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*The effect of experimental variables on the formulation of
ophthalmic inserts, fabricated by solvent casting method*

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LIST OF ABBREVIATIONS

mm – Millimetre

mg – Milligram

kg – Kilogram

µl – Microliter

mN – Millinewton

Da – Daltons

kDa – Kilodaltons

MPa – Megapascal

mOsm – Milliosmole

min – Minute

pH – Potential hydrogen

% w/w – Percentage weight per weight

% w/v – Percentage weight per volume

kV – Kilovolt

cp – Centre point

API – Active pharmaceutical ingredient

BAB – Blood-aqueous barrier

BRB – Blood-retinal barrier

RCE – Retinal capillary endothelial cells

RPE – Retinal pigment epithelial cells

ALG – Sodium alginate

k-Carr – Kappa Carrageenan

PVA – Polyvinyl alcohol

GLY – Glycerine

PBS – Phosphate-buffered saline

DSC – Differential scanning calorimetry

DSA – Drop shape analysis

BCOP – Bovine Cornea Opacity/Permeability

SEM – Scanning electron microscopy

DoE – Design of experiment

1. ABSTRACT

Background and Aim: Ocular drug delivery is a very challenging process due to the complex and selective anatomy of the eye. Precorneal loss factors such as solution drainage, induced lacrimation, tear film, tear turn over, blinking, conjunctival and non-productive absorptions, and also layered structure of the cornea are the main restrictions of the ocular drug absorption. Conventional ocular drug delivery systems are not good enough to overcome the challenges. Ocular inserts are one of the novel therapeutics with many advantages including controlled sustained drug release and increased retention time resulting in better bioavailability, reduced eye irritation and less administration frequency resulting in better patient compliance. The aim of the study was to examine the effect of the polymer and the plasticiser concentration on the formulation of ocular inserts to produce better featured inserts.

Methods: Experimental grid was generated by design of experiment (DoE), Minitab 17 software. Ocular inserts containing sodium alginate (ALG), polyvinyl alcohol (PVA), k-Carrageenan (k-Carr) and glycerol (GLY) were produced by solvent casting method with varying polymer and plasticiser concentrations. Casted inserts were characterised for weight and thickness variation, pH, percentage moisture uptake, contact angle, folding endurance, tensile strength, SEM, mucoadhesion and BCOP tests. The obtained data were fed into DoE to analyse the effect of each variable on the responses.

Results: The results showed that increase in PVA concentration and GLY amount resulted in more elastic and hydrophilic ocular films whereas increase in ALG concentration produced more hydrophobic, tough and rigid films with higher tensile strength. Mucoadhesion, on the other hand, was found to be directly proportional with ALG concentration and GLY amount whereas the inverse relation was present with PVA concentration. Acquired data demonstrated that k-Carr concentration had minor effect on responses compared to other excipients, but it was observed that it facilitated the moisture uptake and increased the contact angle of the films.

Conclusion: The study demonstrated that the design of experiment is a very crucial application to optimise the formulation of the ocular inserts. Ideal inserts with better bioavailability could be produced by manipulating the concentrations of the polymers and the plasticiser.

2. INTRODUCTION

2.1. CHALLENGES OF THE OCULAR DRUG DELIVERY

The eye is the organ of vision with 24.2 mm anteroposterior diameter along the geometrical axis which is composed of two main parts: anterior and posterior (1). The accessibility of the surface of the eye makes it very suitable for topical administration of the ophthalmic drugs. However, the unique and the layered anatomy, shown in *Figure1*, of it challenges the efficient drug delivery. Although conventional eye drops are the most common treatment method, only less than 5% of the drug can penetrate through the highly impermeable cornea (2). That is why the main challenge is the bioavailability of the drug during the ocular drug delivery process. So that the major purpose of the ocular studies is the maintenance of the optimum concentration of the drug at active site within the convenient time duration to achieve the highest therapeutic effect (3).

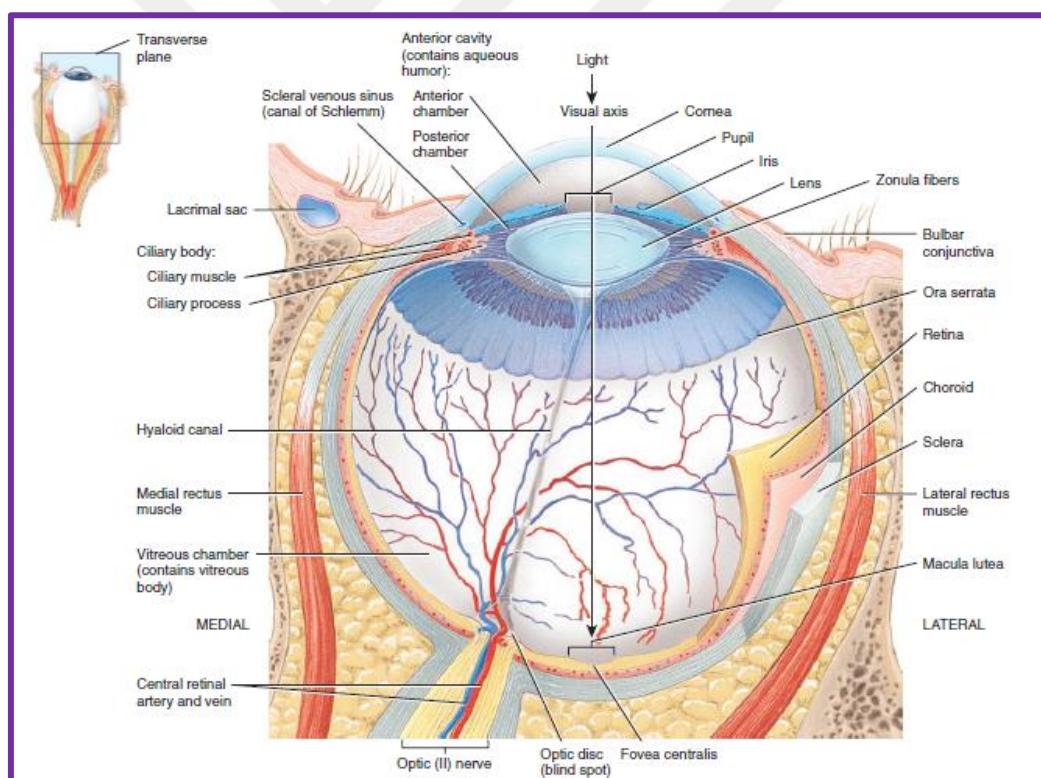


Figure 1: Transverse section of right eyeball (1)

2.1.1. Anatomical and Physiological Considerations

Complex structure of the eye makes the drug delivery very hard and restricts the bioavailability of the drug. One of the main reasons of low bioavailability is the precorneal loss of the drug due to many different factors shown in *Figure 2*.

Precorneal area called tear film, the first place that applied drug meets, lubricates the cornea and makes the surface of the eye uniform. It has three layers; from outer to inner, superficial oily layer, lachrymal or tear fluid and mucoid layer (4). Superficial oily layer mainly prevents the evaporation of the tear and is composed of sterol esters, triacylglycerols and phospholipids. Tear fluid creates 98% of the tear film and contains water, electrolytes and certain proteins including lipocalin, lysozyme and lactoferrin (2). Due to the layered structure of the tear film, it is the first place that the drug is exposed to selective barriers.

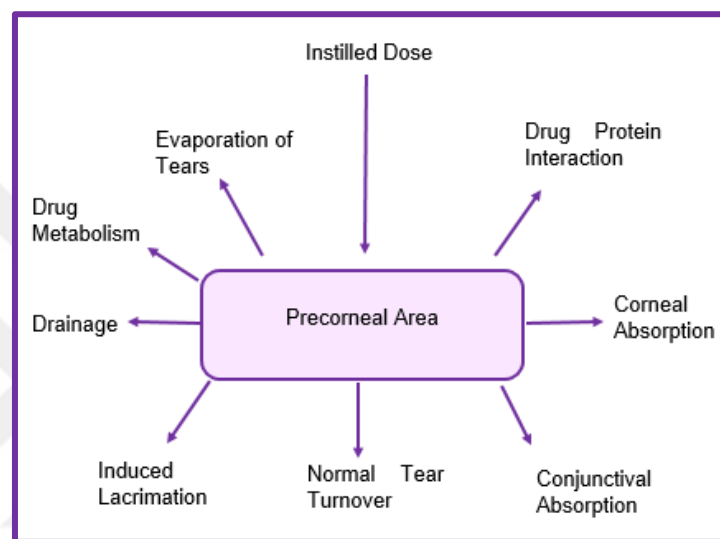


Figure 2: Precorneal loss factors that affect the bioavailability of the drug, adopted from Patel et.al. (3)

Precorneal loss factors include solution drainage, induced lacrimation, tear film, tear turn over, tear dynamics, and tear dilution, blinking, conjunctival absorption, non-productive absorption, transient residence time in cul-de-sac and impermeability of the corneal epithelium (2, 5). Solution drainage is caused by the nasolacrimal drainage system shown in Figure 3. Tears are produced by lacrimal gland and spread towards to puncta during the blinking. Due to blinking, tears flow from lacrimal canaliculi into the lacrimal sac. From the lacrimal sac, tears pass to nasolacrimal duct which is 12 mm long and finalise at the inferior nasal passage. This passage contains lots of blood vessels which make it the place of systematic drug absorption (2). Systemic drug absorption is one of the causes of non-productive absorption and it absorbs the drug rapidly. Drug absorption by the systemic circulation reduces the drug concentration in lacrimal fluid extensively. This

absorption mechanism decreases the ocular bioavailability of the drug to almost 10% that is released from the solid delivery system to the tear fluid (6).

Tear capacity of cul-de-sac is 7–9 μl and the tear flow rate is 1.2–1.5 $\mu\text{l}/\text{min}$. Since the drainage rate is faster than the ocular absorption rate, a big portion of the tear fluid is lost within the first minute. That is why precorneal loss including drainage, tear turnover, and systemic absorption is a highly effective matter that determines the bioavailability of the drug (2).

Pulse entry is another undesired condition during the application of ocular drugs. Pulse entry is the rapid increase of drug concentration followed by the rapid reduction after the application of ocular solutions. It is mainly caused by extensive precorneal loss resulting in low corneal bioavailability. Pulse entry can be prevented either by high dose and frequent drug administration or by using controlled drug delivery systems such as ocular inserts (7).

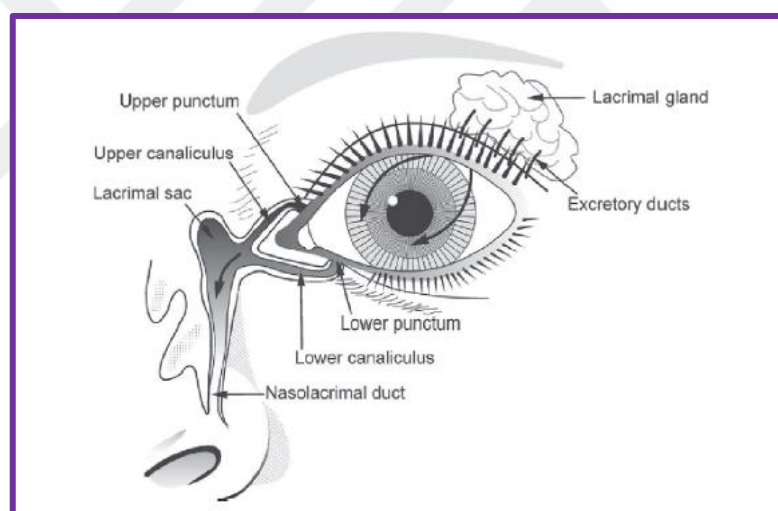


Figure 3: *Nasolacrimal drainage system (2)*

The two main ocular barriers of the eye are blood-aqueous barrier (BAB) and blood-retinal barrier (BRB) which presents highly selective permeation to drugs. Both layers have tight junctions that prevent free diffusion of the molecules. BAB blocks the passage of plasma albumin to the aqueous humour and restricts the penetration of hydrophilic drugs from the plasma to the aqueous humour. It also allows the passage of small lipophilic molecules to the uveal blood circulation and triggers the rapid elimination of them whereas larger and more hydrophilic drugs are barely eliminated by the aqueous humour turnover across the BAB (8, 9).

Additionally, BRB has retinal capillaries composed of tight walls. For instance, molecules larger than 20–30 kDa are made impermeable through the retinal vessels by the inner BRB (1). It basically inhibits the entrance of toxic molecules, plasma components and water into the retina (2). However, the vasculature of the choroid has high blood flow rate and has walls with holes. Drugs can pass to choroidal extravascular space easily due to leaky structure of the choroid veins. Nevertheless, the tight structure of the retinal capillaries reside in BRB limits the amount of drug that pass into the retina (6). It has poor permeability to macromolecules and small hydrophilic molecules whereas it has better permeability to lipophilic compounds (8).

Other main challenge of the ocular drug delivery is the multifaceted anatomy of the cornea. Sandwich like structure of the cornea has hydrophilic stroma in between the highly lipophilic epithelium and less hydrophilic endothelium. That is why lipophilic drugs are more permeable than the hydrophilic ones on the cornea. The rate of the penetration of drugs depends on their *n*-octanol-water partition coefficient (1, 2). This exceptional structure makes the drug penetration very selective and challenging. Prof. Alany states that “Only drugs with a partition coefficient between 10 and 100 that show both lipid- and water- soluble properties can readily pass through the cornea” (2). Moreover, the presence of tight junctions that surround the superficial cells of the cornea makes the epithelium rate-limiting barrier for hydrophilic substances (2, 10). Although stroma, highly hydrophilic and porous, allows the transfer of the molecules with molecular weight lower than 500,000 Da, it presents a diffusion barrier structure to lipophilic compounds (2).

In addition to the cornea, conjunctiva, sclera, iris-ciliary body and lens are also other parts of the eye that forms limiting barrier for the drug absorption. Conjunctiva has several layers and tight junctions as the cornea. Tight junctions prevent penetration of the molecules larger than 20,000 Da. Presence of collagen fibres in both sclera and iris-ciliary body generates protective barriers for foreign substances (2). As it is seen, layered structure of the eye's sections makes drug penetration harder and very selective. But structure is not only barrier for the effective drug absorption, pharmacokinetics aspect should also be considered carefully.

2.1.2. Pharmacokinetic Considerations

Applied drug is immediately diluted by the tear fluid which reduces the concentration gradient resulting reduction in the trans-corneal flux. There are two absorption routes through the anterior chamber for the drug that remain on the ocular surface for a certain time period: corneal absorption and conjunctival-scleral absorption routes (2).

Corneal absorption is the main penetration route which has two different mechanisms: paracellular and transcellular diffusion. Lipophilic drugs penetrate via transcellular route whereas hydrophilic drugs are absorbed with the paracellular way. Transcellular route includes diffusion, active transport and endocytosis. In contrast, paracellular route is passive transport that depends on only to size of the pores and the charge of the intracellular spaces. Small molecules, positively charged and neutral molecules pass easier through the pores (2, 11).

On the other hand, conjunctival-scleral absorption route is not that effective since most of the drugs enter into systemic circulation before they get access to intraocular tissues. Amount of drug loss from this route may be noticeably high since the surface area of the conjunctiva is larger than the cornea. Also, conjunctiva is highly vascularised which increases permeability especially to large hydrophilic molecules. That is why this route is not an optimum absorption way (2).

Absorption process is one of the important limitations of the ocular drug delivery since it is highly affecting the amount of the drug that is absorbed by the systemic or ocular circulation. In the light of these information, it is also important to formulate the ideal drug with optimum physical and chemical properties to prolong the retention time and increase the effect of the drug.

2.1.3. Formulation and Manufacturing Considerations

The challenge of the ocular drug delivery is not only related to the anatomy of the eye, but it is also related to the formulation and physico-chemical properties of the drug since the drug can irritate the eye. Moreover, osmolality of the drug, pH and the applied volume can also irritate the eye and induce the reflex tearing which affects the bioavailability of the drug (2). That is why these factors should be examined well before the manufacture of drugs.

First of all, both the chemical structure and the concentration of the drug may induce reflex tearing and cause lower bioavailability. But more importantly, lipophilicity, hydrophilicity, molecular size and shape, degree of ionization and charge of the drug are the factors that affect both the absorption rate and the absorption route. Lipophilicity of the drug affects the corneal permeability. *n*-octanol-water partition coefficient, the charge status, molecular size and shape, and acidity/basicity defined by drug's pKa are the factors that define the lipophilicity of the drug (2). Some studies suggest that the drugs with octanol/buffer distribution coefficient in the range of 100–1000 have the ideal solubility for the corneal absorption (12). Furthermore, it is known that the drugs with *n*-octanol-water partition coefficient in the range of 10–100 have the optimum lipophilicity for the corneal absorption (2).

For the charged molecules, the equilibrium state between ionised and unionised form is the decisive factor for the absorption. But mostly, unionised molecules can pass lipid membranes easier than the ionised molecules (2, 12). As the charge of the molecule is compared, cationic molecules can penetrate through cornea easier than the anionic molecules. The reason is that the anionic molecules are repelled by both the negative charges of mucin layer of the ocular surface and the negatively charged pores (2). In the case of any ionic interaction between the positively charged molecules and the negatively charged carboxylic acid groups of the tight junction proteins, cationic molecules can also disturb the corneal absorption (2).

Buffer capacity and pH of the drug are important factors for the conformity between the drug and the eye. pH of the tear fluid is around 7.4 (13) therefore pH of the drug should be in the range of 7–7.7 to prevent the irritation but in most of the cases pH of the drug that is required for the maximum solubility is out of stated range. Dissolved bicarbonate and carbon dioxide makes the buffer capacity of the tear fluid small so it is better to formulate drugs with buffer that has low buffering capacity to permit tear fluid return back to their normal pH range rapidly (2).

As the ocular solution is placed into the eye, it mixes with the tear fluid which generates osmotic pressure depending on the concentration and the amount of the drug instilled and osmolality of the tears. The osmolality of tears is in the range of 310–350 mOsm/kg which is determined by inorganic ions Na⁺, K⁺, Cl⁻, HCO₃⁻ and

proteins (13). Hypotonic solutions are more acceptable than the hypertonic solutions for the ocular tissues since hypertonic solutions may increase the lacrimation rate. Furthermore, the tonicity of the drug should be in the range of 260–480 mOsm/kg not to become irritant which induces reflex tearing and blinking (2).

As a last factor, the volume of the instilled drug is another significant matter for the efficient drug delivery. The tear capacity of the cul-de-sac is around 7–9 μl as it is stated in section 2.1.1. but it can carry 30 μl of liquid without overflowing. Generally, commercial eye drops install approximately 50 μl where the excess volume is immediately lost by the nasolacrimal drainage or conjunctival sac. The study of Chrai *et.al.* showed that the drainage rate is directly proportional with the volume of the installed drug by using rabbits (14). Therefore, it is recommended to keep the amount of applied drug constant while reducing the instilled volume to decrease the drainage rate resulting increase in the bioavailability of the drug (2).

All the factors that are described above make the ocular drug delivery very challenging. Before passing to currently active ocular drug delivery systems, common ocular diseases will be reviewed.

2.2. COMMON OCULAR DISEASES

Ocular diseases are very prevalent among the population. In 2016, 2 million people in UK are registered as having sight loss (15). And this number is reflecting only the sight loss, not all the patients suffer from the eye diseases. Some of the most common ocular diseases are conjunctivitis, cataract, iritis, glaucoma and keratitis (11).

Conjunctivitis is the inflammation or the infection of the conjunctiva emerged from the dilatation of conjunctival vessels. It can be caused by bacteria, virus which are the most common sources, some external irritants such as shampoo or allergens e.g. pollen or dust that mostly depends on the season. Virus and bacteria caused ones are infectious whereas allergic and toxic sources are non-infectious. There is no effective treatment for the viral conjunctivitis but the symptoms can be eased with artificial tears, topical antihistamines or cold compresses. Antibiotics are not useful for viral conjunctivitis while they reduce the duration of the bacterial conjunctivitis. Even though some studies revealed that there is not much difference between the placebo and patients treated with antibiotics, they are used to ease the

symptoms and shorten the time of the disease. Allergic conjunctivitis is treated with saline solution or artificial tear to clear the allergens (16).

Cataract is clouding of the lens that reduces the light transmitting ability of the lens. It is one of the most common ocular diseases, especially over the age fifty, that causes loss of clear vision gradually. The only treatment is the removal of the lens surgically which may include replacement of new artificial lens or not. The success of treating cataract via surgery is more than 95% (17).

Glaucoma is an ocular disease in which the optic nerve is damaged. It may result in irreversible loss of vision (18). The damage occurs at the superior and inferior poles of the optic disc (19). It is mainly caused by the increase of eye's intraocular pressure and it is the number one cause of the blindness. The most common sub-class of glaucoma is known as primary open-angle glaucoma while the second common one is angle-closure glaucoma. There is no sign of open-angle glaucoma until irreversible damage occurs. Only sign that shows something is wrong could be weakening of peripheral vision. Available treatments are ointments, eye drops, pills and surgery. For open-angle glaucoma, besides the eye drops, ointments and oral tablets, ocular inserts can also be used (20). For instance, timolol-bimatoprost ocular insert is used to treat glaucoma (21).

Iritis, sub-class of uveitis, is the inflammation of the iris. Since the iris is a part of the middle layer of the eye known as uvea, it is a sub-class of uveitis which is the inflammation of the uvea (22). The disease is also known as anterior uveitis which is the most common intra-ocular inflammation. The symptoms of the iritis are redness of the eye, blurred vision, pain in the eye, light sensitivity and sometimes loss of peripheral vision. The disease could be infectious, non-infectious, human leukocyte antigen (HLA-B27) related, acute or chronic. Causes could be the injury of the eye, infections or infectious diseases e.g. tuberculosis, toxoplasmosis, genetic disorders affecting immune system and immune related diseases such as sarcoidosis. Widely used treatments are steroids and dilating eye drops. If it is not treated correctly, it can induce cataract or glaucoma (23).

Keratitis is basically the inflammation of the cornea which can be caused by infectious bacteria, fungi, virus or parasites. In addition, some minor injuries including wearing contact lenses too long or some other non-infectious disease may

stimulate non-infectious keratitis. The symptoms of the keratitis could be itching, redness of the eye, tearing, irritated or blurred vision, sensitivity to light, non-clear appearance of the cornea and sticky eyelids. If keratitis is not treated, it may destroy the corneal tissues which may cause to insensitivity of the cornea. There are different ways to treat keratitis depending on the cause of the disease. Antiviral eye drops, eye drop or oral antibiotics can be used besides the surgical clearance of the cornea. Vitamin A supplementation can also benefit (24).

There are varying ocular drug delivery systems with different advantages to treat the ocular diseases. Recent studies are focusing on delivery of the ocular drugs via ocular inserts for many ocular diseases including keratitis. For instance, Shukr's study showed that *in situ* gelling ocular inserts loaded with antifungal drugs can be used for ocular irritations (25).

2.3. OCULAR DRUG DELIVERY SYSTEMS

The eye is a highly complex organ with very selective barriers therefore it is very hard to deliver the drugs to the eye with high efficiency. There are many different ocular drug delivery systems to overcome the challenges. All the possible routes of an ocular drug from application to elimination are shown in *Figure 4*. Currently active delivery systems are reviewed in this section.

Topical eye drops are the most traditional ocular drug delivery systems due to high patient compliance and low range of side effects. It is immediately active and non-invasive way of ocular drug delivery for the treatment of the diseases in the anterior part (3). The biggest problem of the eye drops is the retention time of the drug on the cornea (12). That is why many additives are used to formulate eye drops. For instance, viscosity enhancers such as hydroxyl methyl cellulose prolong the precorneal residence time whereas the permeation enhancers such as polyoxyethylene glycol ethers advance the corneal uptake of the drug (26).

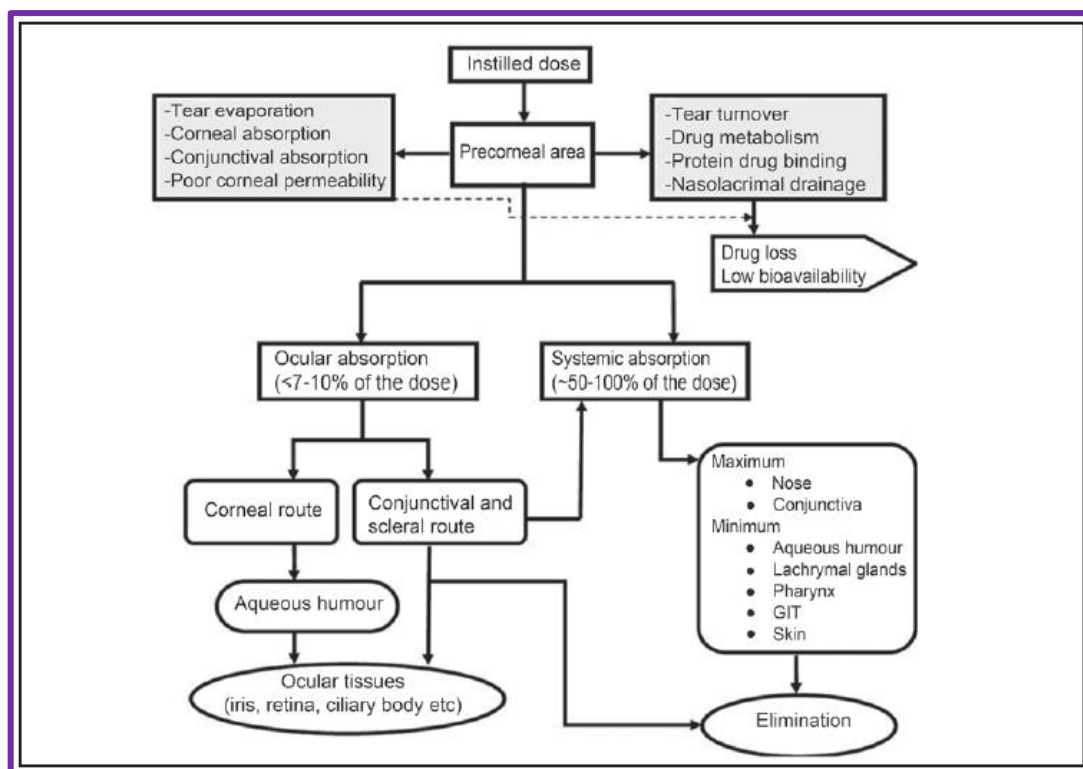


Figure 4: Possible routes of an ocular drug during the absorption process (3)

Emulsions which are the mixture of more than one liquid, are another topical delivery systems that are classified as water in oil (w/o) and oil in water (o/w) systems. Oil in water emulsions are more convenient for the ocular drug delivery due to less irritation and better tolerance (26, 27). Suspensions are another non-invasive topical drug delivery systems that contain insoluble active pharmaceutical ingredient (API) in an aqueous solvent with suitable suspending and dispersing agents. Suspension particles reside in the precorneal pocket and release the drug slowly which extends the drug contact time. Particle size affects the drug release time. Bigger particles release the drug slower than the small particles (26, 27). Ointments are the last class of conventional ocular drug delivery systems. They are viscous structure since they contain mixture of semi-solid and solid hydrocarbon such as paraffin (26).

The most important and the common problem of all conventional delivery systems is the rapid precorneal loss of the applied drug therefore recent researches focus on the novel delivery systems (12). Novel ocular drug delivery systems include nanoparticles, nanomicelles, nanosuspensions, *in-situ* gelling systems, liposomes, contact lenses, implants and microneedles. Nano-based systems focus on low

irritation, high ocular tissue compatibility and good bioavailability. Liposomes are lipid vehicles that surround an aqueous core. They are highly compatible with the ocular tissues due to similar structure to the cell membrane and ability to enclose both hydrophilic and hydrophobic drugs.

In-situ gels are polymeric systems that convert itself from solution to viscoelastic gel when they are stimulated by the change in pH, temperature or ions etc. Contact lenses are plastic films that cover cornea and they can be used as a vehicle by loading the drug. Implants are used as drug delivery devices to induce the localised drug release and they can be biodegradable or non-biodegradable. In contrast to conventional methods, microneedles are invasive ocular drug delivery systems that are used to treat posterior ocular diseases (26). Many different ocular drug delivery systems are briefly defined to give an idea about existing treatment ways. Even though there are different delivery systems, there is a certain number of drug release ways to the eye which should be analysed in detail to produce better delivery methods.

2.3.1. Mechanisms of Controlled Drug Release into Eye

There are three mechanisms of the controlled drug release into the eye: diffusion, osmosis and bioerosion.

Drug is constantly released at a controlled rate to the tear fluid by passing the membrane in diffusion mechanism. When the insert is formed by a solid non-erodible body and pores around, the release occurs via diffusion. Another form of the diffusion is the invasion of aqueous solution into the solid dispersed drug. Diffusion happens through the polymer swelling in the soluble delivery devices. For instance, as the ocular insert is located into the eye, water of the tear fluid flow into the matrix which triggers swelling and polymer relaxation followed by diffusion of the drug (11).

In osmosis, ocular insert is composed of two parts where the first section is surrounded by a semipermeable and an impermeable elastic membrane, and the second part is rounded by an elastic membrane with impermeable material inside. The first section contains solute that is not able to penetrate semi-permeable membrane. When the insert is placed into the eye, water enters into the first part

and expands the elastic membrane which narrows the second part so that the drug can pass through the aperture of the impermeable walls (11).

In the bio-erodible mechanism, the core material, matrix, of the insert should be bio-erodible where the drug can disperse. As the insert meets with the tear fluid, it induces the bioerosion of the matrix that results in controlled sustained drug release (11).

2.4. OCULAR INSERTS

Ocular inserts are solid dosage forms that are defined as “sterile, thin, multi-layered, drug-impregnated, solid or semisolid consistency devices placed into the cul-de-sac or conjunctival sac, whose size and shape are specially designed for ophthalmic application” (28). They are composed of different polymers that may or may not be integrated with an ophthalmic drug. They can also be described as biodegradable polymeric systems.

The main objective of the ocular inserts is to increase the contact time of the insert on the eye to supply controlled and sustained drug release. This provides the maintenance of the effective drug concentration in the target tissues. They have many advantages over the conventional ocular drug delivery systems. Some of these advantages are: better mucoadhesive property that lengthens the retention time (29), better bioavailability due to increased contact time, slow rate drug release, reduced administration frequency resulting in better patient compliance, ease of integration with many different chemicals including viscosity enhancers, prodrugs, mucoadhesives etc., prolonged shelf life, reduced risk of eye irritation and eye reactions (11, 28). It is not possible to provide all the listed advantages in a single insert. Desired advantages may cause different disadvantages e.g. difficulty in placement to the correct place, interference with the vision, dislocation within the eye etc. (11, 28).

Ocular inserts can be classified as shown in *Figure 5*. Insoluble inserts generally contain drug encapsulated by a membrane like structure or polymers. Like insoluble inserts, bio-erodible inserts are also coated by bio-erodible polymers (28).

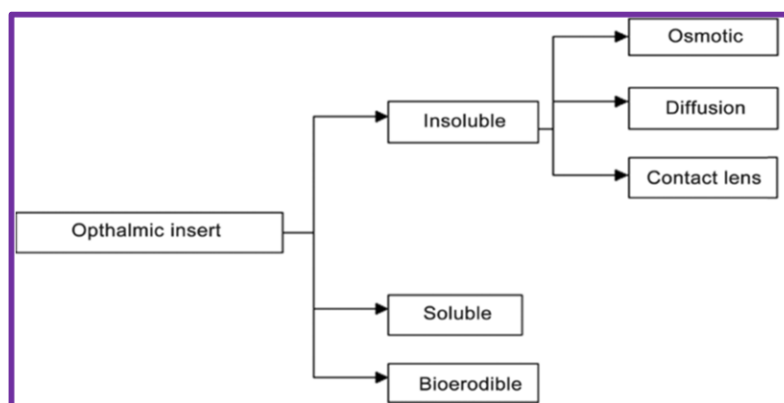


Figure 5: Classification of ocular inserts (28)

In the project, sodium alginate (ALG), Kappa carrageenan (k-Carr), polyvinyl alcohol (PVA) and glycerol (GLY) were used to produce the inserts via solvent casting method. The polymers and the plasticiser used are well known with their good film forming properties. GLY is a hydrophilic plasticiser that is used to lubricate, to increase elasticity and to improve smoothness of the films. It is highly hygroscopic which facilitates the moisture uptake. Its high water solubility, low molecular weight, good miscibility due to polyhydric alcohol structure and high hygroscopicity properties makes GLY very favourable for film making. It can easily penetrate into polymers and weaken the intermolecular interactions resulting in reduction of polymer chain stiffening and increase of elasticity (30).

Carrageenan is a high-molecular weight polysaccharide with good gelling, thickening and stabilising features. There are different forms of carrageenan depending on the arrangements of chains and ion types. Kappa carrageenan (k-Carr) is the one used in the project. k-Carr is also non-toxic, water-soluble, biocompatible and hydrophilic. Sulphate groups in the structure makes k-Carr negatively charged polymer and enhances the swelling index (31, 32). All those features make k-Carr very useful to produce ocular films.

PVA is a widely used water soluble, non-toxic, viscosity enhancer polymer that prolongs the retention time of the insert and reduce the drug loss caused by the drainage which increases the bioavailability of the drug (2). Hydrophilicity of it makes PVA available for mixing with other biopolymers. It is also lipophilic which can pass through the cornea easily. The presence of hydroxyl groups in the structure makes PVA biodegradable and available for the cross-link with other polymers (33).

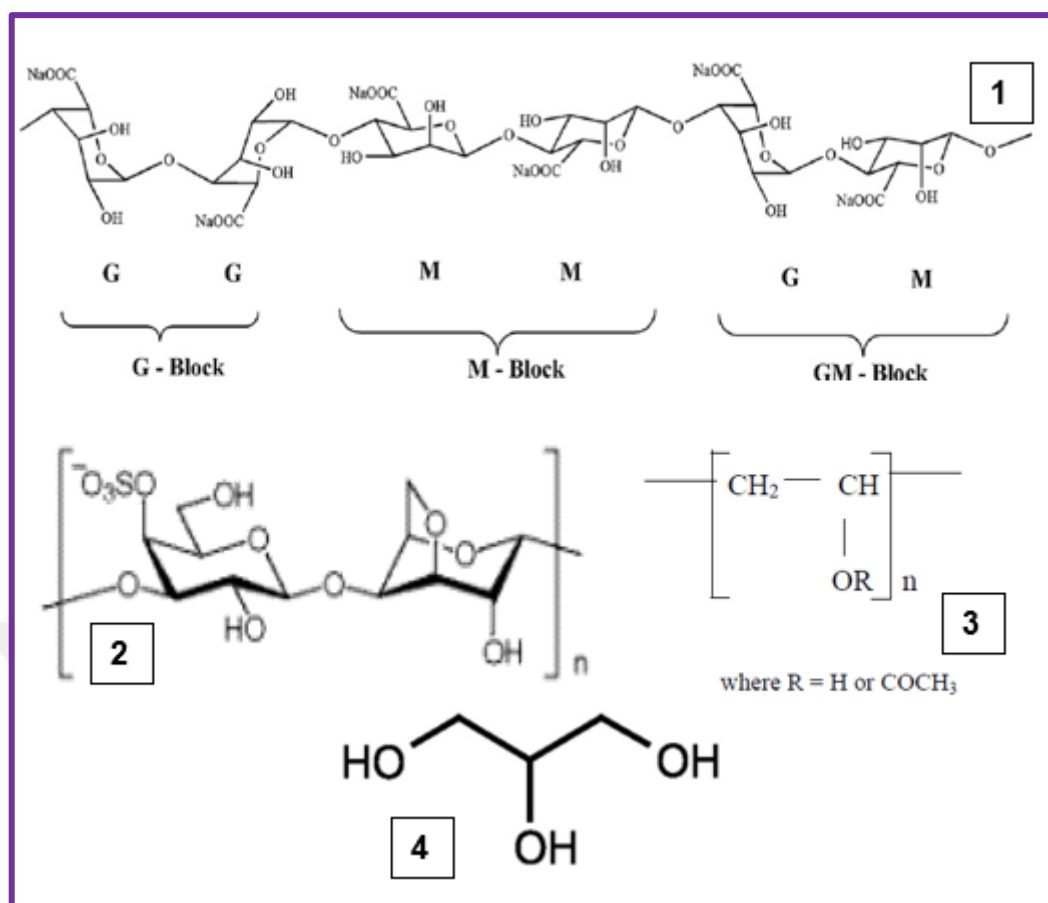


Figure 6: Structure of the polymers and the plasticiser used

***Numbers in squares refer to 1:** Sodium alginate (34), **2:** k-Carrageenan (35), **3:** Polyvinyl alcohol (36) **4:** Glycerol (37)

ALG is widely used polysaccharide for its good gelling and mucoadhesive properties in pharmaceutical industry. It is formed by the sequence of two α-L-guluronic acid (G) and β-D-mannuronic acid (M) monomers. The backbone of ALG contains three different structures: homogenous G and M homopolymers and heterogeneous MG blocks. The amount and the sequence of the blocks determine the properties of the polymer (34). The length and the proportion of the G blocks are the main contributors of gel forming property (38). Presence of sodium (Na) makes ALG available for hydrogel forming by acting as cross-linker between the functional groups of ALG chains (39). It is also known that ALG is a good drug release retarding polymer which is an important factor for the formulation of ocular films (30). ALG is hydrophilic and amorphous polymer which affect the interaction of it with other polymers and film forming property (30).

As it is seen, all the selected polymers and the plasticiser are widely used in pharmaceutical industry due their favourable features. The study examined how the concentration change of both polymers and the plasticiser is affecting the certain responses by using design of experiment analysis method.

2.5. DESIGN of EXPERIMENT

Minitab is a statistical software that is used for the data analysis to make the experimental process better and to improve the results. It has many different statistical features including basic statistics e.g. outlier test, normality test, paired t-test and one or two sample Poisson rate test, all the probability distribution graphs, scatterplots, histograms, interaction plots, linear or non-linear regression, residual and factorial plots, variance analysis e.g. ANOVA, and design of experiments with response prediction and optimisation (40). Design of experiment (DoE) application is the one used in this project for the optimisation and the analysis of the outcomes.

DoE is a very useful statistical tool to design the experimental grid, to predict the outcomes and to analyse the relationship between the variables and the responses. The effect of one variable on the outcome, the interaction between the variables and their effect on the outcome can be analysed with design of experiment. The results are presented in the form of special mathematical model where the change in outcome is estimated with respect to changing variables (41). In general, variables set at two levels i.e. high (+1) and low (-1) are used in factorial design (42). Full factorial design feature of DoE generates 2^k experiments where k is the number of variables and the number 2 represents 'two level variables'. It is better to use full factorial design when the number of variables is four or less (41).

As the number of variables increases, the outcome may be affected by high number of variables. That is why if the number of variables is higher than five, fraction factorial design which runs a fraction of the full factorial is recommended to use. Reducing the number of experiments can be more effective to predict more effects (41). In this project, since the number of variables is four, both designs could be used. It was decided to use $\frac{1}{2}$ factorial design in which the variables are set at two levels and 2^{k-1} experiments are generated. Experimental matrix is randomised to eliminate the effect of external factors. Additional two experiments are generated for the centre points which are the controls of the experiment. Centre points are

used as guides to detect any instability during the process and they are generated in certain intervals in contrast to randomised order of experimental matrix (41, 43).

2.6. AIMS and OBJECTIVES

The aim of the study was the investigation of the effect of certain experimental variables on the formulation of ophthalmic inserts produced via solvent casting method with the purpose of providing information about optimum formulation content for the further studies.

Experimental variables subject to investigation were sodium alginate concentration, polyvinyl alcohol concentration, kappa carrageenan concentration and glycerol amount. Mainly the effect of change in the polymer concentration and the plasticiser amount was tested on certain responses by using design of experiment software. The objectives of the study were:

- To do optimisation study by generating different experimental grids to find optimum content for the formulations by using design of experiment, Minitab software.
- To formulate the ocular films by solvent casting method.
- To examine physical and mechanical characteristics of the films by doing weight and thickness variation, surface pH analysis, percentage moisture uptake measurement, folding endurance and tensile strength measurements.
- To study morphology of the films by scanning electron microscopy (SEM)
- To investigate the wettability, hydrophilicity/hydrophobicity and mucoadhesive characteristics of the films by measuring the contact angle and doing mucoadhesion test.
- To apply irritation test to detect whether the films are biocompatible with the eye by doing BCOP assay.
- To analyse the results by using design of experiment in Minitab.

3. MATERIALS AND METHODS

3.1. MATERIALS

Poly(vinyl) alcohol (MW: 146.00–186.00 g/mole / 99+% hydrolysed), sodium alginate (MW: 216.121 g/mole), fluorescein sodium salt, phosphate buffered saline (PBS) tablets, pH 4 and pH 7 buffer tablets were purchased from Sigma Aldrich (Dorset, UK). k-Carrageenan (MW: 788.647 g/mole) was purchased from Tokyo Chemical Industry (Oxford, UK) (35). Glycerol (MW: 92.09 g/mole) and sodium hydroxide were purchased from Fisher Scientific (Loughborough, UK).

3.2. METHODS

Factorial design of experiment was used to analyse the effect of four variables (Sodium alginate (ALG) concentration, polyvinyl alcohol (PVA) concentration, k-Carrageenan (k-Carr) concentration and glycerol (GLY) amount) on six responses, i.e. pH, percentage moisture uptake, folding endurance, tensile strength, mucoadhesion, contact angle, shown in *Figure 7*.

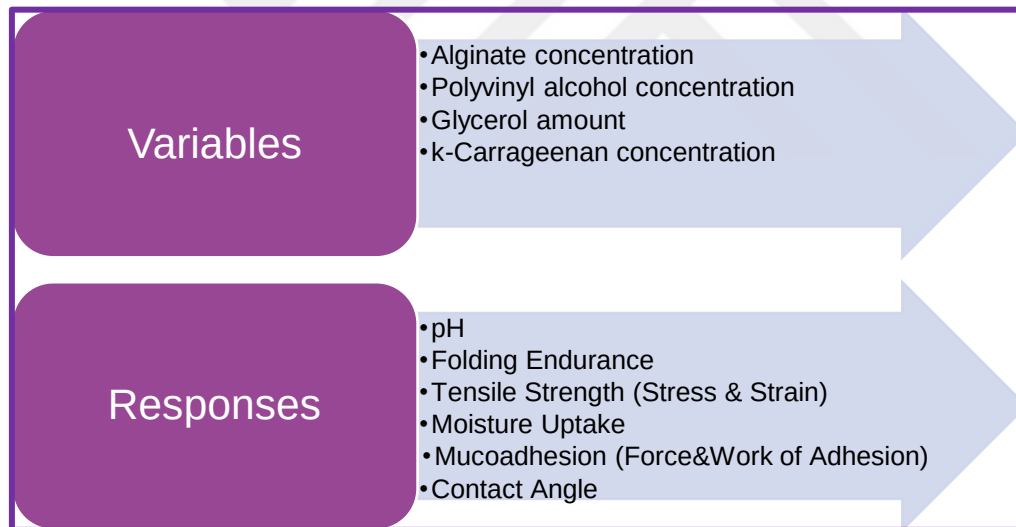


Figure 7: The variables and the responses examined in the project

The experimental grid was created by using $\frac{1}{2}$ fractional factorial design which generates 2^{k-1} experiments, where k is the number of variables (44). Since the study had four variables, ten experiments were generated by the software to optimise the experiments where two of the experiments were centre points (cp) stated with different colour in *Table 1*. The experimental runs were randomised to eliminate the external factors (44).

Table 1: The experimental grid generated by DoE in Minitab 17

Formulation Number	StdOrder	ALG % (w/w)	PVA % (w/w)	k- Carr % (w/w)	GLY (mg)
F1	1	5	2	0.1	500
F2	3	5	4	0.1	1000
F3 (cp)	9	7.5	3	0.2	750
F4	6	10	2	0.3	500
F5	8	10	4	0.3	1000
F6	4	10	4	0.1	500
F7 (cp)	10	7.5	3	0.2	750
F8	2	10	2	0.1	1000
F9	7	5	4	0.3	500
F10	5	5	2	0.3	1000

Maximum and minimum concentrations of the variables were determined as following: ALG concentration of 5–10% (w/w), PVA concentration of 2–4% (w/w), k-Carr concentration of 0.1–0.3% (w/w) and GLY amount of 500–1000 mg. The generated experiments are shown in *Table 1*. StdOrder was the randomised order of the experiments by DoE automatically while the formulation numbers were named by the experimenter manually.

3.2.1. Preparation of Polymer Solutions

ALG solutions of 5%, 7.5% and 10% (w/w) were prepared in water at room temperature. k-Carr solutions of 0.1%, 0.3% and 0.5% (w/w) were prepared by adding the chemicals to water at room temperature and then heating up to 80–85 °C on a hot plate by stirring. PVA solutions of 2%, 3% and 4% (w/w) were prepared with cold water and left overnight for hydration. After at least 24 hours, solutions were dissolved in approximately 90 °C hot water.

3.2.2. Preparation of Ocular Films via Solvent Casting Method

Specified amounts of GLY, 500 mg, 750 mg and 1000 mg, were added to ten different 250 ml beakers. Amounts of the polymers and the plasticiser in the formulations were fixed to 20 grams. Concentrations of the polymers specified by DoE were added to the beakers while stirring on the magnetic stirring plate. First, PVA and k-Carr were added since they are more viscous than ALG. As a last step, specified concentrations of ALG were added into the beakers. Solutions were stirred more than 4 hours to mix equally. Mixed solutions were poured into Petri dishes of 120 mm diameter and left to dry for almost 2 days under ambient conditions. Dried

films were cut with disposable scalpel and then they were cut in small circular ocular discs of 6 mm diameter by hole puncher to be suitable for the lower conjunctival sac (30). Three batches were prepared for each formulation to get statistically more reliable results.

3.2.3. Physical Characterisation

3.2.3.1. Weight & Thickness Variation and pH Analysis

Ten random discs were cut from each formulation and then weighed individually by using digital balance. Mean and standard deviation of ten values were calculated to evaluate variation. This procedure was applied for all ten formulations of three batches. Similar to weight analysis, same ten discs were used to evaluate thickness variation. Thickness of discs was measured by using Cocraft Vernier calliper (Clas Ohlson, Surrey, UK) and, mean and standard deviation were calculated to determine the variation.

Three ocular discs of 6 mm diameter from each formulation were placed into a plastic 12-hole mini-Petri dishes. 500 µl of artificial tear fluid with a pH range of 7.35–7.5, was pipetted onto each disc and waited for 3–5 minutes to allow discs to keep the tear fluid. pH of the fluid was measured by using digital 570 pH meter (Jenway, Staffordshire, UK).

3.2.3.2. Percentage Moisture Uptake

Three ocular discs from different batches were weighed individually and placed onto Petri dishes of 120 mm diameter and labelled. Then they were incubated for 3 days in the stability chamber at 37.0–37.5 °C, 66.0 ± 5% relative humidity. After 3 days, all discs were weighed individually again to compare with the initial weights based on the following equation.

$$\% \text{ moisture uptake} = \frac{\text{final weight} - \text{initial weight}}{\text{initial weight}} \times 100 \quad (45)$$

Equation 1: Percentage moisture uptake calculation formula

3.2.3.3. Contact Angle Measurement

Two ocular discs of 10 mm from each formulation were cut and each disc was tested from one side. Contact angle of the films was detected by using Drop Shape Analyser (Krüss GmbH, Hamburg, Germany). The test was applied by using Drop Shape Analyser software. Volume of deionised water drops was set up as 8 µl

and computation settings was fixed to manual computation. After pressing 'run' button, baseline was determined as quickly as possible and then profile detection and computation were clicked respectively.

3.2.4. Mechanical Characterisation

3.2.4.1. Folding Endurance

Three ocular films from each batch was cut into 20 mm x 20 mm diameter individually. Each film was folded from the same place repetitively up to 300 times or till the breaking point (30). The number of folding till the breaking point was recorded as the folding endurance. If they were not broken up to 300 times, they were recorded as no break in 300 times.

3.2.4.2. Tensile Strength Measurements

Ocular films were cut into rectangles of 30 mm x 10 mm diameter. Before the test, thickness of the films was measured using Vernier calliper from three different points and the mean of three values was considered. Strain (%) and stress (MPa) were measured by using TA-XT plus texture analyser (Stable Micro Systems, Godalming, UK). Two tensile grips were calibrated to starting position of 20 mm and the test speed was fixed to 1 mm/s. After fixing the ocular film between the two tensile grips, the test was started in tension mode. Strain (%), stress (MPa) and area of the graph (%*MPa) were calculated using Exponent Lite software (30).

3.2.5. Surface Morphology Analysis using Scanning Electron Microscopy (SEM)

Films were cut into discs of 6 mm diameter. Before the imaging, the surfaces that will be analysed were gold sputtered. Both up and down surfaces of the formulations were analysed individually using Zeiss EVO 50 scanning electron microscope (Carl Zeiss Ltd., Cambridge, UK) with an accelerated voltage of 5.0 kV.

3.2.6. Mucoadhesion

TA-XT plus texture analyser (Stable Micro Systems, Godalming, UK) used for tensile strength was used in a specific mucoadhesive mode. Sclera of the excised bovine eyes taken from ABP Food Group (Guildford, UK) was used in the test. The eyes were kept in 37 °C water bath to prevent drying of the surface. Cleaned eyes were put in plastic sample weighing boats. One eye was used in each time. Ocular inserts were attached to the 10 mm-diameter Delrin cylinder probe with

double-sided adhesive tape. Test settings were arranged as following: test speed of 0.1 mm/s, applied force of 5 g, and the contact time of 120 seconds. The probe was allowed to move towards to the eye and stick to the sclera. Adhesion force (mN) which is the force required for the detachment of the insert from the sclera, the debonding distance (mm) which is the distance moved by the probe while detaching from the sclera and the area under the force-distance curve (mN*mm) were recorded. Each eye was used for maximum three testing and in each test the new area that is not used in the previous test was used.

3.2.7. The Bovine Cornea Opacity – Permeability (BCOP) Test

Freshly excised bovine eyes were placed into plastic sample weighing boats where the cornea is facing up. The eyes were placed in shaking water bath set to 37 °C, 90 rpm. After ten minutes, one drop of sterile saline was dropped onto the eyes. Then they were incubated in water bath five more minutes. Ocular discs were placed onto the cornea for 30 seconds. After taking the discs off, the eyes were washed with 10 ml of saline solution and incubated in water bath for ten minutes. The eyes were treated with one drop of sodium fluorescein (2% (w/v) pH 7.4) and then washed with PBS. As a last step, the eyes were examined under a cobalt blue filter (465 – 490 nm) to detect any irritation or damage.

3.2.8. Analysis of the Results by Using Minitab Software

The effect of four variables on six responses, *Figure 7*, were analysed by using the statistical factorial analysis option of design of experiment. The results of the responses are entered in individual columns of the same experiment sheet where design of experiment was generated. Main effects plots with fitted means were plotted for each response. With that plot, individual effect of the variables on the responses is analysed. Pareto chart of standardised effects with 95% confidence interval (where $\alpha=0.05$) is the other analysis tool that was used. It demonstrates if the variables have statistically significant effect on the responses and also which variable affects the response most.

4. RESULTS & DISCUSSION

4.1. OPTIMISATION STUDY

The first grid generated by DoE was different than the one in *Table 1*. Concentrations of the polymers and the amount of plasticiser fed into DoE were different. As a first step, formulations shown in *Table 2* were casted in a same way described in the methods section and exposed to some tests. Obtained results were deemed to be inappropriate for the reproducibility of the ocular inserts. Some of the formulations were too thin, too sticky and too elastic. For instance, formulations that did not have ALG were too sticky and did not dissolve at all. Because of the physical properties of the formulations and due to disintegration problems, it was decided that ALG must be added to all formulations in the second experimental grid, *Table 1*. Concentration of PVA was 1–3% (w/w). PVA is known with its good film forming property that is why it was estimated that 1% (w/w) of PVA was not enough concentrated to form more convenient films based on the disintegration test results. That is why the minimum concentration was set as 2% (w/w) in the second experimental design, *Table 1*. The amount of GLY was kept constant to eliminate the plasticiser effect but then it was decided to analyse the effect of amount of plasticiser as well.

Table 2: First experimental grid generated by DoE for the optimisation study

Formulation Number	StdOrder	ALG % (w/w)	PVA % (w/w)	k-Carr % (w/w)	GLY (mg)
F1	8	10	3	0.5	875
F2	3	0	3	0.1	875
F3	2	10	1	0.1	875
F4	4	10	3	0.1	875
F5 (cp)	9	5	2	0.3	875
F6 (cp)	10	5	2	0.3	875
F7	7	0	3	0.5	875
F8	6	10	1	0.5	875
F9	5	0	1	0.5	875
F10	1	0	1	0.1	875

Optimum formulation contents were tried to be found by trying different concentrations and doing tests. The process is called optimisation which took almost two weeks during the project. Then based on the results, final grid, *Table 1*, was prepared and proceeded to further analysis steps.

4.2. PHYSICAL CHARACTERISATION

4.2.1. Weight, Thickness and Surface pH Analysis

Average weight of the ocular inserts was in the range of 10.64 ± 1.53 mg – 17.80 ± 5.01 mg while average thickness of the inserts was in the range of 0.17 ± 0.02 mm – 0.28 ± 0.05 mm (Table 3).

The results of the thickness analysis with low standard deviation confirmed the reproducibility and uniformity of the inserts for the further investigations. It is obvious that increase in the concentrations of the polymers and the amount of the plasticiser increases both weight and thickness. F3 was detected as the thinnest film while F5 was the thickest one. As weight is directly proportional with thickness, F3 was determined as the lightest and F5 as the heaviest one.

Table 3: Average weight and thickness values of the formulations

	Average Weight (mg)	Average Thickness, (mm)
Formulation 1	13.33 ± 4.08	0.22 ± 0.05
Formulation 2	15.94 ± 2.93	0.27 ± 0.03
Formulation 3 (cp)	10.64 ± 1.53	0.17 ± 0.03
Formulation 4	14.33 ± 2.60	0.21 ± 0.04
Formulation 5	17.80 ± 5.01	0.28 ± 0.08
Formulation 6	16.75 ± 4.10	0.25 ± 0.06
Formulation 7 (cp)	14.72 ± 3.89	0.23 ± 0.06
Formulation 8	16.50 ± 1.86	0.25 ± 0.03
Formulation 9	14.54 ± 1.48	0.23 ± 0.02
Formulation 10	12.37 ± 1.89	0.24 ± 0.01

**Results are demonstrated as (mean $\pm$ standard deviation), n= 30*

F3 has same content with F7 which are the centre points determined by DoE. The difference between the thickness and weight values of both formulations indicates that weight and thickness could also be affected by the external conditions such as temperature and humidity. Even the slope of the surface that the Petri dishes were left for drying affects the equal and smooth distribution of the formulations. This may result in unequal distribution of the polymers so that it gives different thickness and weight values in different parts of the inserts resulting in high standard deviation. High standard deviation was observed for the weight of F1, F5, F6 and F7 which indicates that the polymers and the plasticiser were not uniformly distributed (46). It could be due to insufficient stirring or unfavourable drying

conditions. Or the stated concentrations of the polymers may not be properly mixing with each other.

Surface pH analysis is another test which gives information about the mechanical properties of the films. The analysis showed that pH of the inserts was in the range of 7.81–8.15 as shown in *Table 4*.

Table 4: Average pH values of the formulations

	F1	F2	F3(cp)	F4	F5	F6	F7(cp)	F8	F9	F10
pH	7.94 ± 0.03	8.06 ± 0.12	7.95 ± 0.04	8.09 ± 0.12	7.99 ± 0.15	8.07 ± 0.13	8.15 ± 0.27	8.18 ± 0.23	7.81 ± 0.25	7.99 ± 0.20

*Results are demonstrated as (mean ± standard deviation), n= 9

Surface pH analysis gives an idea about the compliance of the inserts with the tear fluid and the surface of the eye. pH of the tear fluid is in the range of 7.2–7.5 (21). However, pH values between 5.2 and 9.3 are also measured depending on age and diseases (13). Additionally, there are many alkaline eye drops that are commercially available (47). So that the films with obtained pH values were compatible with the eye. Based on the results depicted in *Figure 8*, it could be said that ALG concentration and GLY amount are directly proportional with pH whereas it is inversely proportional with PVA and k-Carr concentration. pH of 1% ALG varies between 5 and 8 which could explain the results in *Figure 8* (48). pH of 1.5% of k-Carr solution is between 7 and 10 (49). Since the prepared concentrations of k-Carr (0.1%, 0.3% and 0.5%) are too low, decrease in pH from 8.03 to 7.95 could be expected. pH of 5% of PVA solutions is in the range of 5–6 which explains the decrease in pH (36). Normally pH of used GLY is 5 but increase in pH could be explained by the interactions of it with the polymers (37).

In addition, it could be stated that the addition of more concentrated ALG and GLY generated more basic films whereas the more concentrated k-Carr and PVA generated more acidic ocular films.

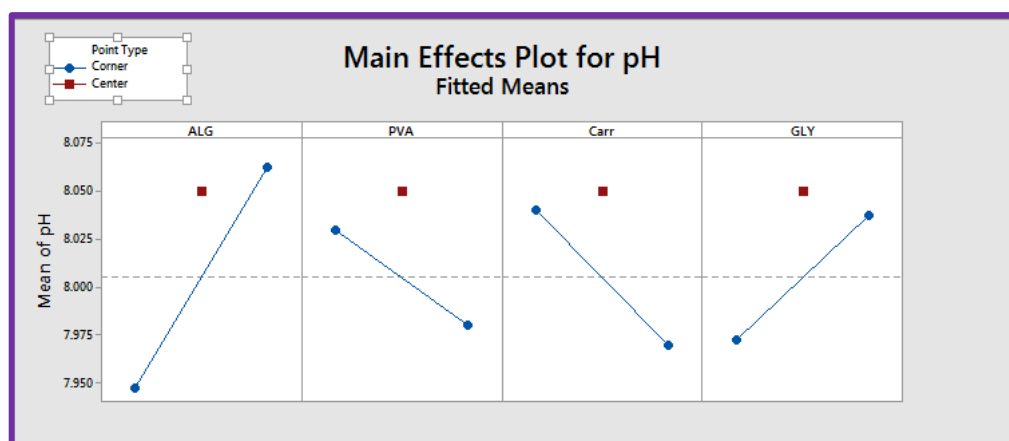


Figure 8: Main effects plot for pH by DoE

Pareto chart of the standardised effects (Figure 9) indicates that the pH was mainly affected by ALG and ALG-GLY interaction which could be caused by the strength of the possible interaction between -OH groups of GLY and NaOOC groups of ALG. Even though they affect the pH slightly, the effect of concentration of polymers/plasticiser was not statistically significant within 95% confidence interval, where $\alpha=0.05$.

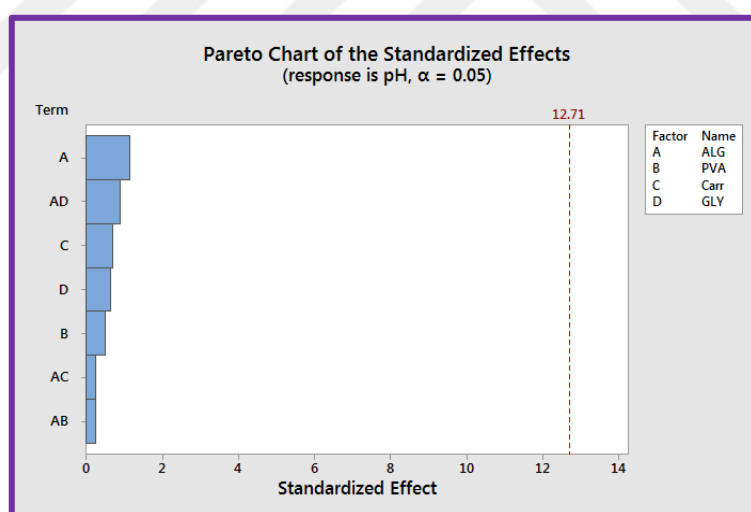


Figure 9: Pareto chart for pH by DoE

4.2.2. Percentage Moisture Uptake

Moisture uptake analysis was applied to test the physical stability and the integrity of the ocular films (18, 30). The test was repeated two times during the experimental process because some of the films lost weight which is not possible under high humidity conditions. Different stability chamber was used to test whether the problem was in the first stability chamber or not. Same inconvenient results were

obtained with the second chamber as well. Fluctuations in humidity and temperature for three days were observed because the chambers were opened several times by the students. Temperature and humidity must be kept constant to get reliable results. The results shown in *Table 5* were picked from the results that match with each other however they may not reflect 100% accurate results due to the inconvenience occurred. Results in *Table 5* were calculated by using *Equation 1*.

Table 5: Average percentage moisture uptake of the formulations, $n=1$

	F1	F2	F3(cp)	F4	F5	F6	F7(cp)	F8	F9	F10
Moisture Uptake (%)	0.54	2.93	5.08	3.57	1.75	1.79	1.91	2.01	1.95	6.54

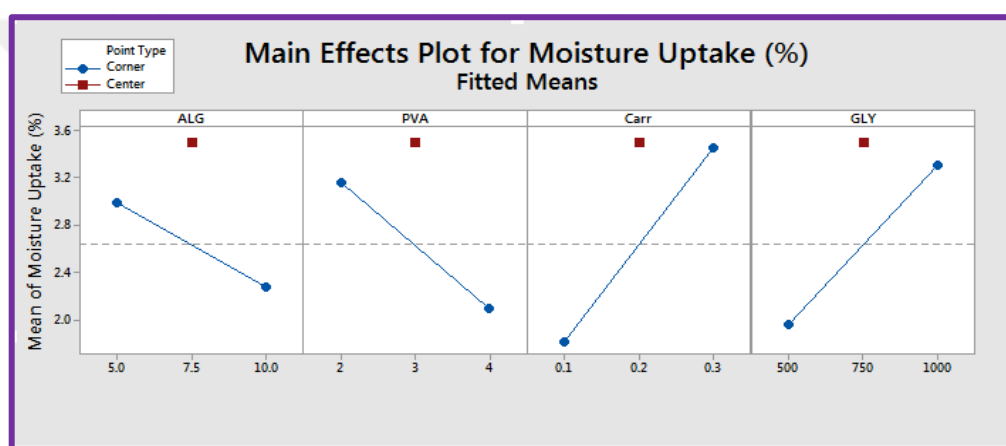


Figure 10: Main effects plot for moisture uptake by DoE

As shown in *Figure 10*, increase in concentration of ALG and PVA reduced the moisture uptake while k-Carr concentration and GLY amount were directly proportional with it. Difference in moisture uptake could be due to different porosity of the films which depends on the concentration and type of the polymers and the plasticiser used (18). k-Carr with its sulphate groups is known with its good swelling index which is well-correlated with the increase in moisture uptake (31). Normally moisture uptake increases with the increase in concentration of hydrophilic polymers (50, 51). Even though ALG and PVA are hydrophilic polymers, inverse proportion could be attributed to high concentration of the polymers. 10% of ALG made the films very rigid which could be decreasing the porosity of the films that might prevent the ability to absorb water. Another possibility could be any possible interaction between ALG and PVA because normally PVA has good swelling index (52). As the number and the strength of -OH bonds increase, the ability to absorb water

decreases due to presence of strong network by -OH bonds (29). PVA with large number of hydroxyl groups has ability to make strong -OH bonds with other polymers so that the water is not able to penetrate easily (53). This could explain the inverse effect of PVA on the moisture uptake. However, the inconvenient results may only be due to the problems occurred in the stability chambers.

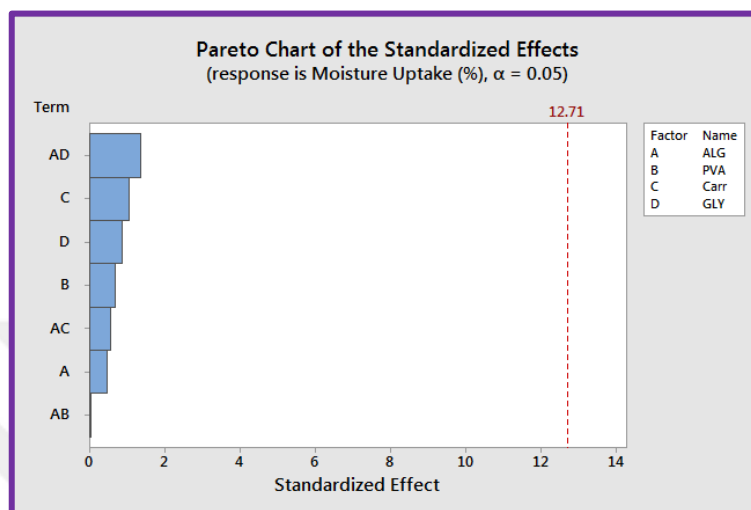


Figure 11: Pareto chart for moisture uptake by DoE

Based on *Figure 11*, moisture uptake was mainly affected by ALG-GLY interaction and k-Carr concentration itself. Vieira *et.al.* stated in their paper that increase in GLY amount in ALG-based films increased the moisture uptake which supports the findings (54). This could be one of the reasons why ALG-GLY interaction was the most effective factor on the moisture uptake. Although the effect was not deniable, concentrations of the polymers and the amount of the plasticiser did not have statistically significant effect on the moisture uptake within 95% confidence interval, where $\alpha=0.05$. Even the quantitative results may not reflect 100% accuracy, there was no sign of any disintegration in none of the formulations during the moisture uptake analysis which was observed from the physical appearance of the films. This agrees with that the formulations preserve their integrity under high humidity conditions which supports the reproducibility of the films.

4.2.3. Contact Angle Measurement

Contact angle is the angle between a liquid drop and a solid surface where liquid-vapour interface meets with the liquid-solid interface as shown in *Figure 12* (55). Contact angle measurement is a tool to analyse the surface chemistry,

wettability and hydrophilicity/hydrophobicity of the compounds. Wettability is the characteristic of a liquid that helps to remain in contact with the solid surface which results from the intermolecular interactions between the surfaces (56).

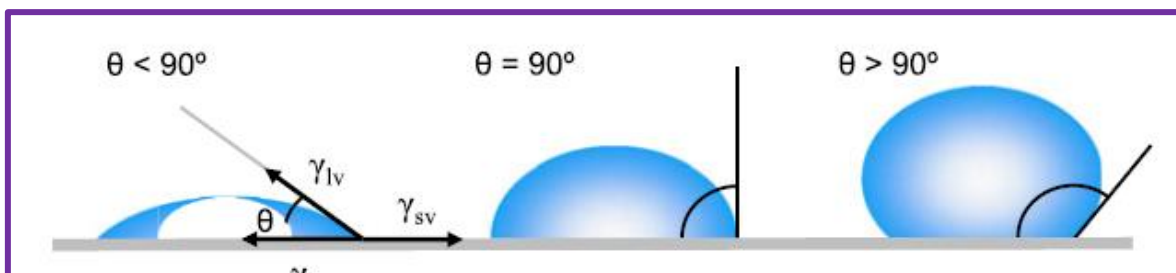


Figure 12: Contact angle measurement illustration (56)

When the liquid spreads over the surface, smaller contact angle is observed while larger contact angle is formed when the liquid beads on the surface. Contact angle is calculated by using Young's equation shown in *Equation 2*, where γ_{lv} , γ_{sv} , and γ_{sl} represent the liquid-vapour, solid-vapour, and solid-liquid interfacial tensions, respectively, and θ_Y is the contact angle.

$$\gamma_{lv} \cos \theta_Y = \gamma_{sv} - \gamma_{sl}$$

Equation 2: Young's equation (56)

If the contact angle is less than 90° , the solid is said to be hydrophilic whereas hydrophobic solid surfaces give contact angle higher than 90° . In addition, contact angle between $0-90^\circ$ states that the solid has high wettability while it has low wettability above 90° (55, 57). The solid with high wettability has stronger surface energy than the surface tension of the liquid that keeps the droplet in shape, so that the droplet cannot resist to surface energy of the solid and spreads resulting in lower contact angle (58).

Table 6: Average contact angle measurements of the formulations

	F1	F2	F3(cp)	F4	F5	F6	F7(cp)	F8	F9	F10
Contact Angle	24.4°	21.6°	26.0° ±	73.8°	23.4°	28.6°	28.7° ±	34.0°	24.7°	20.4°
	±	±	12.37	±	±	±	2.73	±	±	±
	0.71	0.85		7.95	8.97	3.00		2.87	6.29	6.21

*Results are demonstrated as (mean ± standard deviation), n= 30

Drop Shape Analyser (DSA) is an imaging tool to determine the contact angle. It is determined from the shadow image of a sessile drop which is the standard arrangement of a drop by Drop Shape Analyser, and the interfacial tension from the shadow of a pendant drop which is the drop supplied by the needle of the Drop Shape Analyser resulting from the relation between the surface tension and the gravity (59). DSA software is used to measure the contact angle and to obtain the images.

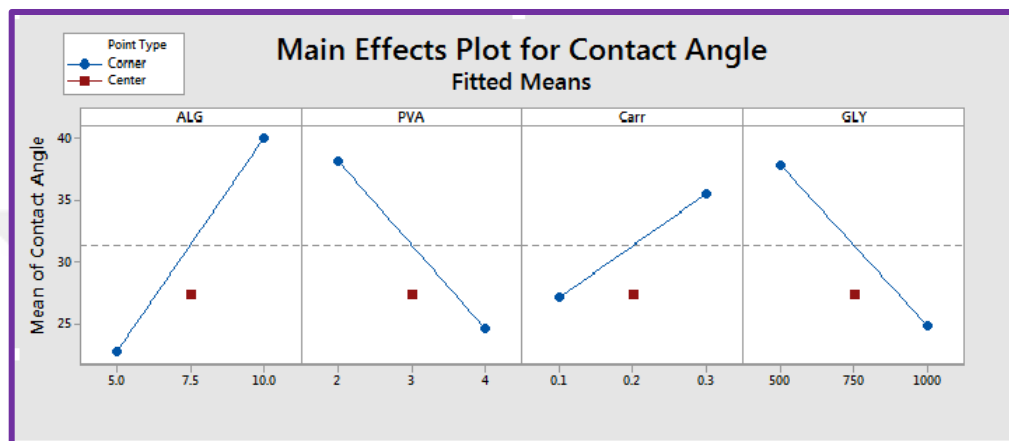


Figure 13: Main effects plot for contact angle by DoE

From Figure 13, it could be interpreted that increase in ALG and k-Carr concentrations raised the contact angle of the films whereas PVA concentration and GLY amount were inversely proportional with the contact angle. In other words, increase in ALG and k-Carr concentrations resulted in enhancement of hydrophobicity while increase in PVA concentration and GLY amount facilitated hydrophilicity of the formulations. Since GLY is a hydrophilic compound, decrease in contact angle due to increasing hydrophilicity was expected in response to increasing amount of GLY. PVA is also hydrophilic polymer which explains the revealed findings. It was known that ALG is making the films more rigid so that the increase in contact angle could be attributed to reduction in the surface energy of the films due to high ALG concentration. So that the surface tension of the liquid becomes higher than the surface energy of the film and the droplet is held by the surface tension which prevents spreading of the liquid over the ocular film. Although k-Carr is also hydrophilic, the interaction between ALG and k-Carr could be repressing the hydrophilicity of k-Carr or charged sulphate ions in the structure could be affecting the spreading of droplets.

Pareto chart of the standardised effects for the contact angle showed that the contact angle was mostly affected by ALG concentration and ALG-PVA interaction. ALG concentration had statistically significant effect on the contact angle within 95% confidence interval, where $\alpha=0.05$. Although they were not statistically significant, the effect of PVA concentration and GLY amount could not be denied.

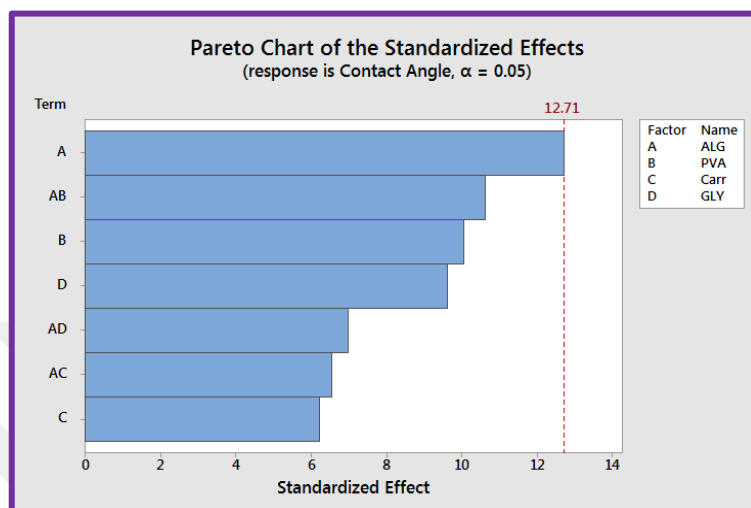


Figure 14: Pareto chart for contact angle by DoE

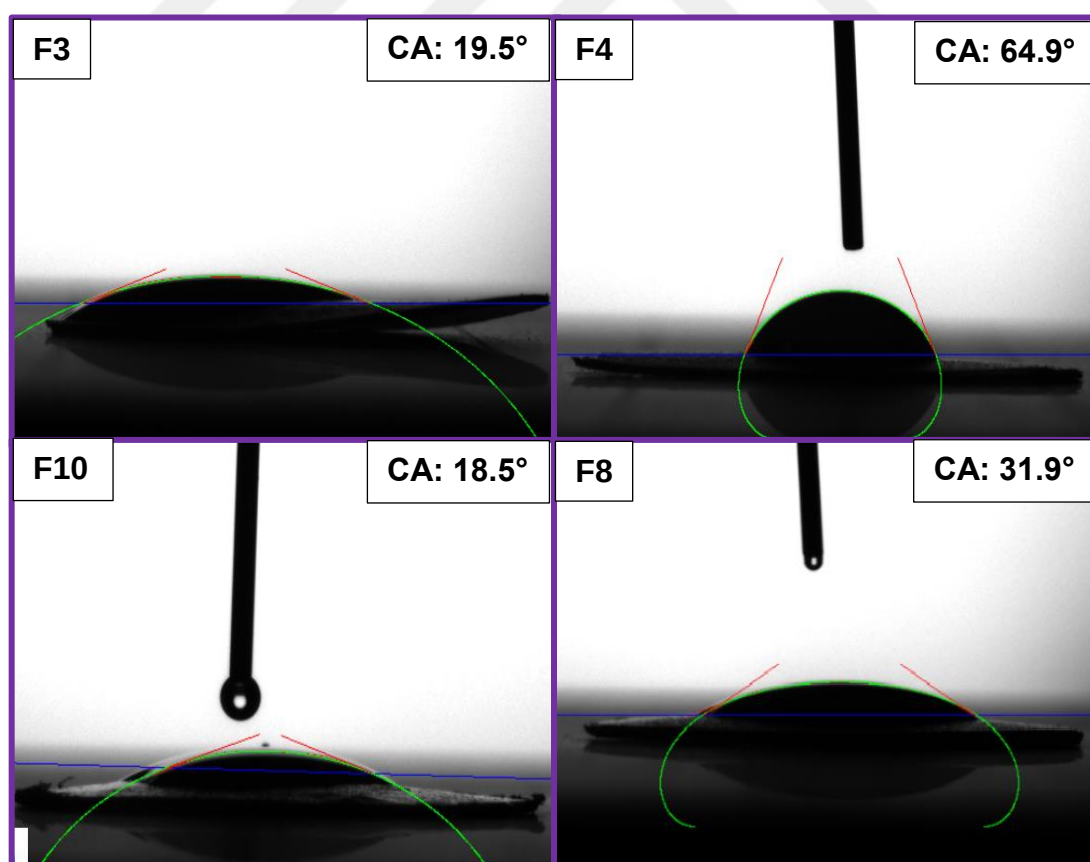


Figure 15: Contact angle images obtained from Drop Shape Analyser

F4 showed the least hydrophilic characteristic with a considerable difference compared to other formulations, as seen in *Figure 15* and it has the least wettability. Contact angle of all the other formulations varies between 20° and 35°. The most hydrophilic formulation was F10 with the smallest contact angle which was in agreement with low ALG concentration and high GLY amount. This test was important to analyse the wettability and the surface energy of the films which makes the films proper to be held by the surface of the eye. In conclusion, it could be said that all the formulations except F4 showed good ability of spreading and wettability based on the contact angle results.

4.3. MECHANICAL CHARACTERISATION

4.3.1. Folding Endurance and Tensile Strength

Both folding endurance and tensile strength tests are analysis tools that give information about the mechanical properties of the films. Folding endurance is an analysis that helps to examine flexibility and fragility of the films (18). Only F2 and F10 did not break in 300 times whereas all other formulations cracked at some certain point within 300 times as shown in *Table 7*.

Table 7: Average folding endurance values of the formulations

	F1	F2	F3(cp)	F4	F5	F6	F7(cp)	F8	F9	F10
Folding Endurance	171± 53.27	300 ± 0.00	202± 53.97	43± 3.51	67± 16.04	36± 15.72	48± 15.95	47± 19.92	163± 24.11	300± 0.00

*Results are demonstrated as (mean ± standard deviation), n= 3

Based on *Figure 16*, it could be said that PVA and k-Carr concentration did not affect folding endurance. It is certain that as the GLY amount increased, folding endurance increased as well. GLY is a plasticiser which is mainly used to increase the flexibility of the films (54). As it is seen in *Figure 16*, as the amount of plasticiser increases, the flexibility of the films escalates resulting in higher folding endurance. The study of Jethava *et.al.* about *in-situ* gelling ocular inserts containing glycerol, sodium alginate and chitosan proved that as the amount of plasticiser increased, the inserts resulted in higher folding endurance (46). The results are perfectly matching with the expected results obtained from the literature.

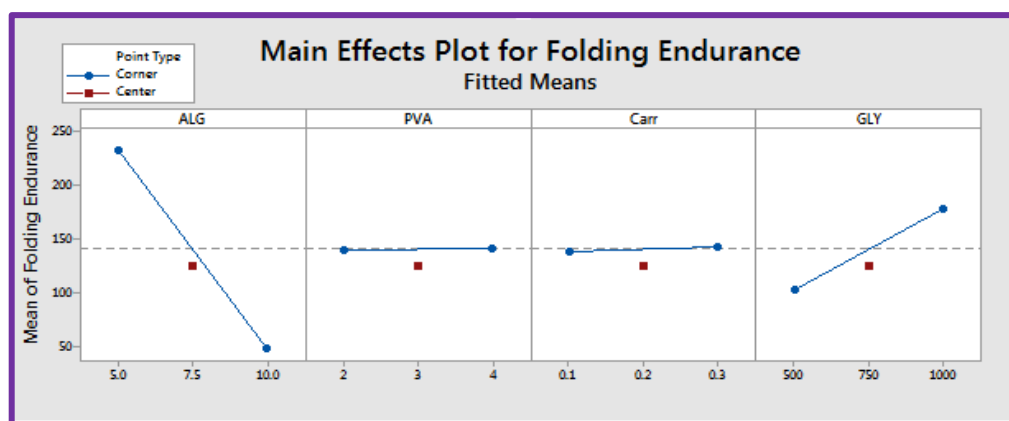


Figure 16: Main effects plot for folding endurance

In contrast to GLY result, ALG concentration was affecting the folding endurance inversely which means that as the concentration of ALG increased, flexibility of the films diminished. This result is also compatible with the physical characteristics of the films. It was obvious that the films with higher concentration ALG was more fragile and rigid based on the physical examination. Therefore, it was expected that ALG concentration is inversely proportional with the flexibility of the films. For instance, although F4 and F5 had same concentration of ALG, their rigidity was different. F5 was softer than F4 which could be correlated with the higher concentration of PVA or higher amount of GLY. Figure 17 demonstrated that the folding endurance was mostly affected by a change in ALG concentration and GLY amount even though none of the variables had statistically significant effect on folding endurance within 95% confidence interval ($\alpha=0.05$)

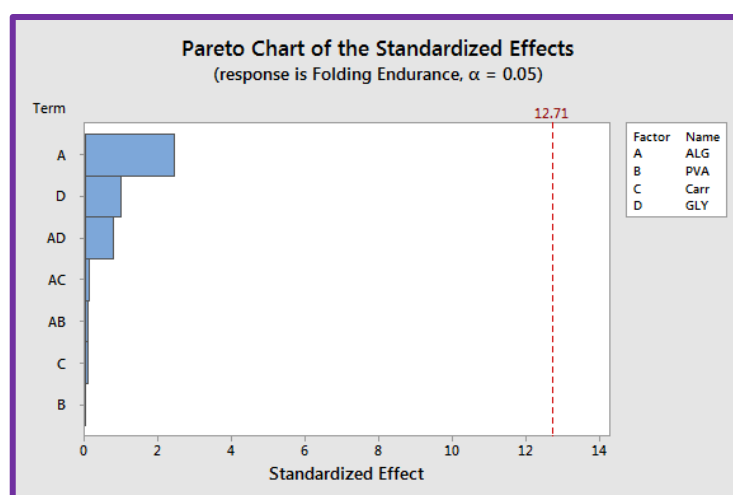


Figure 17: Pareto chart of the standardised effects for folding endurance

Tensile strength analysis is a test to determine the breaking resistance of a material when certain tension is applied (60). Tensile stress is the measurement of breakability where the strain is the measurement of elasticity (30). Tensile stress of the films was in the range of 1.76–14.75 MPa where the strain was in the range of 8.86–25.74%. The study done by Abdelkader *et.al.* about *in-situ* gelling ocular films suggests compatible tensile stress values for the ocular films which have almost similar content with the formulations of this study (30).

Table 8: Average tensile strength test results of the formulations

	Stress (MPa)	Strain (%)	Area (MPa*%)
Formulation 1	4.41 ± 1.48	11.52 ± 3.61	49.19 ± 19.31
Formulation 2	3.6 ± 0.34	21.62 ± 7.30	101.45 ± 18.33
Formulation 3 (cp)	11.41 ± 1.39	21.87 ± 4.31	193.65 ± 50.37
Formulation 4	13.52 ± 2.97	9.86 ± 5.37	148.20 ± 61.59
Formulation 5	6.81 ± 1.33	25.74 ± 9.86	131.36 ± 45.11
Formulation 6	14.75 ± 5.64	20.46 ± 8.20	292.53 ± 18.66
Formulation 7 (cp)	3.71 ± 1.07	18.13 ± 4.48	65.35 ± 27.64
Formulation 8	6.93 ± 3.46	21.89 ± 11.36	155.13 ± 139.17
Formulation 9	10.23 ± 2.62	24.71 ± 5.33	174.3 ± 65.56
Formulation 10	1.76 ± 0.37	8.86 ± 1.55	16.51 ± 2.35

*Results are demonstrated as (mean ± standard deviation), n= 3

F10 had the lowest tensile stress value while F6 had the highest stress. In *Figure 17*, it is seen that tensile strength (stress) increased in respect to increment in ALG and PVA concentration. Since ALG makes the films more rigid and tough, increase in stress was already expected. The effect of k-Carr on tensile strength was negligible. Increase in the amount of the plasticiser results in enhancement in elasticity and reduction in tensile strength normally since the purpose of GLY is to provide elasticity. Moreover, the study of Vieira *et.al.* obtained that increase in the amount of GLY in ALG containing films reduced the tensile strength which supports the findings in *Figure 18* (54). PVA was facilitating the elasticity, in the meantime it was slightly increasing the breakability. It is known that PVA has substantial tensile strength and flexibility which supports the revealed data (52). The results of tensile strength and folding endurance were compatible with each other.

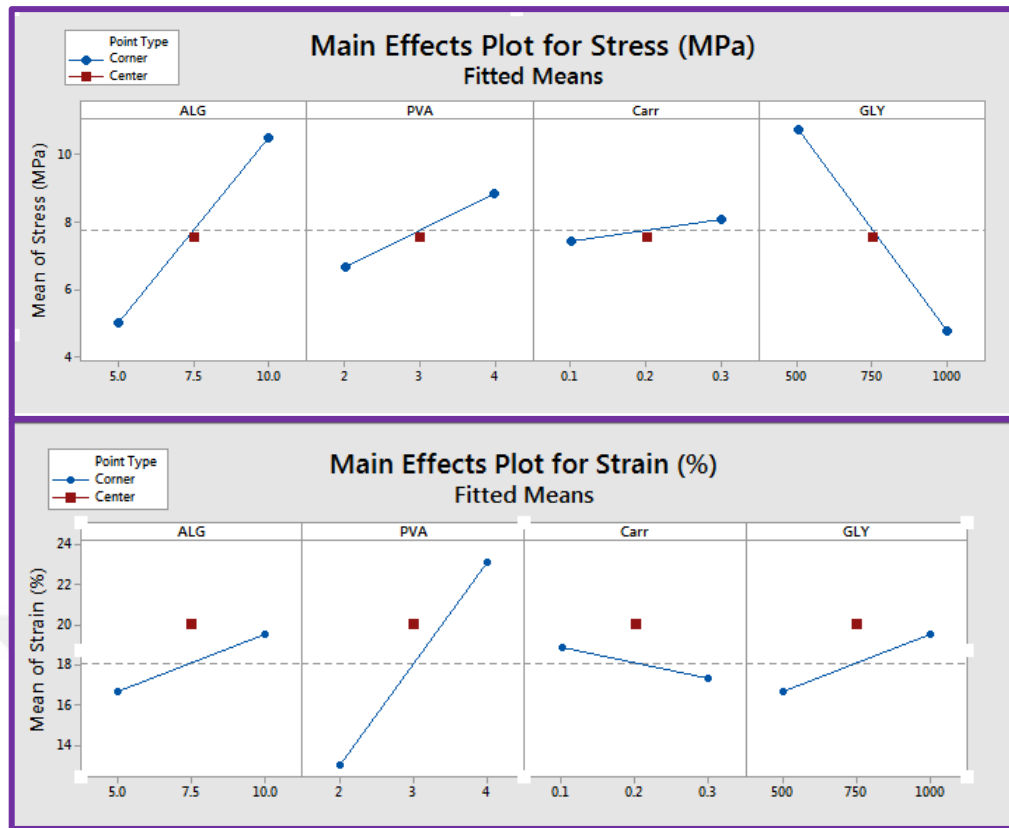


Figure 18: Main effects plots for stress and strain

Tensile strength graph has another important feature that helps to examine toughness and stiffness of the materials tested. Slope of the stress-strain curve is known as Young's modulus that can be expressed as stiffness as well. Young's modulus is described as the response of the substance to deformation. It is calculated by dividing stress over strain (61). Stiffness of the material increases as the stress increases where strain is constant or increasing with a lower rate than the stress. For instance, F10 had higher Young's modulus than F3, shown in *Figure 19*, so that F10 was stiffer than F3. In addition, area under the curve is defined as toughness which is the energy capacity of the material before fracture (62). Although F3 was less stiff than F10, F3 was able to absorb more energy than F10 before break. Based on the average values in *Table 8*, F6 had the highest toughness whereas F4 had the highest stiffness.

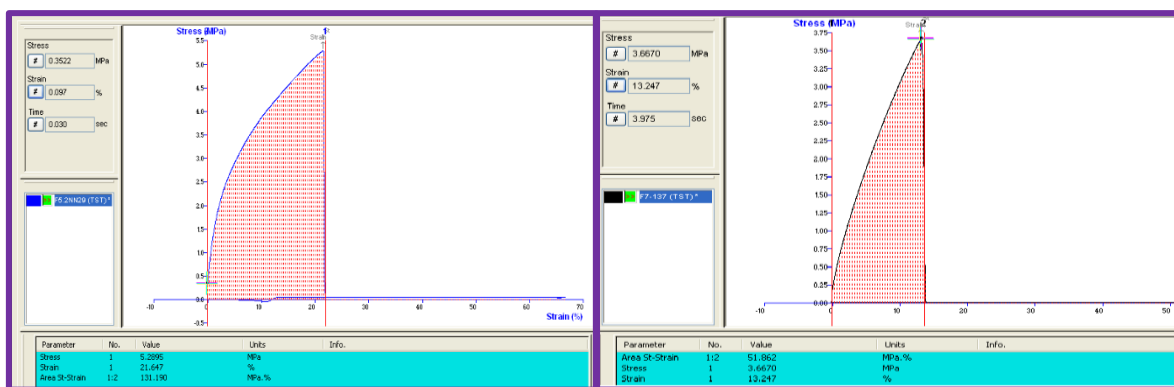


Figure 19: Tensile strength measurement graphs of the F3 (left) and F10 (right)

Based on the Pareto charts, stress was mainly affected by GLY amount and ALG concentration but none of the variables had statistically significant effect on the stress. Although strain was mainly affected by PVA concentration and ALG-GLY interaction, the effects of the variables were not statistically significant within 95% confidence interval ($\alpha=0.05$).

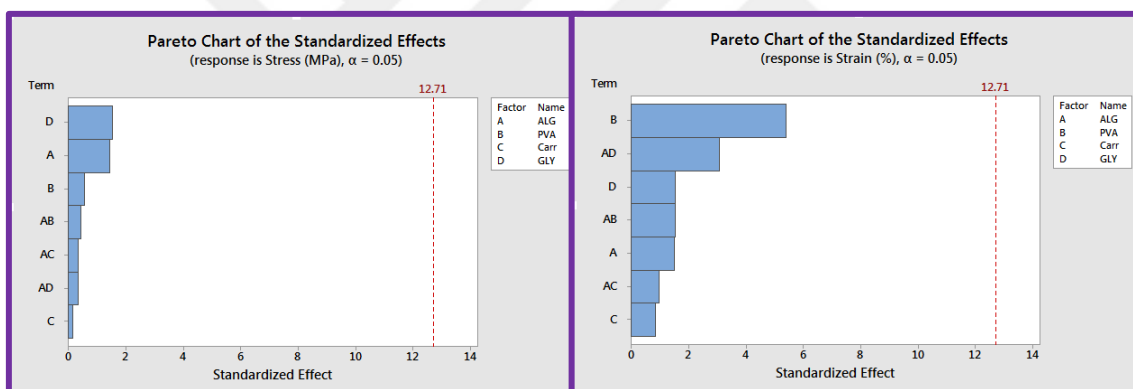


Figure 20: Pareto charts for stress (left) and strain (right)

4.4. SEM ANALYSIS

Surface morphology analysis images of the formulations are depicted in Figure 21. SEM analysis was applied to examine the phase separation and to understand how equally the excipients were distributed within the inserts (30). Almost all the formulations had smooth surface. White spots seen in some images, e.g. F2 and F3, correspond to bubbles that remained in the films during drying. Research done by Abdelkader *et.al.* suggests that differential scanning calorimetry (DSC) analysis of the polymers i.e. ALG and PVA showed that stated polymers are amorphous and able to form uniform films (30). This research also supports the findings.

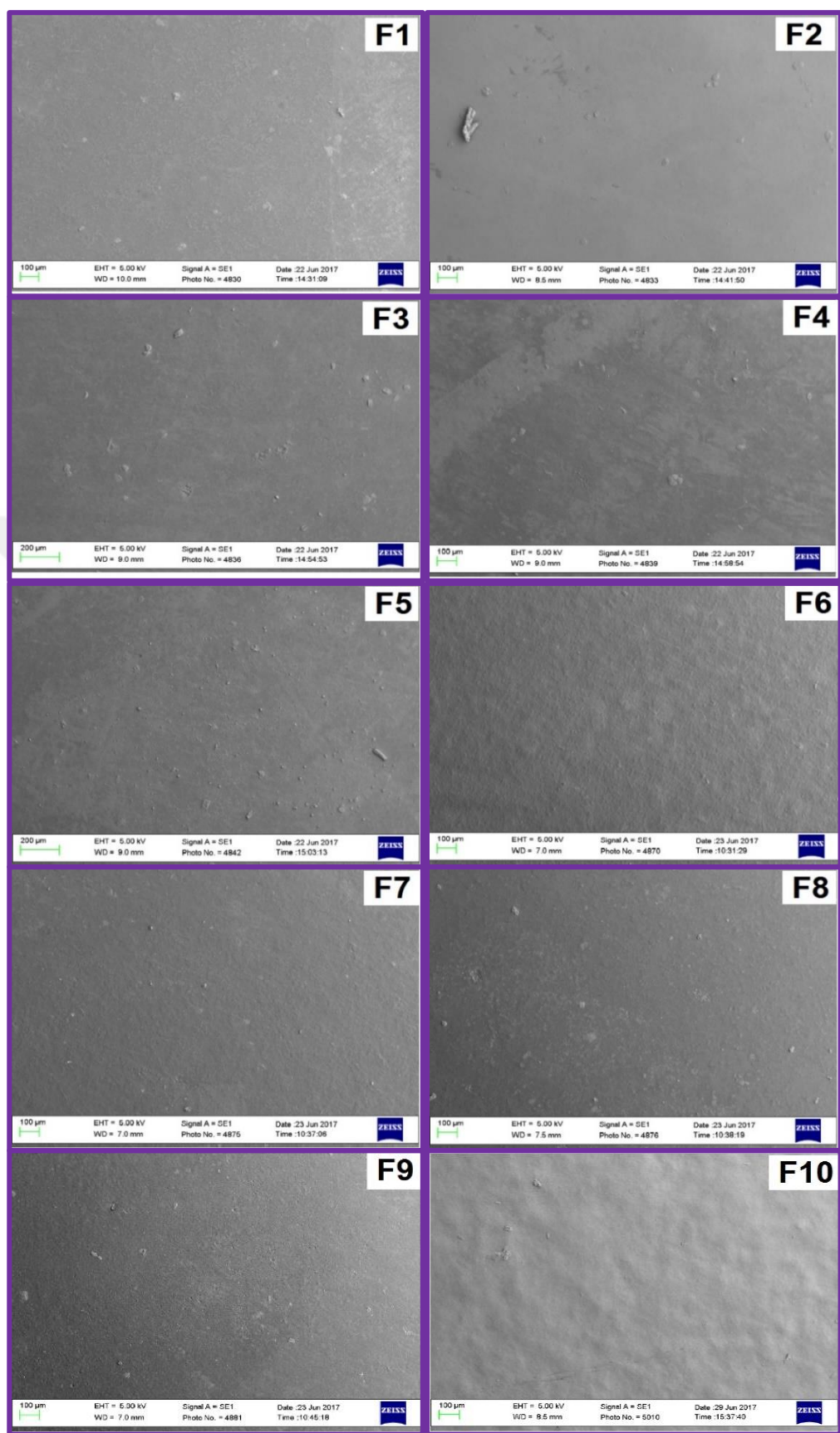


Figure 21: SEM imaging results of all the formulations

None of the formulations showed any phase separation and any texture. The smooth surface formation was due to good film-making property of the polymers and the plasticiser that were used. In addition, GLY is a well-known plasticiser that helps providing smooth surface to the films (30) and k-Carr has good gelling property which facilitates smoothing (32). F10 had slightly rough surface compared to other formulations which may be resulted from the concentration ratio of the polymers and the plasticiser or could be due to drying conditions. SEM images of the films suggested that the films were suitable for the further production in terms of surface characteristics.

4.5. MUCOADHESION

Mucoadhesion test was applied to determine how much force is needed to detach the films from the conjunctival surface. It is important to have a good mucoadhesive characteristic to prolong the retention time of the films on the ocular surface. The results and the graphs of mucoadhesion test obtained from the special mucoadhesive mode of TA-XT texture analyser are depicted in *Table 9* and *Figure 22*, respectively.

Table 9: Mucoadhesion data of the formulations, $n=1$

	Force (mN)	Travel (mm)	Work of Adhesion (mN*mm)
Formulation 1	104.59	2.27	119.61
Formulation 2	113.93	1.88	114.90
Formulation 3 (cp)	279.56	5.98	770.69
Formulation 4	225.47	2.30	274.22
Formulation 5	189.52	4.23	436.16
Formulation 6	177.25	2.04	209.34
Formulation 7 (cp)	94.38	2.09	121.34
Formulation 8	299.00	3.50	630.73
Formulation 9	102.74	1.39	85.01
Formulation 10	193.11	2.45	291.28

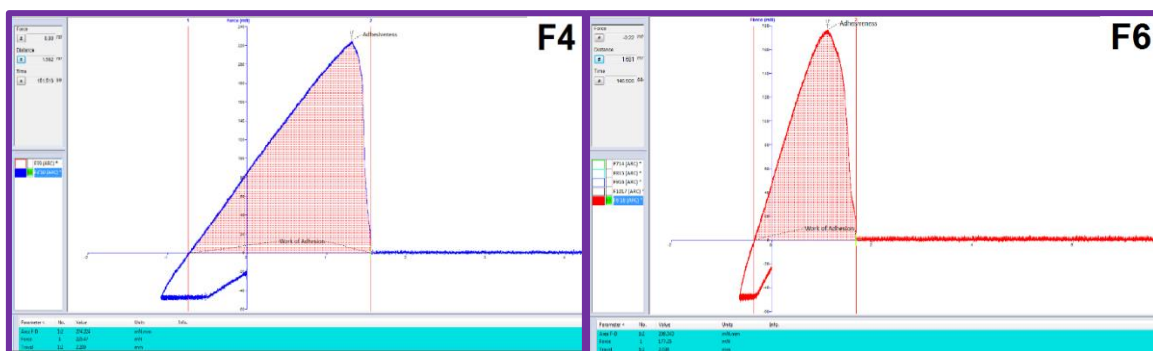


Figure 22: Mucoadhesive graphs of the formulations F4 and F6

At least one of the following characteristics should be present in the polymers to be able to have a good mucoadhesive property for the conjunctival surface: high hydrogen bonding capacity, strong anionic charge, strong hydrogen bonding groups (-OH and -COOH), adequate chain flexibility, high molecular weight, good spreading ability on mucous coat and good swelling behaviour (30, 41, 63). Polymer chains should be flexible enough to penetrate to an adequate depth on mucin coat. This connection is generated by hydrogen bonding, hydrophobic, electrostatic and van-der-Waals interactions which varies with pH and ionic strength of the polymers (2, 13).

Based on the findings, mucoadhesive force increased with respect to increment in concentration of ALG and amount of GLY. Direct correlation between ALG concentration and the force is well-matched with the findings of Abdelkader *et.al.* study. They found that the ALG-based films show better mucoadhesive property (30). One of the possible reasons could be the presence of free hydroxyl group in ALG which is available for hydrogen binding. ALG is an anionic polymer due to carboxylate functional group which can interact with the conjunctival mucin coat when it is ionised. Higher density of the carboxylate groups and high molecular weight of the polymer (100 kDa) in which long flexible chains have ability to connect with mucin better, are other contributors of good mucoadhesive characteristic. Moreover, conformational arrangements of D-mannuronic acid (M blocks) and L-guluronic acid (G blocks) of ALG are very suitable for the interaction with mucin coat. All these features make ALG a good mucoadhesive polymer (30, 41).

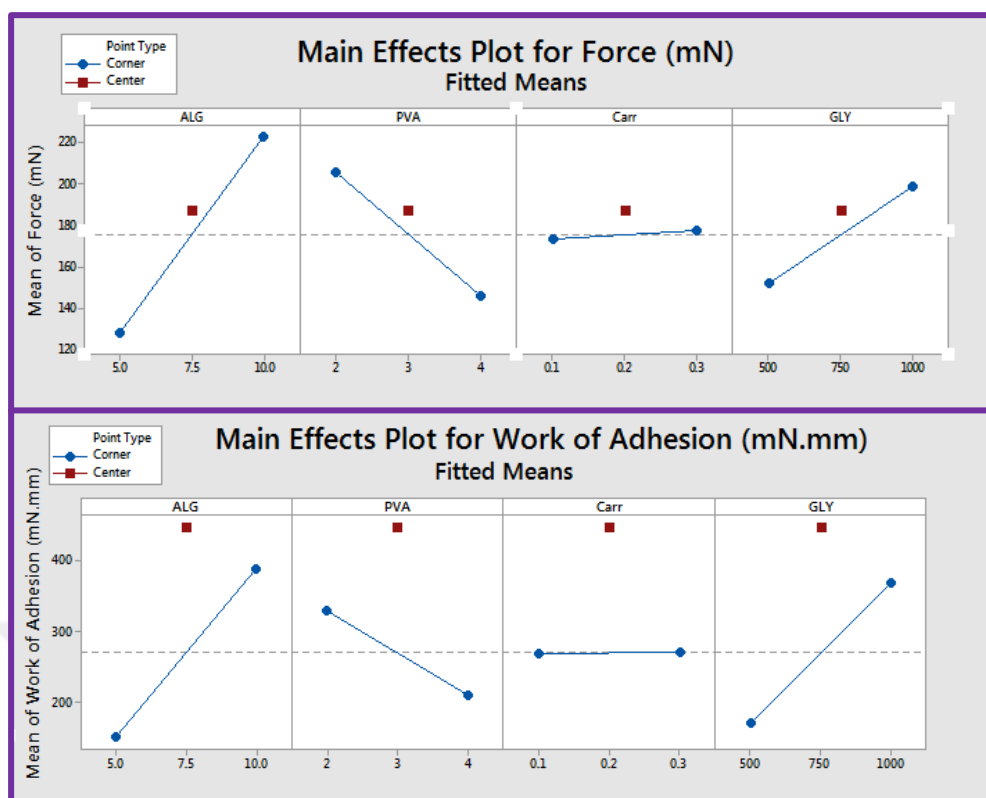


Figure 23: Main effects plots for force and work of adhesion

k-Carr seemed to be ineffective in mucoadhesion and there is no certain statement about carrageenan's mucoadhesive feature. PVA concentration was inversely proportional with the mucoadhesive force. It was an expected result because it is proposed in Ludwig's study that the mucoadhesive property of PVA is very poor compared to ALG and other charged polymers (13). Since PVA is a non-ionic polymer, it is not able to interact with the mucin surface as ALG does. Force and work of adhesion was found to be directly correlated (30). Formulations with higher force had higher work of adhesion, as well.

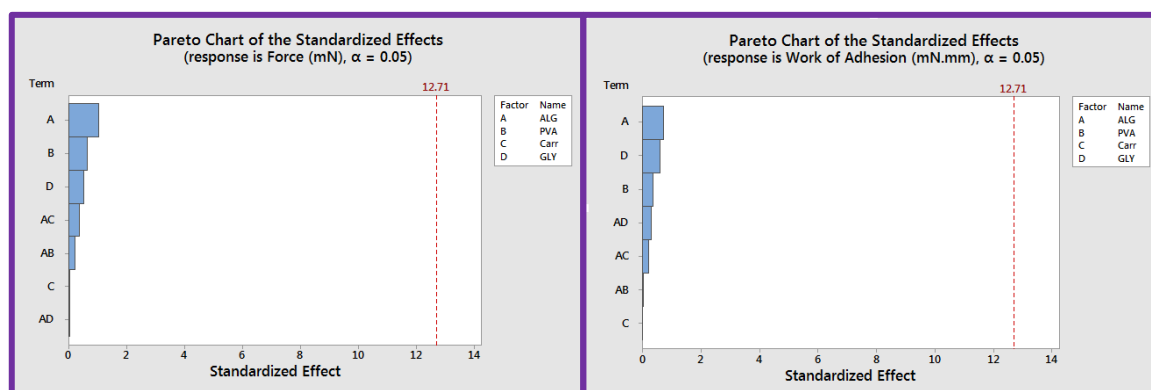


Figure 24: Pareto charts of the standardised effects for force and work of adhesion

4.6. BCOP ANALYSIS

BCOP analysis is one of the ocular irritation assays to determine any potential irritation or corrosive risk of the ocular films (64). Corneal opacity and permeability were analysed to test any possible irritation. BCOP assay is widely used as an alternative to Draize Eye Test which uses rabbits for the irritation test because the similarity between the results of BCOP and Draize Test is 73% with 8% false negative and false positive (64). BCOP assay examines the irritation by measuring the fluorescein permeability of the cornea after exposing to an ocular irritant (65).

All ten formulations were tested by incubating the eyes with the films and then by evaluating the eyes under cobalt blue filter after sodium fluorescein treatment. None of the formulations showed any irritation under the cobalt blue filter. Normally, yellow spots are produced in the presence of irritation as it is seen in positive control image of *Figure 26*. BCOP images of F1 and F3 are presented in *Figure 25* and *Figure 26* with positive and negative controls, respectively. Produced films were not irritant and not toxic without any yellow spot on the cornea. These results support further production of the films. BCOP images of all formulations can be found in the appendices section.

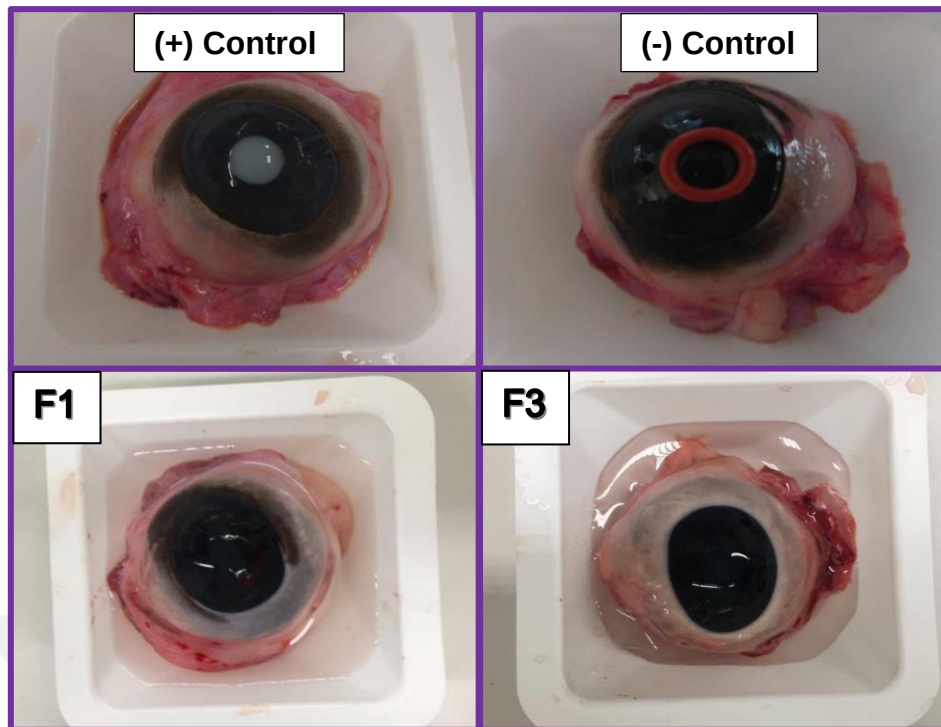


Figure 25: Positive and negative controls of excised bovine eyes before fluorescein treatment, and demonstration of F1 and F3 after the BCOP test

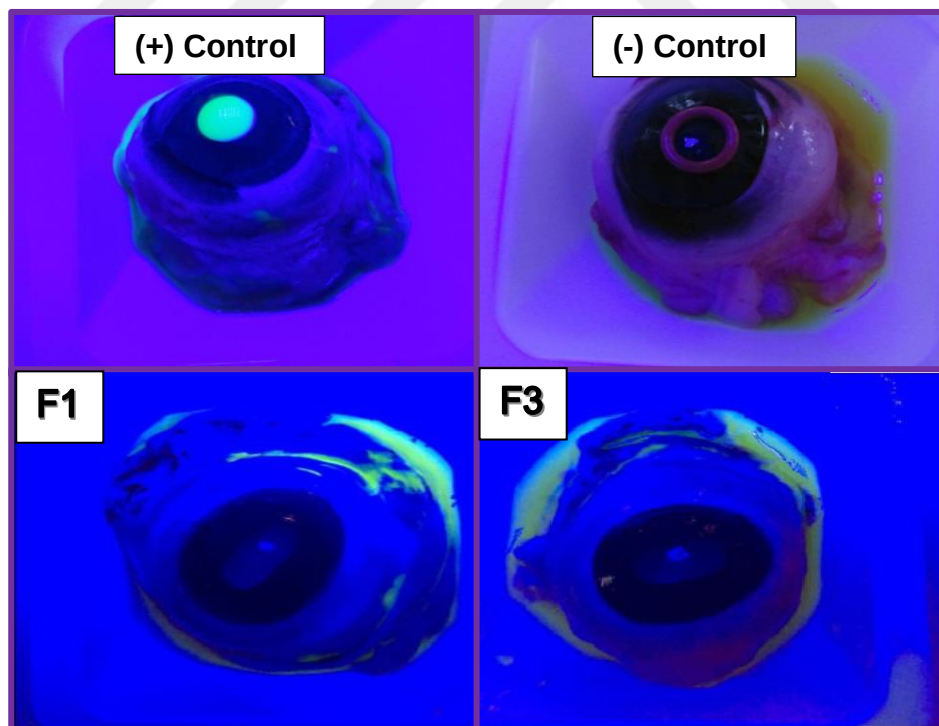


Figure 26: Positive and negative controls of excised bovine eyes under cobalt blue filter after fluorescein treatment, and demonstration of F1 and F3 after the BCOP test

5. CONCLUSION

The main aim of the study was to investigate the effect of the change in the polymer concentrations and the plasticiser amount on the formulation of ocular inserts produced by solvent casting method. So that the obtained data could be used to optimise the formulations for the future development of ocular films with better characteristics. Sodium alginate, polyvinyl alcohol, k-Carrageenan were the polymers and glycerol was the plasticiser that were tested. The effect was studied by doing weight and thickness variation, surface pH analysis, percentage moisture uptake measurement, tensile strength and folding endurance analysis, contact angle measurement and SEM imaging, mucoadhesion test and BCOP analysis. The analysis part was examined by using design of experiment, Minitab software in which main effects plots and Pareto charts were plotted.

Design of experiment was the most significant tool of the study. It was a very useful application to optimise the formulation of the ocular inserts. The effects of each individual variable on the responses were demonstrated by main effects plot. Additionally, statistical significance of the effects was determined by Pareto charts. Pareto charts analysed both the individual variable effect and the effect of the interaction between the variables on the response.

Weight and thickness variation results were in the acceptable range and verified that the films were uniformly distributed for the further production. Surface pH was in the range of 7.81–8.15 which was compatible with the tear fluid. DoE revealed that increase in ALG concentration and GLY amount generated more basic films whereas increase in PVA and k-Carr concentration produced more acidic films. Moisture uptake analysis was revealed that the films kept their integrity and stability under high humidity conditions.

The results showed that increment in PVA concentration and GLY amount generated more elastic and hydrophilic ocular films while increase in ALG concentration produced more hydrophobic, tough and rigid films with higher tensile strength. SEM imaging revealed that there was no phase separation and texture, and all the films had smooth surface. In overall, all the films showed compatible mechanical and morphological properties.

Mucoadhesion test, on the other hand, obtained that ALG concentration and GLY amount increased the mucoadhesive force whereas PVA concentration slightly reduced the force. Acquired data demonstrated that k-Carr concentration had minor effect on the responses compared to other excipients, but it was observed that it facilitated the moisture uptake and increased the contact angle of the films. BCOP test proved that none of the films was causing any irritation. All the formulations with specified features were found safe to be used and biocompatible with the surface of the eye.

In conclusion, the effect of the concentration of three polymers and the amount of the plasticiser were studied by analysing different responses individually on the formulation of the ocular inserts. The broad objective of the study was to provide background information about the optimisation of the formulations for the further studies and film manufacturing. Although the effects were found to be statistically insignificant except contact angle, the obtained data constructed a background guidance for the future studies. Future studies may also focus on the amount of polymers, besides the concentration effect.

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7. APPENDICES

7.1. APPENDIX I

Table 8: Results of all the analysis for the formulations 1 – 5

Overall Evaluation	F1	F2	F3	F4	F5
Av. Weight	13.33 mg	15.94 mg	10.64 mg	14.33 mg	17.80 mg
Av. Thickness	0.22 mm	0.27 mm	0.17 mm	0.21 mm	0.28 mm
pH (7.4-7.5)	7.93	8.06	7.95	8.09	7.99
Folding Endurance	171 times	No break (300 times)	202 times	43 times	67 times
Contact Angle	24.4°	21.6°	26.0°	73.8°	23.4°
Stress (MPa)	4.41	3.6	11.41	13.52	6.81
Strain (%)	11.52	21.62	21.87	9.86	25.74
Area (Stress*Strain) (MPa*%)	49.19	101.45	193.65	148.20	131.36
Moisture Uptake (%)	0.54	2.93	5.08	3.57	1.75
Force (mN)	104.59	113.93	279.56	225.47	189.52
Travel (mm)	2.27	1.88	5.98	2.30	4.23
Work of Adhesion (mN*mm)	119.61	114.90	770.69	274.22	436.16

Table 9: Results of all the analysis for the formulations 6 – 10

Overall Evaluation	F6	F7	F8	F9	F10
Av. Weight	16.75 mg	14.72 mg	16.50 mg	14.54 mg	12.37 mg
Av. Thickness	0.25 mm	0.23 mm	0.25 mm	0.23 mm	0.24 mm
pH (7.4-7.5)	8.07	8.15	8.11	7.81	7.99
Folding Endurance	36 times	48 times	47 times	163 times	No break (300 times)
Contact Angle	28.6°	28.7°	34.1°	24.7°	20.4°
Stress (MPa)	14.75	3.71	6.93	10.23	1.76
Strain (%)	20.46	18.13	21.89	24.71	8.86
Area (Stress*Strain) (MPa*%)	292.53	65.35	155.13	174.3	16.51
Moisture Uptake (%)	1.79	1.91	2.01	1.95	6.54
Force (mN)	177.25	94.38	299.00	102.74	193.11
Travel (mm)	2.04	2.09	3.50	1.39	2.45
Work of Adhesion (mN*mm)	209.34	121.34	630.73	85.01	291.28

7.2. APPENDIX II

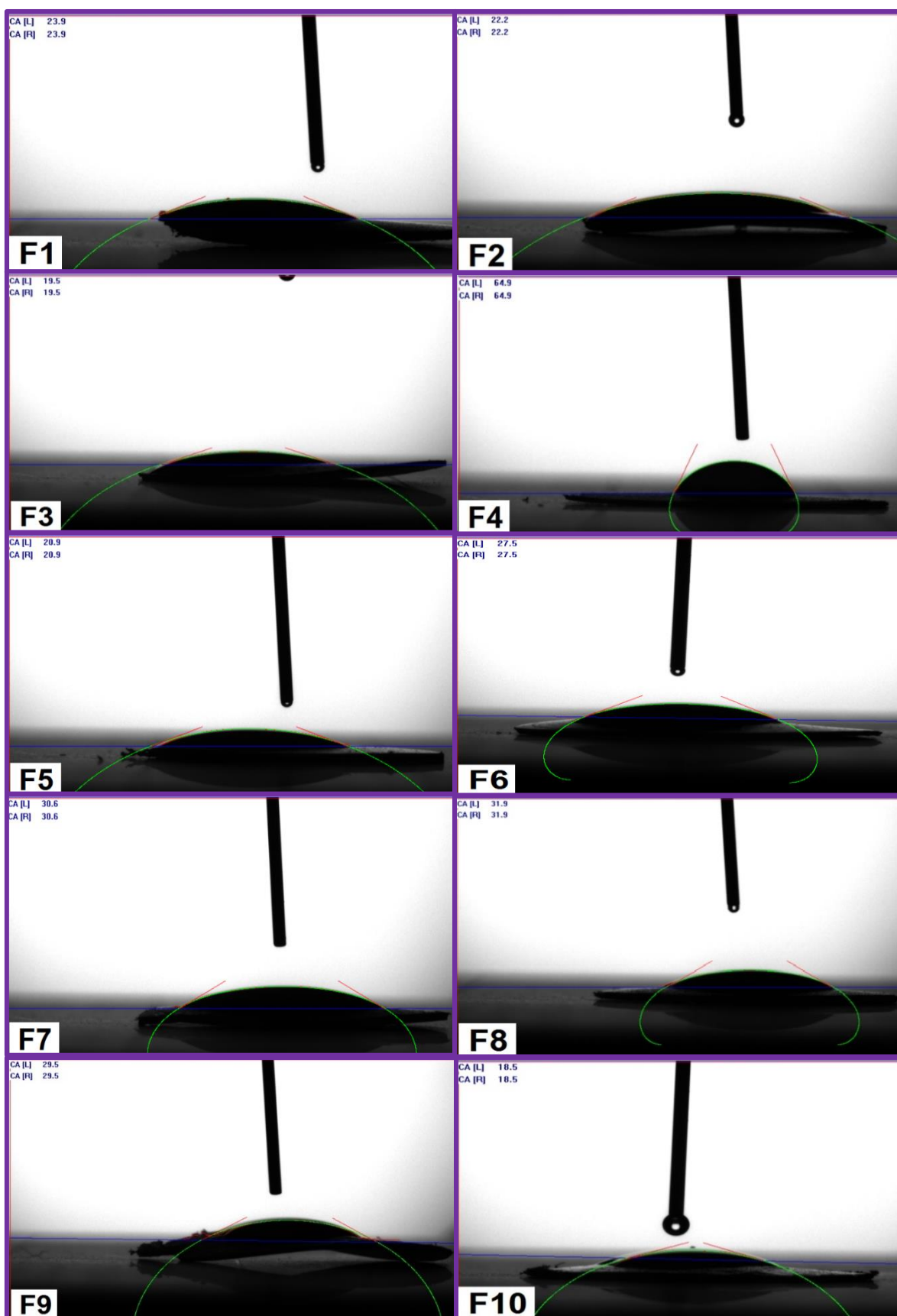


Figure 27: Contact angle images of all the formulations by Drop Shape Analyser

7.3. APPENDIX III

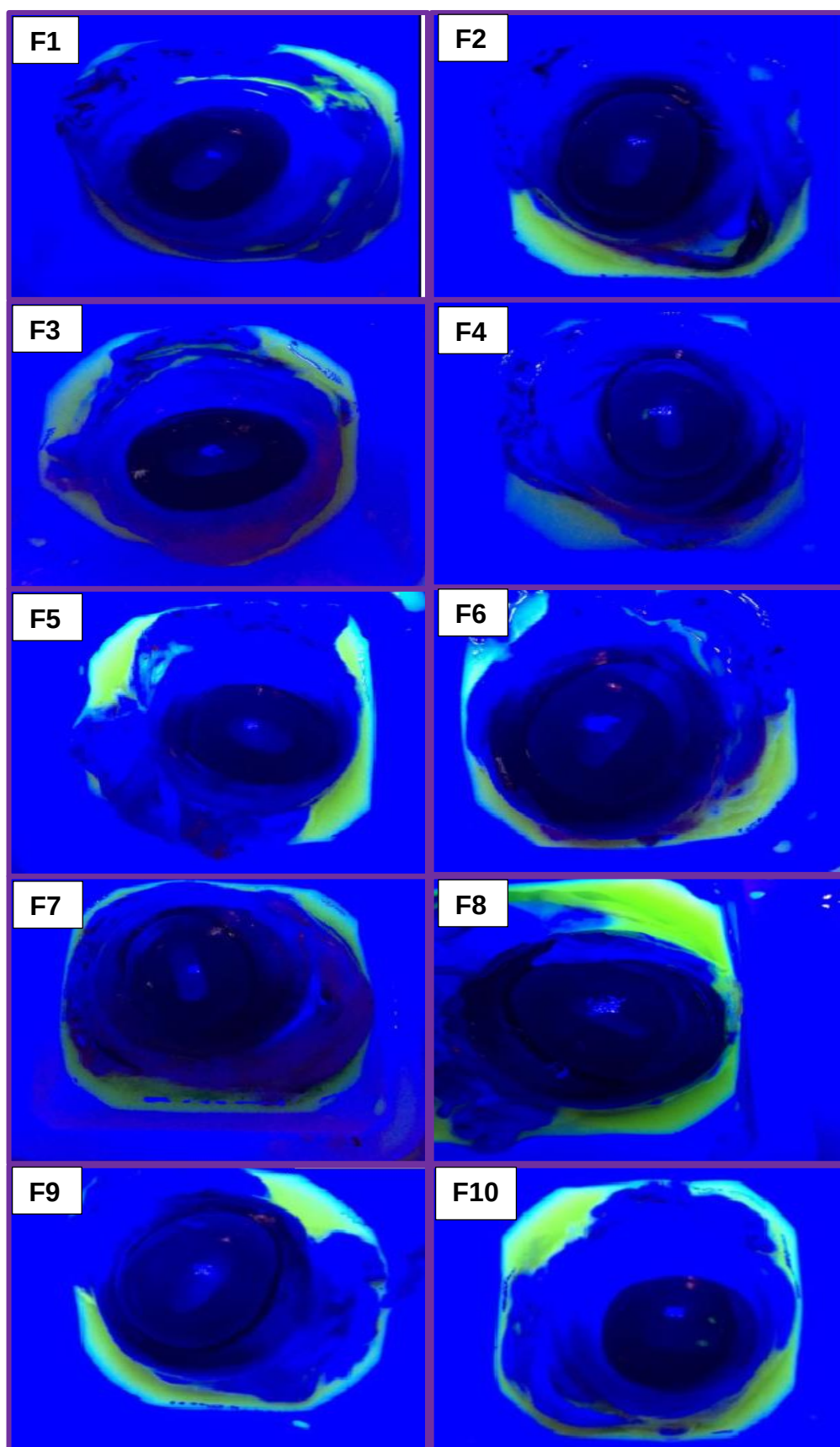


Figure 28: BCOP images of all the formulations after fluorescein dye treatment