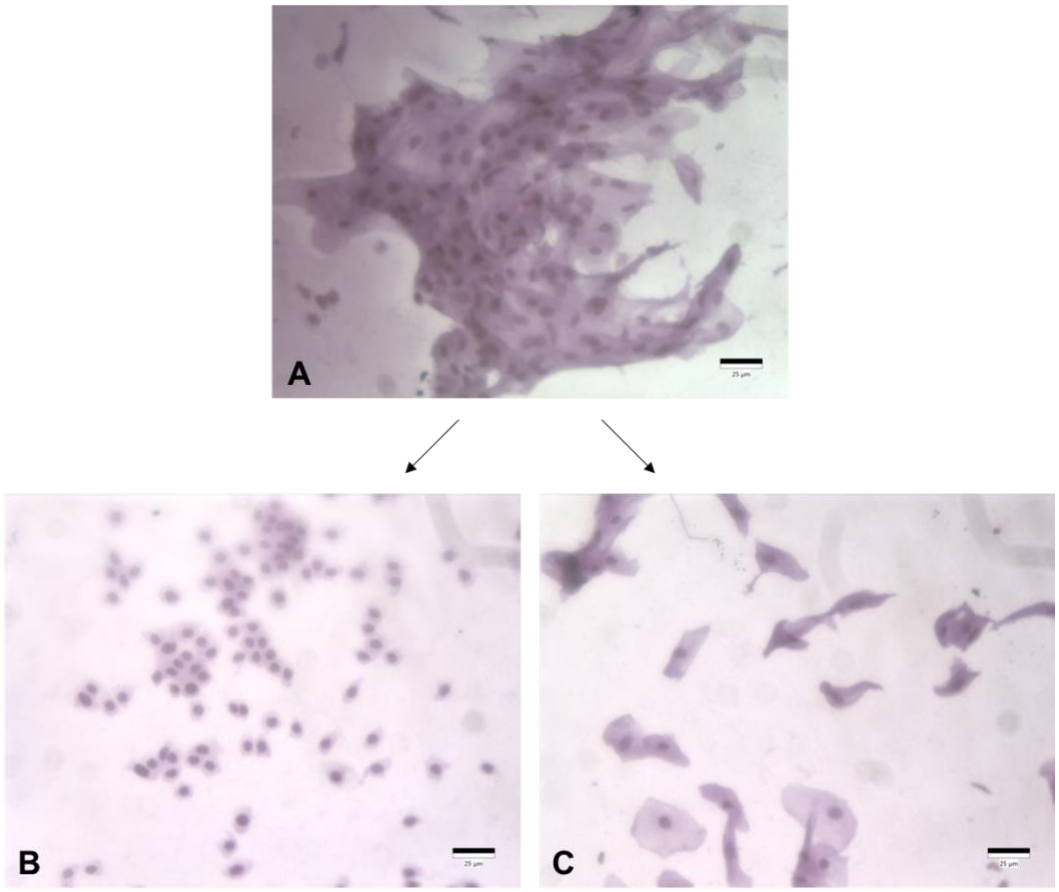

Figure 4.10. *Representative images of conjunctival impression cytology in porcine eyes.* ***A:*** *Freshly enucleated porcine eye (baseline). Nelson grade: 2. Epithelial cells (violet): large, mostly abnormally shaped, and moderate in number. Cell-cell contact: high. Nucleus/cytoplasm ratio: >1/5. Goblet cells: absent.* ***B:*** *Porcine eye irrigated with 20-second intervals for 2 hours (normal state). Nelson grade:2. Epithelial cells: smaller, and scarce to moderate in number. Cell-cell contact: moderate. Nucleus/cytoplasm ratio: 1/3–1/4. Goblet cells: absent.* ***C:*** *Porcine eye irrigated with 40-second intervals for 2 hours (dry eye state). Nelson grade:3. Epithelial cells: large, abnormally shaped, and scarce. Cell-cell contact: low. Nucleus/cytoplasm ratio: >1/5. Goblet cells: absent. Scale bars: 25 μm.*

4.4. Preliminary efficacy studies of the artificial tear formulations

Preliminary efficacy studies of the artificial tear formulations developed in this thesis were performed in the Aston Biological Anterior Eye Model with CIC. Freshly enucleated porcine eyes (n=4), not subjected to scalding process, were mounted in the model and irrigated with the irrigation medium with 40-second intervals for 2 hours, in order to establish dry eye conditions as previously described. 30 µL of Formulations 1a, 2a, and 3 were instilled in the model eyes (n=1 for each formulation) upon reaching dry eye conditions (at the end of 2 hours), and for a second time, 4 hours after the first instillation. One model eye was left untreated and used as control. The model eyes with the type of treatments they received during the study are illustrated in Figure 4.11. The irrigation frequency was kept constant and set to every 40 seconds to continue simulating dry eye conditions throughout the study.

CIC was performed in freshly enucleated porcine eyes (baseline; n=4), after the 2-hour irrigation period with 40-second intervals (dry eye state; t=0 of the treatment; n=4) and at 18 hours of the treatment (t=18h; n=1 for each formulation and the control) in the Aston Biological Anterior Eye Model.

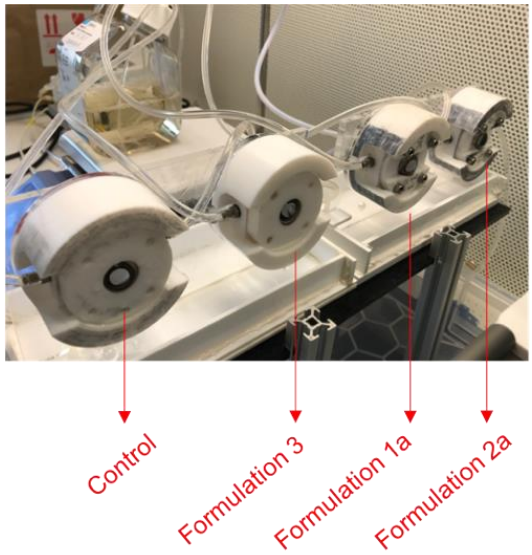

Figure 4.11. *Illustration of the model eyes with the corresponding treatments they received during the preliminary efficacy studies.*

Between the hours 4-18 of the treatment (overnight), the model was run unsupervised in the automated system. At hour 18, the spray bottles irrigating the eyes that received treatment with Formulations 1a and 2a were encountered in a rotated position, not providing the eyes with the irrigation medium. As the unintended cessation of the irrigation at an undetermined time point between the hours 4-18 caused excessive drying of the model eyes, the results relative to Formulations 1a and 2a were considered improper and are not included here.

Cytological scores and the corresponding Nelson grades of the model eyes at baseline (n=4), dry eye state (n=4), and at 18 hours of the treatment (only for Formulation 3 and the control; n=1 for each), obtained by the semi-quantitative estimation of five cytological criteria as described in Section 3.2.7.1, are reported in Table 4.2.

	Cytological score	Nelson grade
Baseline (n=4)	8.25 ± 0.50	2
Dry eye state; 0h (n=4)	10.25 ± 0.50	3
Control at 18h (n=1)	10.00	3
Formulation 3 at 18h (n=1)	4.00	1

Table 4.2. *Cytological scores and the corresponding Nelson grades in the Aston Biological Anterior Eye Model during preliminary efficacy studies.*

With two instillations of Formulation 3 in the model eye in dry eye state, an improvement in the cytological score and the Nelson grade was observed after 18 hours; while the control eye maintained its grade. Statistical analysis was not performed due to the small sample size. Goblet cells, previously absent, were observed in the treated eye after 18 hours. Representative CIC images are illustrated in Figure 4.12.

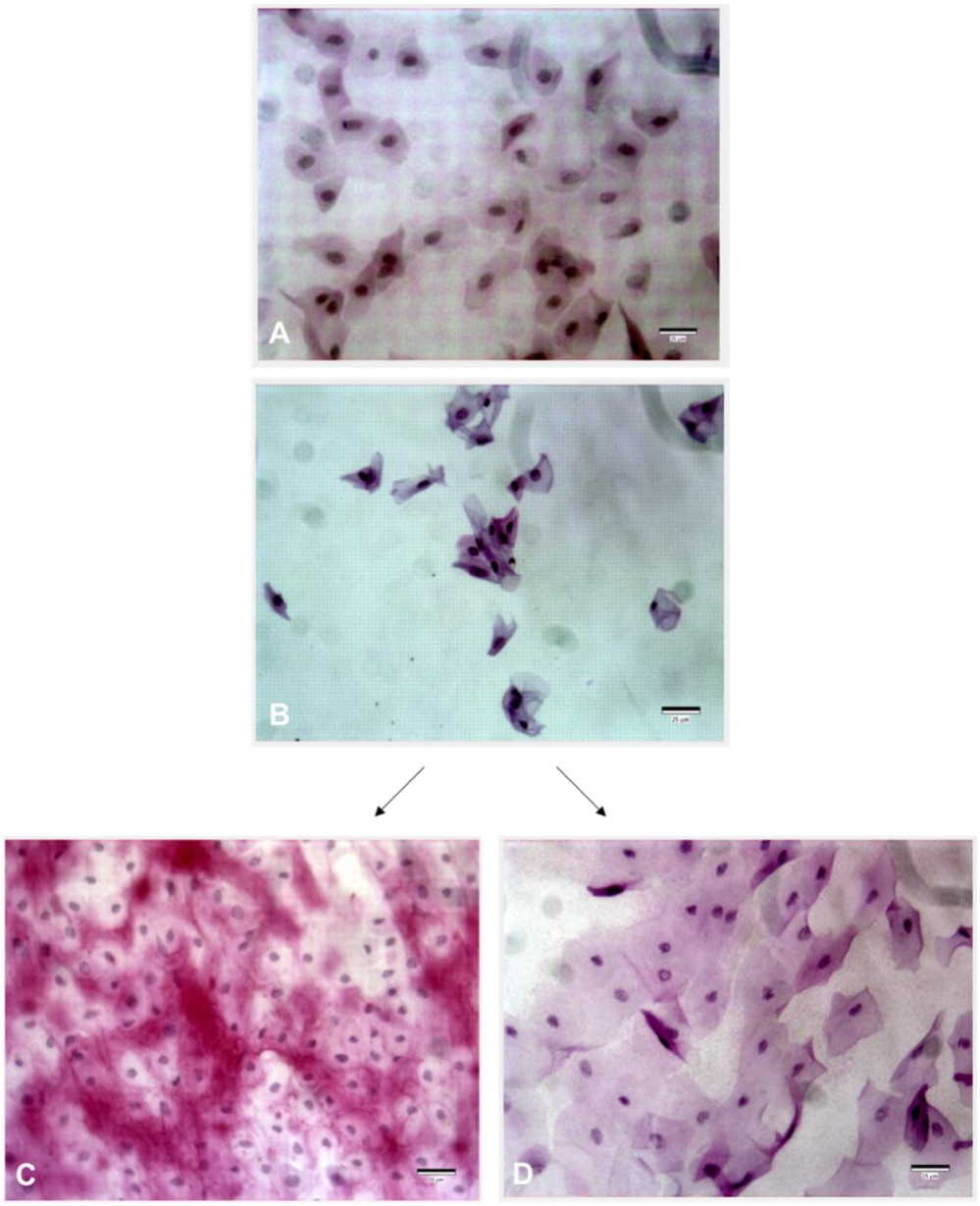

Figure 4.12. *Representative images of conjunctival impression cytology in the Aston Biological Anterior Eye Model during preliminary efficacy studies.* ***A:*** *Freshly enucleated porcine eye (baseline). Nelson Grade: 2. Epithelial cells (violet): large, mostly polygonal, and scarce to moderate in number. Cell-cell contact: low. Nucleus/cytoplasm ratio: varying from 1/3 to >1/5. Goblet cells: absent.* ***B:*** *Porcine eye irrigated with 40-second intervals for 2 hours (dry eye state; t=0 of the treatment). Nelson grade: 3. Epithelial cells: large, abnormally shaped, and scarce. Cell-cell contact: low. Nucleus/cytoplasm ratio: >1/5. Goblet cells: absent.* ***C:*** *Porcine eye treated with Formulation 3, t=18h. Nelson grade: 1. Epithelial cells: smaller and abundant. Cell-cell contact: high. Nucleus/cytoplasm ratio: 1/3–1/4. Goblet cells (pinkish-purple): poorly defined and moderate in number.* ***D:*** *Control eye, t=18h. Nelson grade: 3. Epithelial cells: large, abnormally shaped, and scarce to moderate in number. Cell-cell contact: moderate. Nucleus/cytoplasm ratio: >1/5. Goblet cells: absent. Scale bars: 25 μm.*

This preliminary study gives an insight into the use of Aston Biological Anterior Eye Model as a platform to study the effects of topical ophthalmic dry eye formulations, and into the efficacy of an artificial tear formulation developed in this thesis in improving the ocular surface in dry eye. The study had some limitations such as the small sample size and short duration, due to the technical problems related to the eye model; however, the preliminary results are promising. The findings suggest that the parameters of the Aston Biological Anterior Eye Model may be adjusted to mimic dry eye conditions and the platform may be a useful tool and a potential alternative to animal testing for the efficacy studies of artificial tear formulations. CIC findings related to Formulation 3 support the findings obtained in Chapter 3; suggesting that the artificial tear may be beneficial in improving the ocular surface in dry eye, with potential future application in the treatment of dry eye disease. Further experiments with a larger sample size/more repeats and longer duration are needed to obtain more conclusive results and establish the efficacy of the formulations.

5. NETOSIS STUDIES

5.1. Introduction

Neutrophil extracellular traps (NETs) were described in 2004 as extracellular fibers of nuclear and granule components released by neutrophils, and were shown to disarm and kill bacteria.[222] Over the past 15 years, NETs have attracted considerable attention; several aspects of these structures such as their morphology, components, and formation have been elucidated to an extent. Implications of NETs in health and disease continue to be explored.[245]

Morphological changes such as loss of the multilobulated structure of the nucleus, chromatin decondensation, disintegration of nuclear and granular membranes, mixing of nuclear and cytoplasmic contents have been observed during NET formation, denominated NETosis.[246,247] Extracellular DNA (eDNA), histones, and several proteins such as neutrophil elastase and myeloperoxidase have been identified as components of NETs.[5,248] Different mechanisms have been described for NET formation, including suicidal (or lytic) NETosis, resulting in the death of the neutrophil releasing the NET material, and vital NETosis, where the neutrophil survives the process and remains viable.[249] The cell death associated with NET formation is considered to be a new active cell death mechanism, different form apoptosis and necrosis.[246]

Beside the defense against pathogens as part the innate immune system, NETs are considered to be involved in other physiological events such as coagulation and inflammatory processes.[245,250] However, NET accumulation due to excessive production and/or impaired clearance may have negative effects; it has been associated with pathophysiological processes in autoimmune, autoinflammatory, and lung diseases, such as systemic lupus erythematosus, rheumatoid arthritis, diabetes, and cystic fibrosis.[251,252]

While NETs may be involved in the defense against pathogens in healthy eye, excessive amounts of NETs and eDNA have been found on the ocular surface and

in the tear fluid of dry eye disease (DED) patients.[5,6] Hyperosmolarity was shown to induce NET formation by neutrophils and postulated as a possible explanation of the elevated NET levels on the ocular surface of DED patients.[28] Furthermore, tear nucleases that can degrade and clear eDNA from the ocular surface were shown to be deficient in severe DED patients, resulting in the accumulation of NETs in the tear film.[5] The Tear Film and Ocular Surface Society Dry Eye WorkShop II that took place in 2017 with the participation of experts in dry eye field drew attention to the possible role of NETs in the pathogenesis of DED, emphasizing the need for further studies for better understanding the role of NETs in the disease and for the development of new therapeutic strategies.[4] In fact, DNase I (a nuclease normally found in the tear fluid) eye drops are already being investigated as a new therapeutic approach in DED.[6]

In the present thesis, a collaboration with the University of Birmingham, UK (with Prof. Graham Wallace from the Institute of Inflammation and Ageing at the University of Birmingham) was established to assess the effects of the artificial tear formulations developed in this thesis on NET formation.

5.2. Ethics

This study was performed at the research laboratories of the University of Birmingham in Queen Elizabeth Hospital Birmingham, UK, under existing ethics "Inflammation in Ocular Surface Disease" HRA rec. ref. 08/H1206/125, and with the written informed consent of the study volunteer.

5.3. Materials and methods

5.3.1. Materials

Dextran, Trypan blue solution, Triton™ X-100, phorbol 12-myristate 13-acetate, dimethyl sulfoxide (DMSO), and sodium chloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phosphate buffered saline (PBS; Calbiochem®) was supplied by Merck KGaA (Darmstadt, Germany), Percoll® plus by GE Healthcare (Buckinghamshire, UK), and VectaMount® mounting medium by Vector Laboratories (Peterborough, UK). Roswell Park Memorial Institute medium (with L-glutamine; Gibco), paraformaldehyde (4% in PBS; Alfa Aesar), Sytox™ Green nucleic acid stain (Invitrogen), and glass microscope slides ("Superfrost Plus", Thermo Scientific) were supplied by Thermo Fisher Scientific (Waltham, MA, USA). 24-well culture plates (Costar®) were purchased from Corning (Corning, NY, USA), 13 mm round glass cover slips from Paul Marienfeld GmbH & Co. KG (Lauda-Königshofen, Germany), and heparinized vacutainer (Vacuette® tube) from Greiner Bio-One (Kremsmünster, Austria).

5.3.2. Experimental procedure

Neutrophils were isolated from the blood of a healthy volunteer, counted, and seeded into the wells of culture plates. Artificial tear formulations prepared in this thesis were incubated with unstimulated neutrophils, to determine whether they induce NETosis, and with neutrophils stimulated with the NETosis-inducing agent phorbol 12-myristate 13-acetate (PMA), to assess their effects on PMA-induced NETosis.

Isolation of neutrophils

Neutrophils were isolated from human blood (collected the same day, in a heparinized vacutainer) with the following steps:

- In a laminar flow hood (Labculture Plus, Esco Micro Pte. Ltd., Singapore), the blood sample was mixed with 2% dextran (in 0.9% NaCl solution) at a dextran:blood ratio of 1:6 and left for 40 minutes for the sedimentation of red blood cells.

- The leukocyte rich plasma was removed from the dextran sedimentation, added dropwise to a Percoll gradient (2.5 mL of 72% and 5 mL of 50.4% Percoll in 0.9% NaCl), and centrifuged (Thermo Scientific Heraeus Megafuge 16, Thermo Fisher Scientific, Waltham, MA, USA) at 1100 rpm for 20 minutes.

- The neutrophil layer was then collected from the gradient, added to a centrifuge tube containing 10 mL of Roswell Park Memorial Institute (RPMI) medium, and centrifuged at 1600 rpm for 10 minutes to obtain a pellet of neutrophils. The supernatant was discarded and the pellet was resuspended in 5 mL of RPMI by tapping and swirling gently.

- Neutrophils were stained with Trypan blue (1:1) for live cells and counted with a haemocytometer (Improved Neubauer, Hawksley, Sussex, UK) under microscope (Zeiss Primo Star, Carl Zeiss AG, Oberkochen, Germany).

NET visualization

The artificial tear formulations were tested with unstimulated neutrophils and PMA-stimulated neutrophils. PMA was diluted in RPMI from a 1.6 mM stock solution (in DMSO) and had an in-well concentration of 25 nM.

- In a laminar flow hood, 13 mm round glass cover slips were placed into the wells of 24-well culture plates. $2x10^5$ neutrophils were seeded into each well in RPMI

(total volume was made up to 500 µL or 490 µL, for wells without and with PMA, respectively) and the culture plates were incubated in a CO_2 controlled incubator (Thermo Scientific, Waltham, MA, USA) for 30 minutes at 37°C for neutrophil adherence to the cover slips.

- 10 µL of the NETosis-stimulating agent PMA (2.5 µM) was added to the wells where NETosis stimulation was desired. 500 µL of each test formulation was added into their corresponding wells (3 wells without PMA and 3 wells with PMA for each formulation) and 500 µL of RPMI was added to the control wells (3 unstimulated controls and 3 PMA-stimulated controls), bringing the total volume in each well to 1 mL. The culture plates were incubated in the incubator for 3 hours at 37°C.

- The supernatant from each well was aspirated and discarded. The cover slips were covered with paraformaldehyde (4% in PBS; sufficient volume to cover the surface of the slips) and the culture plates were incubated in the incubator for a further 30 minutes at 37°C.

- The samples were washed with PBS, permeabilized with Triton X-100, and stained with the nucleic acid stain Sytox green. These washes were performed using an apparatus adapted from Brinkmann et al.,[253] made by placing and pressing parafilm strips over a test tube rack to create "craters" (Figure 5.1). A drop of each wash was placed into the craters. The cover slips were removed from the culture plates gently with tweezers, placed face-down onto the first wash and passed through the following washes:

- PBS: 3 times for 5 minutes
- Triton X-100 (0.1% in PBS): 1 minute
- PBS: 5 minutes
- Sytox green (1 µM in PBS): 5 minutes
- PBS: 5 minutes

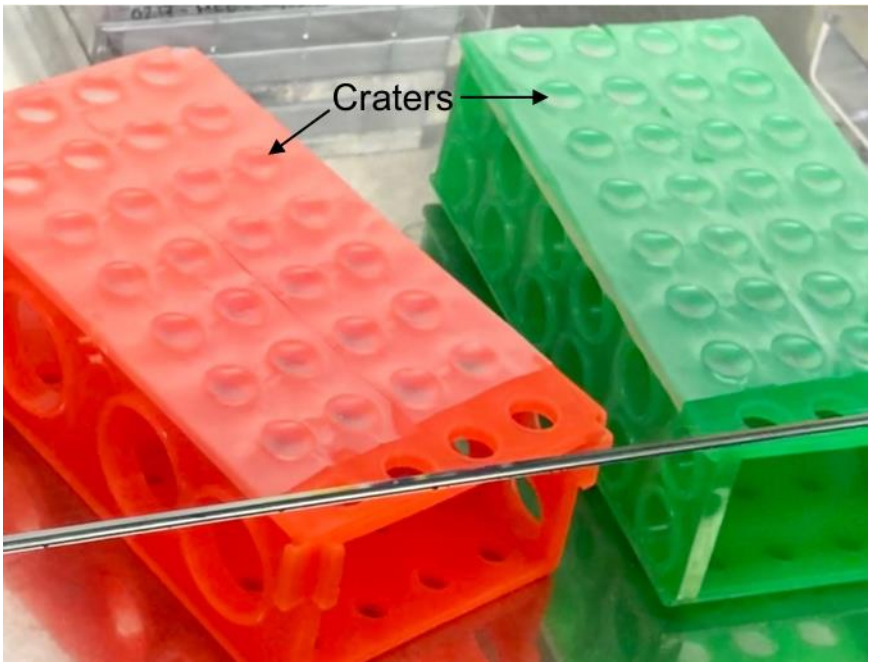

Figure 5.1. *The apparatus used for washing and staining the samples in the NETosis study. The craters contain a drop of the washes.*

- The cover slips were mounted face-down onto microscope slides with the permanent mounting medium Vectamount and left overnight.

- Samples were analyzed under fluorescence microscope (Leica DMI 6000B, Leica Microsystems, Wetzlar, Germany) at 400X magnification (excitation: 490 nm, emission: 523 nm). Three images were taken from different areas of each coverslip and the NETosis rate was calculated for each image as follows:

$$\text{NETosis rate } (\%) = \frac{\text{Number of neutrophils undergoing NETosis}}{\text{Total number of neutrophils}} \text{ x } 100$$

The assessment was made according to the morphology of the stained nucleus and the nucleic acid content; loss of the multilobulated nuclear structure, nuclear swelling indicative of chromatin decondensation, and mixing of nuclear and cytoplasmic contents were accepted to be indicative of neutrophils undergoing NETosis, as demonstrated and recognized as such by previous studies.[246,247] The cells were counted manually with ImageJ/FIJI software (Version 1.52i). The mean

NETosis rate was calculated for each specimen using the corresponding three images.

5.3.3. Statistical analysis

Statistical analyses were performed using the SPSS software (Version 24, Chicago, USA). The Kolmogorov-Smirnov test was performed to determine whether the data was normally distributed; the data showed a non-normal distribution. Therefore, the Mann-Whitney test was used for the comparisons of the NETosis rates. P-values were two-tailed and $P<0.05$ was considered to be statistically significant.

5.4. Results

NETosis rates of unstimulated neutrophils and neutrophils stimulated with the NETosis-inducing agent PMA, incubated with the artificial tear formulations, are reported in Table 5.1.

	NETosis rate (%)	
	Unstimulated neutrophils	**PMA-stimulated neutrophils**
Control	34.74 ± 4.44	100.00 ± 0.00
Formulation 1	21.75 ± 3.23	26.92 ± 10.12
Formulation 1a	27.95 ± 9.46	31.15 ± 6.47
Formulation 2	33.74 ± 13.14	49.73 ± 16.74
Formulation 2a	26.94 ± 13.06	35.56 ± 14.72
Formulation 3	30.37 ± 18.80	49.89 ± 28.03

Table 5.1. *NETosis rates of unstimulated and PMA-stimulated neutrophils incubated with the artificial tear formulations.*

Unstimulated and untreated neutrophils (unstimulated control) had a NETosis rate of 34.74 ± 4.44% whereas PMA-stimulated untreated neutrophils (PMA-stimulated control) had a NETosis rate of 100%. In unstimulated neutrophils (Figure 5.2A), no significant differences were found in NETosis rates between the control and the treatments *(P>0.05)*, except for Formulation 1, which reduced the NETosis rate significantly *(P<0.001)*.

In PMA-stimulated neutrophils (Figure 5.2B), three-hour incubation with each of the artificial tear formulations significantly decreased the NETosis rates compared with the PMA-stimulated control *(P<0.001)*, the greatest decrease being observed with Formulation 1. All of the formulations, except for Formulation 2, reduced the NETosis rates of the PMA-stimulated neutrophils to a degree that was not significantly different than the NETosis rate of the unstimulated control *(P>0.05)*. Formulations 1 and 1a showed a higher efficiency compared with Formulation 2.

Representative images of the PMA-stimulated neutrophils incubated with the artificial tear formulations, stained with the nucleic acid stain Sytox green, and visualized with fluorescence microscope, are illustrated in Figure 5.3.

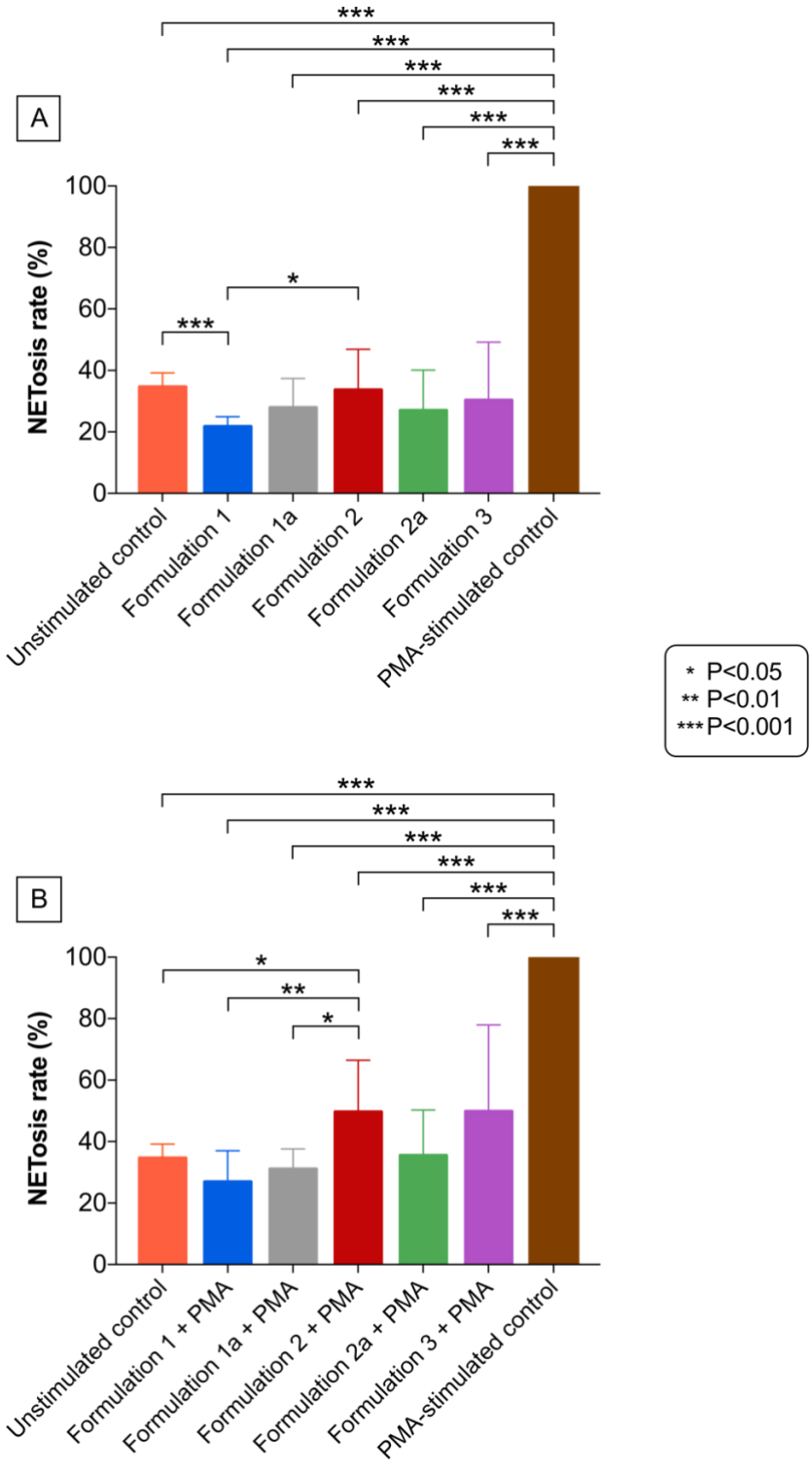

Figure 5.2. *NETosis rates of neutrophils after a three-hour incubation with the artificial tear formulations.* ***A:*** *In unstimulated neutrophils, no increase was observed in NETosis rates for any of the formulations compared with the control.* ***B:*** *All of the formulations significantly decreased the NETosis rates in PMA-stimulated neutrophils compared with the PMA-stimulated control.*

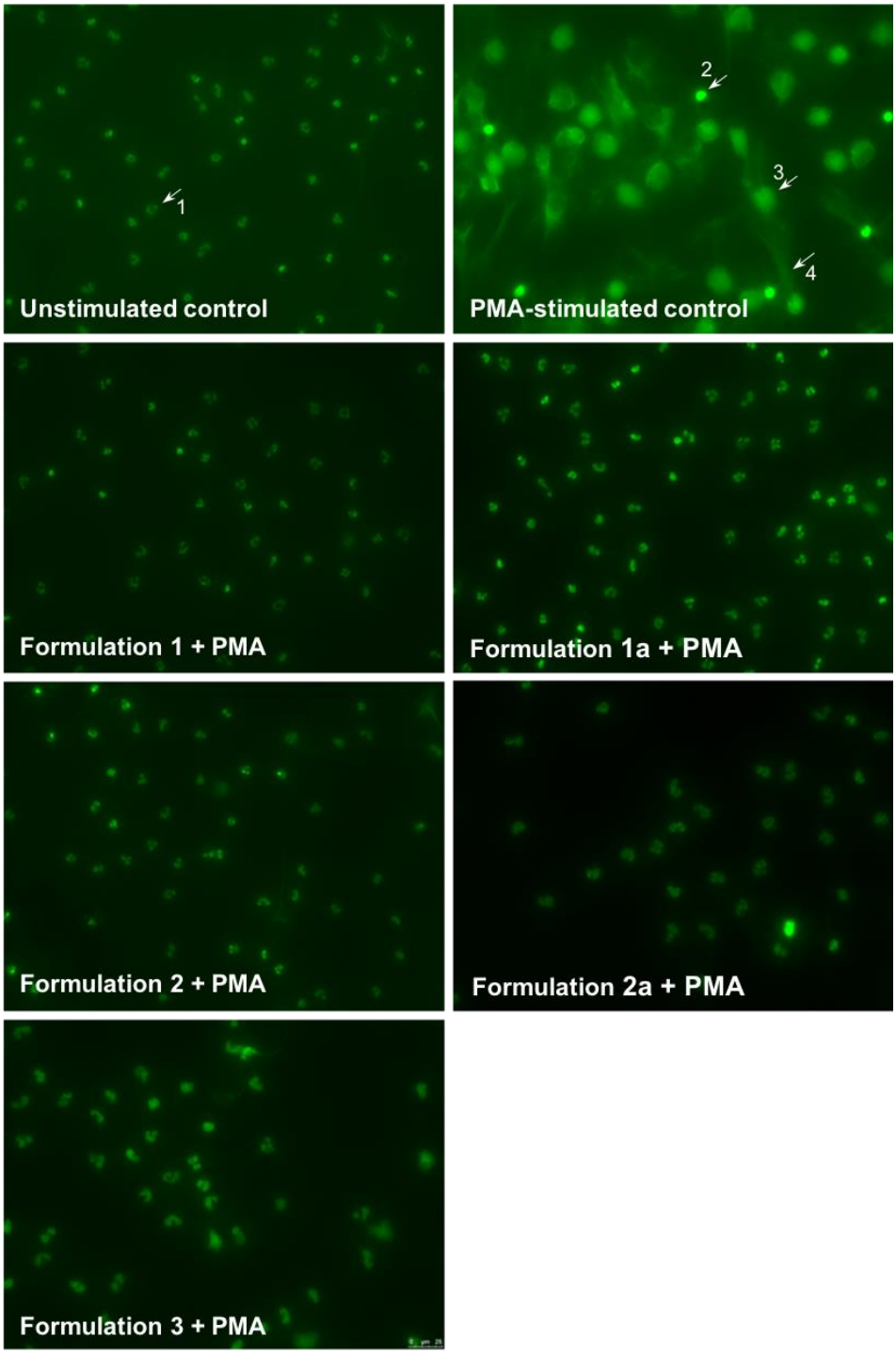

Figure 5.3. *Representative images of the PMA-stimulated neutrophils incubated with the artificial tear formulations. The samples were stained with the nucleic acid stain Sytox green and visualized with fluorescence microscope (magnification 400X).* ***Unstimulated control:*** *Nuclei of the neutrophils are mostly multilobulated (arrow 1).* ***PMA-stimulated control:*** *Nuclei lost their multilobulated form; nuclear swelling (arrow 2), mixing of nuclear and cytoplasmic contents (arrow 3), and NETs (arrow 4) are visible. In PMA-stimulated neutrophils incubated with the artificial tear formulations, nuclei are mostly multilobulated, resembling the unstimulated control. Scale bar: 25 µm.*

5.5. Discussion

NETs and their implications in health and disease have gained much interest since they were first identified in 2004. Excessive amounts of NETs have been found on the ocular surface of dry eye patients and linked to inflammation and the pathogenesis of DED.[5,6] Therefore, decreasing NET formation on the ocular surface may be a useful new strategy in the treatment of DED. The aim of this study was to evaluate the effects of the formulations developed in this thesis on NET formation. With this purpose, the artificial tears were incubated with both unstimulated and PMA-stimulated neutrophils.

The results obtained in this study demonstrated that the artificial tear formulations developed in this thesis did not cause a negative response in neutrophils that would manifest with increased NET formation. Furthermore, all of the five formulations decreased PMA-induced NETosis significantly after a 3-hour incubation, with the most effective being Formulation 1, containing liposomes (phosphatidylcholine 2%), the bioadhesive polymer hydroxypropyl methylcellulose (0.5%), the osmoprotectant levocarnitine (0.1%), the antioxidants vitamin A (0.002%) and vitamin E (0.02%), fructose (1.8%), and borates (boric acid 0.84% and sodium tetraborate anhydrous 0.08%). The present preliminary study was performed with neutrophils isolated from the blood of one healthy donor. Further studies with neutrophils obtained from different donors and with more repeats would help better establish the efficiency of the formulations in decreasing NET formation. It would also be interesting to test the formulation components individually. eDNA quantification as an indicator of the amount of NETs generated might also be a helpful additional technique in future studies.

6. GENERAL DISCUSSION

Dry eye disease (DED) is characterized by a loss of homeostasis of the tear film, where tear film instability and hyperosmolarity, and ocular surface inflammation and damage are involved.[2] Re-establishing the homeostasis of the tear film and restoring the ocular surface are key in the treatment of the disease. Artificial tears are deemed first-line therapy for mild dry eye and may also be employed in more advanced stages of the disease in combination with other therapies.[7] However, most of the commercially available artificial tears replace only the aqueous phase of the tear film and provide a symptomatic relief rather than restoring the tear film and treating the disease. Another shortcoming of such formulations is their short residence time on the ocular surface.[8]

The objective of this doctoral thesis was to design and develop novel artificial tear formulations that effectively replenish the tear film and restore the ocular surface for the treatment of DED. With this purpose, five innovative artificial tear formulations with a range of components were designed and developed in this thesis. The novel formulations were characterized in terms of their physicochemical properties. For the formulations containing an *in situ* gelling agent, *in situ* gelling was simulated and rheological properties were evaluated. Tolerance of the five artificial tears was evaluated *in vivo*. The following steps were the development or optimization of new tools to evaluate the efficacy of the formulations and the subsequent preliminary efficacy studies. Finally, the effects of the artificial tears on NETosis, a recently popular phenomenon, were evaluated as a proof of concept.

Design of novel artificial tear formulations

A comprehensive literature review was conducted to gain knowledge of the advances in dry eye therapies and to select the formulation components. After a thorough investigation, novel artificial tears were designed to incorporate both aqueous and lipid components, taking into consideration key physicochemical characteristics. Different strategies were used to more closely resemble natural

tears, replenish both the aqueous-mucin and the lipid layer of the tear film, and ensure a prolonged ocular residence time. With this purpose, the formulations contained liposomes and/or a bioadhesive polymer (hydroxypropyl methylcellulose) and/or an *in situ* gelling polymer (gellan gum). The artificial tears were further enriched with components having antioxidant properties (vitamins A, C, and E), osmoprotectant properties (levocarnitine and trehalose), and antidessicant properties (trehalose) for an enhanced potential therapeutic efficacy in DED.

Physicochemical characterization

The novel artificial tear formulations were characterized in terms of pH, surface tension, viscosity, osmolarity, and liposome size, to ensure suitability for topical ophthalmic administration. The pH of the artificial tears was neutral to slightly alkaline (7.0–7.6), in an optimum pH range for ophthalmic preparations considering the very similar pH (7.0–7.5) and the buffering capacity of natural tears.[79,80,82–84] The surface tension values of the formulations (44.6–48.7 mN/m) were within or slightly above the physiological range of natural tears (40–46 mN/m)[87] and the viscosity values (3.6–12.1 mPa.s) within the physiological range (1.0–8.3 mPa.s)[17,93] or above the range without being excessively high. These features together would contribute to the uniform mixing of the artificial tears with natural tears and their proper spreading over the ocular surface, without compromising tear film stability or causing adverse effects such as visual disturbance and ocular discomfort.[90] The formulations were slightly hypotonic (222.7–272.7 mOsm/L), constituting a favorable attribute considering the tear hyperosmolarity in DED patients.[233,234] Moreover, the average sizes (191.0–200.1 nm) and the unimodal size distribution exhibited by liposomes were suitable for an ophthalmic formulation. The results show that the artificial tear formulations demonstrate suitable physicochemical properties for topical ophthalmic administration.

Simulation of in situ gelling and rheological studies

In order to simulate *in situ* gelling on the ocular surface, the artificial tears containing an *in situ* gelling agent (gellan gum at 0.25%; Formulations 2, 2a, and 3) were mixed with simulated tear fluid (STF) at a proportion reflecting *in situ* conditions,[226] and their rheological properties were evaluated. When mixed with STF, these formulations showed viscoelastic properties with elastic gel behavior. Furthermore, the gels demonstrated shear-thinning behavior and their dynamic viscosities at elevated shear rates (2.8–3.0 mPa.s; at 800–1100 s^{-1}) were in the physiological range of natural tears. These rheological findings suggest that the gellan gum containing formulations can form viscoelastic gels with suitable viscosities upon ophthalmic administration and may extend the residence time of the formulations on the ocular surface.

In vivo tolerance studies

In vivo tolerance studies of the artificial tear formulations were performed in rabbits (n=6). The formulations were administrated every 30 minutes for 6 hours; the ocular surface and the clinical signs were evaluated macroscopically and graded over a 24-hour period, following a previously described protocol.[227] The animals did not show any clinical signs or indicators of discomfort for any of the artificial tear formulations (grade 0) throughout the experiments, indicating that the formulations are well tolerated in rabbits.

Development or optimization of new tools to evaluate the efficacy of artificial tears

In dry eye research, preclinical research constitutes an important step for investigating the efficiency of new treatments. In this thesis, an *in vivo* dry eye model was developed as a platform to assess the efficacy of the artificial tear formulations. Dry eye was induced in rabbits (n=8) by twice daily topical

ophthalmic administration of the preservative benzalkonium chloride (BAK) at 0.1% for 15 days.[211,212] Corneal fluorescein staining and conjunctival impression cytology assays revealed significant changes on the ocular surface of the animals at the end of the BAK treatment, such as corneal damage, goblet cell loss, and alterations in conjunctival epithelial cells, which are encountered in DED. These findings suggest that the experimental dry eye model obtained with the topical administration of BAK could be a useful platform for the efficacy studies of new DED treatments.

The author collaborated with researchers from Aston University to optimize a complete *ex vivo* anterior eye model developed at Aston University,[244] for its utilization as an alternative tool for the efficacy studies. "Normal" conditions and "dry eye" conditions in the porcine eye model, called the Aston Biological Anterior Eye Model, were optimized by modifying the irrigation (with a tear analogue) frequency of the eyes. An irrigation interval of 20 seconds for normal conditions and 40 seconds for dry eye conditions were selected based on the assessment of non-invasive tear break-up time of porcine eyes and validated by conjunctival impression cytology. This *ex vivo* dry eye model, using waste material and offering the possibility of adjusting several environmental parameters as desired, may be a potential alternative to *in vivo* dry eye models.

Preliminary efficacy studies

Preliminary efficacy studies of Formulation 3, containing the *in situ* gelling agent gellan gum, the bioadhesive polymer hydroxypropyl methylcellulose, levocarnitine, vitamin C, trehalose, electrolytes and borates, were performed *in vivo* and *ex vivo*, in the previously described rabbit dry eye model and the Aston Biological Anterior Eye Model, respectively.

In the rabbit dry eye model, the model eyes were treated with the test formulation three times a day for a period of 21 days (n=6 for the treatment group and n=2 for the control group). Significant improvements in the ocular surface was observed

in as early as 7 days of the treatment (significantly reduced fluorescein and cytological scores compared with baseline), with scores returning to normal levels in 7 days for corneal fluorescein staining and in 14 days for conjunctival impression cytology. These findings showing the repair of corneal damage, increase in goblet cell density, and the return of the conjunctival epithelium to its healthy state, indicate the restoration of the ocular surface with the formulation. An improvement in the ocular surface was observed in the control group as well, though much slower than in the treatment group. The difference between the two groups resulted significant on day 14 (for corneal fluorescein staining) and on day 21 (for both assays). The study had some limitations such as the small sample size, which may have reduced the power of the statistical analysis. Also, due to the time limitation, only one of the artificial tears was tested. Nonetheless, the preliminary findings are promising and suggest that the novel artificial tear formulation developed in this thesis may be beneficial in restoring the ocular surface in dry eye, with potential future application in the treatment of DED. Further studies with larger sample sizes, which would allow for more potent statistical analysis, are needed to draw more concrete conclusions.

In the *ex vivo* dry eye model, the efficacy of the artificial tear formulation in restoring the ocular surface of the physiologically active porcine eye model was assessed. With two instillations of Formulation 3 (at t=0 and t=4h), an improvement in the number and the state of the conjunctival epithelial cells was observed after 18 hours. Goblet cells, previously absent, were observed in the treated eye after 18 hours. In this preliminary study, statistical analysis was not performed due to the small sample size (n=1) for the treatment and the control. Preliminary conjunctival impression cytology findings obtained in this study support the findings obtained in the rabbit dry eye model. Further studies with a larger sample size/more repeats over a longer period of treatment would help obtain more conclusive results.

NETosis studies

The possible involvement of neutrophil extracellular traps (NETs) in inflammation and DED pathogenesis has recently been emphasized.[4,5] On this basis, the effects of the artificial tears on NET formation by unstimulated and stimulated neutrophils (undergoing NETosis) were assessed as a proof of concept, in collaboration with researchers from the University of Birmingham. In unstimulated neutrophils, the formulations did not cause an increase in NET formation compared with the untreated control. In neutrophils stimulated with the NETosis-inducing agent PMA, all of the five artificial tears reduced the formation of NETs significantly after a 3-hour incubation; the greatest reduction was observed with Formulation 1, containing liposomes (phosphatidylcholine 2%), the bioadhesive polymer hydroxypropyl methylcellulose (0.5%), the osmoprotectant levocarnitine (0.1%), the antioxidants vitamin A (0.002%) and vitamin E (0.02%), fructose (1.8%), and borates (boric acid 0.84% and sodium tetraborate anhydrous 0.08%). Testing the formulation components separately would be helpful in understanding the effects of the individual components on NETosis. This study was performed with neutrophils obtained from one healthy donor and constitutes a proof of concept. In light of the promising preliminary results, the artificial tears may be further investigated for their beneficial effects in decreasing NET formation in dry eye, where NETs are found in excessive amounts on the ocular surface.[5,6]

Prospects for future research

This doctoral thesis focused on the development of novel artificial tear formulations for the treatment of DED. With this purpose, five novel artificial tear formulations with suitable physicochemical properties and good *in vivo* tolerance (in rabbits) were developed in the thesis. Preliminary preclinical efficacy studies revealed promising results regarding the efficiency of the tested formulation in restoring an impaired ocular surface. Considerable reductive effects of the

formulations on NET formation were also observed in a preliminary study. Furthermore, the thesis provided insight into the feasibility and the potential use of an *in vivo* and a novel *ex vivo* dry eye model as a platform to evaluate the efficacy of new DED treatments.

Prospects for future research include the establishment of the safety and efficacy of all of the five formulations with more comprehensive preclinical and clinical studies, for their potential future use in the treatment of DED. Moreover, the reductive effects of the formulations and the individual formulation components on NETosis can be further explored as an additional favorable quality in DED treatment. The present research can be further broadened by determination of the ocular residence time of the artificial tears with *in vivo* studies.

7. CONCLUSIONS

1. All of the five novel artificial tear formulations developed in this doctoral thesis, containing different combinations of liposomes (made up of phosphatidylcholine and cholesterol), a bioadhesive polymer (hydroxypropyl methylcellulose), an *in situ* gelling polymer (gellan gum), osmoprotectants (levocarnitine and trehalose), antioxidants (vitamins A, C, and E), and other formulation excipients (fructose, electrolytes, and borates), demonstrate suitable physicochemical properties for topical ophthalmic administration and are well tolerated in rabbits.

2. Incorporation of gellan gum at 0.25% with electrolytes created artificial tears with *in situ* gelling properties, capable of producing viscoelastic gels that suggest an extended ocular residence time of the ophthalmic formulations.

3. Twice-daily topical ophthalmic administration of the preservative benzalkonium chloride at 0.1% for 15 days induces significant alterations on the ocular surface of rabbits that are indicative of dry eye disease. In the conditions described in this thesis, the experimental dry eye model can be used as a platform to evaluate the efficacy of artificial tears.

4. According to the preliminary efficacy study performed in the *in vivo* dry eye model, the novel artificial tear containing the *in situ* gelling polymer gellan gum (0.25%), the bioadhesive polymer hydroxypropyl methylcellulose (0.12%), the osmoprotectants levocarnitine (0.4%) and trehalose (1.68%), the antioxidant vitamin C (0.01%), borates (boric acid 0.84% and sodium tetraborate anhydrous 0.08%), and electrolytes (sodium chloride 0.05% and potassium chloride 0.05%) may be beneficial in the treatment of dry eye disease.

8. CONCLUSIONES

1. Las cinco formulaciones de lágrimas artificiales desarrolladas en la presente tesis doctoral conteniendo diferentes combinaciones de liposomas (compuestos por fosfatidilcolina y colesterol), un polímero bioadhesivo (hidroxipropilmetilcelulosa), un polímero gelificante *in situ* (goma gelano), agentes osmoprotectores (levocarnitina y trehalosa), agentes antioxidantes (vitaminas A, C y E) y otros excipientes como fructosa, electrolitos y boratos presentaron unas propiedades fisicoquímicas que resultan adecuadas para la administración tópica oftálmica. Además, dichas formulaciones presentaron una tolerancia óptima *in vivo* (conejos).

2. La incorporación de goma gelano a una concentración del 0,25% con electrolitos generó la formación de lágrimas artificiales con propiedades gelificantes *in situ,* capaces de producir geles viscoelásticos que sugieren un tiempo de residencia ocular prolongado para las formulaciones oftálmicas.

3. La administración del conservante cloruro de benzalconio 0,1% dos veces al día durante 15 días induce alteraciones significantes en la superficie ocular de conejos que son indicativas de la enfermedad del ojo seco, incluyendo daño corneal, reducción de la densidad de células caliciformes y alteraciones en el epitelio conjuntival. El modelo experimental de ojo seco, en las condiciones ensayadas, puede ser empleado como plataforma para evaluar la eficacia de lágrimas artificiales.

4. De acuerdo con los estudios preliminares de eficacia llevados a cabo en el modelo animal de ojo seco, la nueva lágrima artificial conteniendo el polímero gelificante *in situ* (goma gelano 0,25%), un polímero bioadhesivo como la hidroxipropilmetilcelulosa (0,12%), sustancias con actividad osmoprotectora (levocarnitina 0,4% y trehalosa 1,68%), el antioxidante vitamina C (0,01%), boratos (ácido bórico 0,84% y tetraborato de sodio anhidro 0,08%) y electrolitos (cloruro sódico 0,05% y cloruro potásico 0,05%), podría resultar beneficiosa en el tratamiento de la enfermedad de ojo seco.

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APPENDIX

A. Scientific dissemination

The work developed during this doctoral thesis has been published in a peer-reviewed scientific journal and presented at several conferences.

Publication:

- D. Acar, I.T. Molina-Martínez, M. Gómez-Ballesteros,M. Guzmán-Navarro, J.M. Benítez-del-Castillo, R. Herrero-Vanrell, **Novel liposome-based and in situ gelling artificial tear formulation for dry eye disease treatment**, Contact Lens and Anterior Eye 41 (2018) 93–96. https://doi.org/10.1016/j.clae.2017.11.004

Conference presentations:

- **Novel liposomal artificial tear formulation for dry eye disease treatment**. D. Acar, M. Gómez-Ballesteros, S. Esteban-Pérez, R. Ayesa-Cea, J.M. Benítez-del-Castillo, R. Herrero-Vanrell, I.T. Molina-Martinez. Barcelona 2017 – European Academy of Optometry and Optics (EAOO) Annual Conference, Barcelona, Spain, 12-14 May 2017. Poster presentation.
- **Novel liposomal artificial tear formulation containing gellan gum for dry eye disease treatment.** D. Acar, M. Gómez-Ballesteros, S. Esteban-Pérez, R. Ayesa-Cea, J.M. Benítez-del-Castillo, M. Guzmán-Navarro, R. Herrero-Vanrell, I.T. Molina-Martinez. British Contact Lens Association (BCLA) Clinical Conference, Liverpool, UK, 9-11 June 2017. Oral presentation.
- **Artificial tear with *in situ* gelling properties for the treatment of dry eye disease.** D. Acar, I.T. Molina-Martínez, J.J. López-Cano, J.M. Benítez-del-Castillo, R. Herrero-Vanrell. The Association for Research in Vision and Ophthalmology (ARVO) Annual Meeting, Honolulu, Hawaii, USA. 29 April-3 May 2018. Poster presentation.
- **Artificial tear with *in situ* gelling properties for the treatment of dry eye disease.** D. Acar, I.T. Molina-Martínez, J.J. López-Cano, James S. Wolffsohn, J.M. Benítez-del-Castillo, R. Herrero-Vanrell. Life & Health Sciences (LHS)

Postgraduate Research Day, Aston University, Birmingham, UK, 27 June 2018. Poster presentation.

- ***In situ* gelling artificial tear for dry eye disease treatment.** J. J. López-Cano, D. Acar, M. Caballo-Gonzalez, M.Guzmán-Navarro, J. M. Benítez-Del-Castillo, R. Herrero-Vanrell, I.T. Molina-Martínez. XIV Congreso de la Sociedad Española de Farmacia Industrial y Galénica, Santiago de Compostela, Spain, 23-25 January 2019. Oral and poster presentations.

B. Project authorization for the determination of the *in vivo* tolerance of topical ocular formulations in rabbits

CONSEJERÍA DE MEDIO AMBIENTE, ADMINISTRACIÓN LOCAL Y ORDENACIÓN DEL TERRITORIO
Comunidad de Madrid

REGISTRO DE SALIDA
Ref:10/050571.9/16 Fecha:11/03/2016 13:45
Cons. Medio Amb., Admon. Local y O. T.
Reg C.Medio Amb.Adm.Local y Ord.T.(ALC)
Destino: Facultad de Farmacia - A/A Irene Teresa

Vista la solicitud presentada por IRENE TERESA MOLINA MARTÍNEZ, para la autorización del proyecto de memoria técnica titulada DETERMINACIÓN DE LA TOLERANCIA IN VIVO EN CONEJOS DE FORMULACIONES OCULARES ADMINISTRADAS POR VÍA TÓPICA: a desarrollar en el centro usuario CAI ANIMALARIO UCM con código de registro ES280790000086 y siendo el responsable del proyecto IRENE TERESA MOLINA MARTÍNEZ.

Visto el informe del Área de Protección Animal.

Considerando que el citado proyecto se ajusta a lo establecido en el Real Decreto 53/2013 de 1 de febrero por el que se establecen las normas básicas aplicables para la protección de los animales utilizados en experimentación y otros fines científicos, incluyendo la docencia.

Esta Dirección General ha resuelto: autorizar la realización del proyecto referenciado siempre que se mantengan las condiciones que dieron lugar a la autorización y que el personal que intervenga tenga la preparación y formación adecuada que se especifica en el citado Real Decreto.

Tal y como se establece en el informe de evaluación aportado, este proyecto no deberá ser sometido a la realización de una evaluación retrospectiva.

Contra esta Resolución, que no agota la vía administrativa, cabe interponer recurso de Alzada en el plazo de un mes, contado desde el día siguiente al de la recepción de esta notificación, ante el Viceconsejero de Medio Ambiente, Administración Local y Ordenación del Territorio, conforme a lo establecido en el artículo 114.1 de la Ley 30/1992, de Régimen Jurídico de las Administraciones Públicas y del Procedimiento Administrativo Común .

Madrid, 09 de marzo de 2016.
EL DIRECTOR GENERAL DE AGRICULTURA Y GANADERÍA
P.D.F. Resolución 2 de noviembre de 2015
EL SUBDIRECTOR GENERAL DE RECURSOS AGRARIOS

[redacted]

Ref PROEX 011/16

CONSEJERÍA DE MEDIO AMBIENTE,
ADMINISTRACIÓN LOCAL
Y ORDENACIÓN DEL TERRITORIO
Comunidad de Madrid

REGISTRO DE SALIDA
Ref:10/050571.9/16 Fecha:11/03/2016 13:45
Cons. Medio Amb., Admon. Local y O. T.
Reg C.Medio Amb.Adm.Local y Ord.T.(ALC)
Destino: Facultad de Farmacia - A/A Irene Teresa

Ref PROEX 011/16

FACULTAD DE FARMACIA
Att: IRENE TERESA MOLINA MARTÍNEZ
PLAZA RAMÓN Y CAJAL S/N
28040 MADRID

Se adjunta resolución del Director General de Agricultura y Ganadería conforme a su solicitud de fecha ref. 10/013849.9/16

Madrid, 09 de marzo de 2016.
LA TÉCNICA VETERINARIA
ÁREA DE PROTECCIÓN ANIMAL

[redacted]

C. Project authorization for the development of a DED model in rabbits and the evaluation of new formulations for DED treatment

CONSEJERÍA DE MEDIO AMBIENTE, ADMINISTRACIÓN LOCAL Y ORDENACIÓN DEL TERRITORIO
Comunidad de Madrid

REGISTRO DE SALIDA
Ref:10/027272.9/17 Fecha:03/02/2017 10:42
Cons. Medio Amb., Admon. Local y O. T.
Reg C.Medio Amb.Adm.Local y Ord.T.(ALC)
Destino: Facultad de Farmacia (UCM) - A/A Rocio H

Vista la solicitud presentada por ROCÍO HERRERO VANRELL, para la autorización del proyecto de memoria técnica titulada DESARROLLO DE UN MODELO DE SÍNDROME DE OJO SECO (SOS) EN CONEJOS. EVALUACIÓN DE NUEVAS FORMULACIONES PARA EL TRATAMIENTO DEL SOS: a desarrollar en el centro usuario CAI ANIMALARIO UCM con código de registro ES280790000086 y siendo el responsable del proyecto ROCÍO HERRERO VANRELL.

Visto el informe del Área de Protección Animal.

Considerando que el citado proyecto se ajusta a lo establecido en el Real Decreto 53/2013 de 1 de febrero por el que se establecen las normas básicas aplicables para la protección de los animales utilizados en experimentación y otros fines científicos, incluyendo la docencia.

Esta Dirección General ha resuelto: autorizar la realización del proyecto referenciado siempre que se mantengan las condiciones que dieron lugar a la autorización y que el personal que intervenga tenga la preparación y formación adecuada que se especifica en el citado Real Decreto.

Tal y como se establece en el informe de evaluación aportado, este proyecto no deberá ser sometido a la realización de una evaluación retrospectiva.

Contra esta Resolución, que no agota la vía administrativa, cabe interponer recurso de alzada en el plazo de un mes, contado desde el día siguiente a la recepción de esta notificación, ante el Viceconsejero de Medio Ambiente, Administración Local y Ordenación del Territorio, conforme a lo establecido en el artículo 121 y siguientes de la Ley 39/2015, de 1 de octubre, del Procedimiento Administrativo Común de las Administraciones Públicas.

Madrid, 25 de enero de 2017.
EL DIRECTOR GENERAL DE AGRICULTURA Y GANADERÍA
P.D.F. Resolución 2 de noviembre de 2015
EL SUBDIRECTOR GENERAL DE RECURSOS AGRARIOS

[redacted]

Ref PROEX 316/16

CONSEJERÍA DE MEDIO AMBIENTE, ADMINISTRACIÓN LOCAL Y ORDENACIÓN DEL TERRITORIO
Comunidad de Madrid

REGISTRO DE SALIDA
Ref:10/027272.9/17 Fecha:03/02/2017 10:42
Cons. Medio Amb., Admon. Local y O. T.
Reg C.Medio Amb.Adm.Local y Ord.T.(ALC)
Destino: Facultad de Farmacia (UCM) - A/A Rocio H

Ref PROEX 316/16

FACULTAD DE FARMACIA (UCM)
Att: ROCÍO HERRERO VANRELL
PLAZA RAMÓN Y CAJAL, S/N
28040 MADRID

Se adjunta resolución del Director General de Agricultura y Ganadería conforme a su solicitud de fecha ref. 99/169259.9/16

Madrid, 25 de enero de 2017.
LA TÉCNICA VETERINARIA
ÁREA DE PROTECCIÓN ANIMAL

[redacted]

D. Certificate of competence for the use of animals for scientific purposes (EU functions A and C; in lagomorphs)

CONSEJERÍA DE MEDIO AMBIENTE, ADMINISTRACIÓN LOCAL Y ORDENACIÓN DEL TERRITORIO
Comunidad de Madrid

Ref.: CAP-0379-17

D./D.ª DUYGU ACAR
C/ LONDRES, 18, 1 CT IZ.
28028 MADRID- MADRID

Notifico a Vd. que el Sr. Director General de Agricultura y Ganadería, ha dictado la siguiente Resolución:

"Vista la solicitud formulada por D./DÑA. DUYGU ACAR , con DNI Y4453315T, para obtener el reconocimiento de la capacitación para realizar las funciones de CUIDADO DE LOS ANIMALES, REALIZACIÓN DE LOS PROCEDIMIENTOS, en LAGOMORFOS.

Visto el informe favorable del Área de Protección Animal.

Considerando que el solicitante cumple los requisitos para estimar su solicitud, por lo dispuesto en la Orden ECC/566/2015, de 20 de marzo, por la que se establecen los requisitos de capacitación que debe cumplir el personal que maneje animales utilizados, criados o suministrados con fines de experimentación y otros fines científicos, incluyendo la docencia.

Esta Dirección General ha resuelto: reconocer la capacitación a D./DÑA. DUYGU ACAR , con DNI Y4453315T, para realizar las funciones de CUIDADO DE LOS ANIMALES, REALIZACIÓN DE LOS PROCEDIMIENTOS, en LAGOMORFOS.

El mantenimiento de esta capacitación se debe demostrar al menos cada ocho años, en los términos que establece el artículo 20 de la Orden ECC/566/2015, de 20 de marzo, a partir de la fecha de esta Resolución.

Contra esta Resolución, que no agota la vía administrativa, cabe interponer recurso de alzada en el plazo de un mes, contado desde el día siguiente a la recepción de esta notificación, ante el Viceconsejero de Medio Ambiente, Administración Local y Ordenación del Territorio, conforme a lo establecido en el artículo 121 y siguientes de la Ley 39/2015, de 1 de octubre, del Procedimiento Administrativo Común de las Administraciones Públicas."

Se adjunta Certificado de Capacitación.

Madrid, 03 de marzo de 2017
ÁREA DE PROTECCIÓN ANIMAL
La Jefa de la Sección Técnica I

[redacted]

REGISTRO DE SALIDA
Ref:10/065580.9/17 Fecha:08/03/2017 14:11
Cons. Medio Amb. Admon. Local y O.T.
Reg C.Medio Amb.Adm.Local y Ord.T.(ALC)
Destino: Duygu Acar

CONSEJERÍA DE MEDIO AMBIENTE,
ADMINISTRACIÓN LOCAL
Y ORDENACIÓN DEL TERRITORIO
Comunidad de Madrid

CAP-0379-17

RESOLUCIÓN DE RECONOCIMIENTO DE LA CAPACITACIÓN E INCLUSIÓN EN EL REGISTRO DE PERSONAL QUE MANEJA ANIMALES UTILIZADOS, CRIADOS O SUMINISTRADOS CON FINES DE EXPERIMENTACIÓN Y OTROS FINES CIENTÍFICOS, INCLUYENDO LA DOCENCIA (Orden ECC/566/2015, de 20 de marzo)

Vista la solicitud formulada por D./DÑA. DUYGU ACAR , con DNI Y4453315T, para obtener el reconocimiento de la capacitación para realizar las funciones de CUIDADO DE LOS ANIMALES, REALIZACIÓN DE LOS PROCEDIMIENTOS, en LAGOMORFOS.

Visto el informe favorable del Área de Protección Animal.

Considerando que el solicitante cumple los requisitos para estimar su solicitud, por lo dispuesto en la Orden ECC/566/2015, de 20 de marzo, por la que se establecen los requisitos de capacitación que debe cumplir el personal que maneje animales utilizados, criados o suministrados con fines de experimentación y otros fines científicos, incluyendo la docencia.

Esta Dirección General ha resuelto: reconocer la capacitación a D./DÑA. DUYGU ACAR , con DNI Y4453315T, para realizar las funciones de CUIDADO DE LOS ANIMALES, REALIZACIÓN DE LOS PROCEDIMIENTOS, en LAGOMORFOS.

El mantenimiento de esta capacitación se debe demostrar al menos cada ocho años, en los términos que establece el artículo 20 de la Orden ECC/566/2015, de 20 de marzo, a partir de la fecha de esta Resolución.

Contra esta Resolución, que no agota la vía administrativa, cabe interponer recurso de alzada en el plazo de un mes, contado desde el día siguiente a la recepción de esta notificación, ante el Viceconsejero de Medio Ambiente, Administración Local y Ordenación del Territorio, conforme a lo establecido en el artículo 121 y siguientes de la Ley 39/2015, de 1 de octubre, del Procedimiento Administrativo Común de las Administraciones Públicas.

Madrid, 03 de marzo de 2017
EL DIRECTOR GENERAL DE AGRICULTURA Y GANADERÍA
(P.D.F. Resolución 2 de noviembre de 2015)
EL SUBDIRECTOR GENERAL DE RECURSOS AGRARIOS

[redacted]

CONSEJERÍA DE MEDIO AMBIENTE,
ADMINISTRACIÓN LOCAL
Y ORDENACIÓN DEL TERRITORIO
Comunidad de Madrid

Certificado de reconocimiento de la capacitación para manejar animales utilizados, criados o suministrados con fines de experimentación y otros fines científicos, incluyendo la docencia. Orden ECC/566/2015, de 20 de marzo

D./Dña. **DUYGU ACAR** , con DNI **Y4453315T**, ha obtenido el reconocimiento de la capacitación para realizar las funciones de:

CUIDADO DE LOS ANIMALES

REALIZACIÓN DE LOS PROCEDIMIENTOS

en los siguientes grupos de especies animales:

LAGOMORFOS

Nº de certificado: CAP-0379-17

ORGANISMO QUE EXPIDE EL CERTIFICADO

Dirección General de Agricultura y Ganadería. Consejería de Medio Ambiente, Administración Local y Ordenación del Territorio. Comunidad de Madrid

El reconocimiento de la capacitación para la realización de las funciones relacionadas en este certificado surtirá efecto en todo el territorio nacional.

Fecha	**Sello**
3 de marzo de 2017	COMUNIDAD DE MADRID - DIRECCIÓN GENERAL DE AGRICULTURA Y GANADERÍA - CONSEJERÍA DE MEDIO AMBIENTE, ADMINISTRACIÓN LOCAL Y ORDENACIÓN DEL TERRITORIO

EL DIRECTOR GENERAL DE AGRICULTURA Y GANADERÍA

(P.D.F. Resolución 2 de noviembre de 2015)

EL SUBDIRECTOR GENERAL DE RECURSOS AGRARIOS

Fdo [redacted]