LIPOSOMES AS VEHICLES FOR TOPICAL OPHTHALMIC DRUG DELIVERY AND OCULAR SURFACE PROTECTION

ABSTRACT (maximum 200 words)

Introduction: The development of ophthalmic formulations able to deliver hydrophilic and hydrophobic drugs to the inner structures of the eye and restore the preocular tear film has been a leading topic of discussion over the last few years. In this sense, liposomes represent a suitable strategy to achieve these objectives in ocular drug delivery.

Areas covered: Knowledge of the different physiological and anatomical structures of the eye, and specially the ocular surface are critical to better understanding and comprehending the characteristics required for the development of topical ophthalmic liposomal formulations. In this review, several features of liposomes are discussed such as the essential materials used for their fabrication, basic structure and preparation methods, from already established to novel techniques, allowing the control and design of special characteristics. Besides, physicochemical properties, purification processes and important strategies to overcome delivery or encapsulation challenges are also presented.

Expert opinion: Regarding ocular drug delivery of liposomes, there are some features that can be re-designed. Specific biocompatible and biodegradable materials presenting therapeutic properties, such as lipidic compounds or polymers significantly change the way of tackling ophthalmic diseases. Besides, liposomes entail an effective, safe and versatile strategy for the treatment of diseases in the clinical practice.

Keywords: Ocular topical liposomes dry eye, ocular surface, ophthalmology, ocular drug delivery.

Article highlights box:

- The precorneal tear film preserves ocular surface integrity, cornea and conjunctiva.
- Corneal low permeability entails a challenge to deliver active substances that target both the anterior and posterior segment of the eye.
- Liposomes are biocompatible and biodegradable lipid-made spherical vesicles that resemble cell membranes able to permeate and deliver both hydrophilic and hydrophobic drugs.
- The use of liposomes with similar components to those present in the precorneal tear film entails a novel strategy in the treatment of dry eye disease.
- Methods based on ethanol injection and microfluidics resulted the best options for liposome scaling-up due to their feasibility, robustness and optimization potential.
- Technological strategies such as the incorporation of bioadhesive biocompatible polymers or positively charged phospholipids help to increase mucoadhesion, retention time and permeation of liposomes in the cornea.
- One of the major issues that limits the use of liposomal formulations is the sterilization. A combination of sterilizing filtration and cold methods seems to be the most suitable alternative to industrial fabrication of liposomes.
- A simultaneous administration of topical ophthalmic liposomal formulations with supplements, such as vitamins or fatty acids, represent an important strategy for the recovery of the tear film lipid layer in ocular surface pathologies such as the dry eye syndrome.
- Development of technological strategies that increase the stability of liposomal dispersion is required.

- The attachment of highly specific biomolecules to the liposomal surface and using intrinsic therapeutic materials might entail the next generation of nano-liposome formulations.

1. Introduction.

The development of drug delivery systems for the treatment of ocular diseases is a great challenge mainly owing to the numerous mechanisms of eye protection against exogenous substances that act as effective barriers hindering the entry of drugs [1].

Anatomically, the eye can be divided into anterior and posterior segments. The anterior segment is formed by the first third of the eyeball, made up of structures such as the cornea, conjunctiva, iris, ciliary body, the lens and aqueous humor. The most important pathologies related to the anterior segment include dry eye disease, cataracts, conjunctivitis and keratitis. On the other hand, the posterior segment encompasses the retina, optic nerve, choroid, and vitreous humor. It can be affected by diseases that cause a significant damage in vision, including irreversible blindness, such as glaucoma, age-related macular degeneration and diabetic retinopathy, among others [2]. The treatment of the majority of ocular pathologies, requires that the drug overcomes anatomical barriers such as the cornea, conjunctiva, sclera and retina, and the blood-aqueous and blood-retinal barriers. If the formulation is administered topically physiological barriers such as eye drainage, blinking, dilution in tears and blood and lymphatic flow also limit the access of ophthalmic drugs to the intraocular target tissues.

The routes of ocular drug administration differ according to the desired site of action. The most frequent treatments of the anterior segment diseases consist in the topical administration of eye drops on the ocular surface, which has important advantages over the systemic route: Less toxicity, quicker onset of action and less dose required. Furthermore, the topical route is less invasive than other routes of ocular administration. However, the main problem is often the low bioavailability of the topical administration if the drug has to reach intraocular targets: It is estimated that only 5% of the administered drug reaches the aqueous humor [3][4][5]. For the treatment of posterior segment diseases, the challenge is much greater. Drugs administered by the topical route do not achieve the target site as easily due to the ocular barriers, so intravitreal and periocular administrations such as intravitreal, subconjunctival and retrobulbar injections are preferred. As a drawback, these routes must be used repeatedly to maintain therapeutic drug levels, which entails numerous adverse effects[5][6][7][8].

The main limitations for the topical ocular administration are the tear drainage and dilution of the eye drops, the low residence time of the formulation, the poor corneal and conjunctival absorption of the drug and the drug loss in systemic circulation. To improve topical ocular bioavailability, several resources are used to increase penetration and minimize drug loss (i.e., use of polymers that increase viscosity and mucoadhesion). Another alternative involves the use of drug delivery systems to enhance ocular delivery, such as nanosystems or microsystems capable of raising the bioavailability of the active substance and providing a controlled and sustained drug release [3][9][10].

Micro- and nanotechnologies are widely used for the development of controlled drug delivery systems because they are able to protect the drug from external factors and increase its bioavailability. Some of these systems include microparticles, nanoparticles, nanoemulsions, microemulsions and liposomes.

Microparticles (MPs) are drug delivery systems with sizes between 1 and 1000 μm. For ophthalmic use, the biodegradable polymers polylactic acid (PLA), polyglycolic acid (PGA) and poly (lactic-co-glycolic acid) (PLGA) are the most commonly employed. Microparticles can be classified into microspheres and microcapsules depending on whether the active ingredient is dispersed in the polymeric matrix (microspheres) or is surrounded by the polymeric membrane (microcapsules) [11]. Microparticles are under investigation for the intraocular administration of drugs whose objective is the treatment of diseases affecting the posterior segment of the eye [5]. They have the advantage that can be injected as a suspension in a physiological vehicle, thus allowing the sustained release of the drug and therefore the reduction of the number of administrations, which supposes a reduction of the risks associated to repeated interventions. Furthermore, bioadhesive polymers such as hydroxypropyl methylcellulose (HMPC) or hyaluronic acid (HA) can be used to increase the viscosity of the vehicle and enhance injectability [11]. Nanoparticles (NPs) are smaller in size (1-1000 nm) and can be also classified into nanospheres and nano capsules depending on their structure. The small size of nanoparticles makes them available to be easily taken up by cells and being used to treat retinal pathologies. When used for topical administration, their small sizes also reduce eye discomfort and improves their contact and retention time with the ocular surface, onto the ocular surface [11]. However, they have a more limited sustained release capacity when comparing to microparticles. Microemulsions (ME) and nanoemulsions are capable of incorporating hydrophilic and lipophilic drugs. They are made up of an aqueous phase, an oily phase and surfactants combined in different proportions allowing the system to be stabilized. That is the reason why ME are normally considered thermodynamically stable systems. Thanks to the small droplet sizes (<150 nm) and due to its low viscosity and surface tension these pharmaceutical systems spread easily over the ocular surface, making it a good alternative for topical administration. In addition, positively charged components can be added to increase the retention time in the cornea after topical administration [5][11].

Liposomes are lipidic spherical vesicles biocompatible and biodegradable formed by lipid bilayers with a size range between 10 nm and 10 μm. Its lipid bilayer structure surrounding an aqueous core allows the incorporation of both hydrophilic and lipophilic active substances. In this way, the hydrophilic drugs can be entrapped inside the liposomes or dissolved in the vehicle in which the vesicles are dispersed, while the lipophilic ones are incorporated in the lipid bilayers. Several factors are important in determining the effectiveness of liposomal formulations, such as the properties of the encapsulated active substance, the size of the liposomes and their charge. The use of liposomes for the treatment of ocular diseases has been widely studied due to their good tolerance and their capacity to increase both hydrophilic and lipophilic drugs penetration when applied topically. This is due to their ability to interact with eye tissues such as the cornea. Liposomes present various alternatives for drug release, being able to increase the retention time of drugs on the ocular surface, as well as providing a sustained release after their administration. For example, lipid nanosystems and liposomes in combination with siRNA (lipoplexes) silencing specific genes has been employed for treating some degenerative diseases of the posterior segment of the aye such as diabetic retinopathy through injection [12]. Furthermore, the topical administration of liposomal formulations does not require the use of invasive methods [11][13][14]. Besides, apart from being used as drug delivery systems, liposomes have been also developed to be used as artificial tears, demonstrating their ability to restore the lipid layer of the tear film, improving the symptoms of pathologies such as dry eye disease (DED) [15].

The following sections will review the improvements provided by the use of liposomes in topical ocular administrations, including their composition, technological requirements and methods of preparation and encapsulation of drugs with different properties. In addition, the efficacy of the several developed technological strategies to enhance topical bioavailability and its extended use to different types of active substances will be discussed. In this review, the role of liposomes in tear film recovery and the future prospects in this area will also be emphasized.

2. The ocular surface

2.1. Precorneal tear film

The precorneal tear film is constituted by a thin layer that broadens all over the ocular surface, including cornea, conjunctiva and sclera. This structure plays a decisive function in nurturing and protecting the eye surface. The tear film is composed of an aqueous mucinous gel covering by a lipid layer [16].

The aqueous layer is secreted by the lacrimal gland and accessory lacrimal tissues. Its composition includes salts, glucose, urea, albumin and immune proteins that help to protect the ocular surface [17][18]. The function of the aqueous layer is extremely important providing the cornea with nutrients and oxygen as well as eliminating foreign bodies and toxins from the ocular surface. Furthermore, there are several proteins that play an important antimicrobial role, such as lysozyme that contributes to the stability of the tear film, or lactoferrin [19]. Other proteins present in the precorneal tear film are antibodies, such as Immunoglobulin A (IgA) immunoglobulin G (IgG) and immunoglobulin M (IgM) [18]. IgA is the main immunoglobulin present in the preocular tear film (10–80 mg/dL) playing an important role in immunity protecting against viruses, bacteria and parasites[18]. Besides, lipocalin is worth to be mentioned due to its binding properties to lipids in tears [17].

Mucins present in the preocular tear film are divided in membrane associated mucins and secreted mucins. Secreted mucins are divided into gel-forming and soluble mucins. The presence of soluble mucins has been shown to play an important role in tear film extensibility due to its ability to reduce surface tension[18][20]. Gel-forming mucins, which can reach molecular weights of 40 MDa, provide the necessary rheological properties that allow to adjust the viscosity of the preocular tear film when blinking [21]. One of the most important gel-forming mucins is MUC5AC, which is produced by conjunctival caliciform cells and grant the hydration of the ocular surface. The membrane associated mucins including MUC1, MUC4, and MUC16 are anchored to the plasma membrane of corneal and conjunctival epithelial cells via their hydrophobic terminal transmembrane domain. In addition, they form a glicocalix which main function appears to be the anchoring of a layer made out of secreted mucins. Secreted mucins are hydrophilic and negatively charged, which favors a repulsion that allows the secreted ones to slide over the epithelial mucins. In addition, this facilitates the sliding of the eyelid without adhesion to the epithelium. All these creates a lubricating layer on the ocular surface [17][22][23].

High molecular weight mucins along with proteins confer the tear film a non-Newtonian viscoelastic behavior, regulating tear viscosity when blinking and thus protecting the surface [24]. They are synthesized by the corneal and conjunctival epithelium as well as conjunctival goblet cells. The presence of terminal residues such as sialic acid gives to these glycoproteins a high negative charge, which favors the movement of mucins on the surface and helps to repel pathogens from the epithelium. Further, the high content of sialic acid could favor the blocking of the adhesion of pathogenic bacteria by binding to the adhesins to which these pathogens bind [18][25].

The production and volume management of tears is controlled by the lacrimal gland and accessory lacrimal tissues as previously mentioned and also by regulating the water flow through the cornea. Fluid removal occurs through drainage caused by the eye blink and evaporation. Thus, when the ocular surface is exposed to adverse environmental conditions evaporation occurs with a consequent increment in the tear tonicity. These events will create a flow of water through the corneal epithelium due to the channels of aquaporin, recovering the initial tone [17][26].

The lipid layer is the outermost layer of the preocular tear film and has been widely associated to the reduction of the surface tension favoring the spread of the tear film over the entire surface and the protection against tear evaporation [22][27]. Its production occurs in the Meibomian glands [24][28], and include a complex variety of lipids. The tear lipidome contains amphiphilic and nonpolar lipids which have different function. The group of amphiphilic lipids is composed of phospholipids including phosphatidylcholine, lysophosphatidylcholine and phosphatidylethanolamine and others such as sphingolipids [28][29][30]. Amphiphilic lipids appear to form a sublayer capable of interacting with polar and nonpolar tear compounds. The polar heads are oriented towards the aqueous layer and the apolar ones interact with the non-polar lipids. In this way, the amphiphilic sublayer allows the formation of a stable non-polar lipid sublayer on the surface and its spreading [30][31]. Non-polar lipid sublayer comprehends mainly wax esters, cholesteryl esters, and triglycerides. This sublayer is directly in contact with the air and when it is in proper amounts prevents the evaporation of aqueous layer. If the lipid layer is destabilized, aqueous evaporation increases, causing pathologies such as dry eye disease (DED) [31][32].

The stability of the lipid film is also related to the presence of proteins. An example of this is lipocalin, which as previously mentioned plays an important role related to the lipid layer. Tear lipocalin (TLc) is able to bind lipids such as cholesterol, fatty acids and phospholipids enabling its solubilization and transport. Therefore, lipocalin acts as a scavenger removing lipids from the corneal surface and transporting them to the lipid layer stabilizing the tear film by reducing the surface tension [27][30][33][34]. Lipocalin also binds with lipids and reinforces tears viscosity [24][35].

2.2. Cornea and conjunctiva

The cornea, a transparent tissue with refractive properties [36], is the central structure of the ocular surface. It performs essential functions such as allowing vision, protecting against damage and preventing infections [37]. The cornea is divided in five layers: Epithelium, Bowman's layer, stroma, Descemet's membrane and endothelium [38][39].

The stratified, scaly and not keratinized corneal epithelium is composed by 5 to 7 layers. The deepest layer is composed of basal cells with mitotic properties, followed by wing cells and finally superficial cells [40]. One of its characteristics is that it is constantly renewed [41]. It is responsible for protecting the cornea and can rebuilt itself after injury by sliding epithelial cells to cover the region followed by a mitotic process [42][43]. In addition, epithelial corneal cells express aquaporin-5 channels in charge of transporting water through the epithelium [44]. The epithelial basement membrane (BM), with a high content of type IV collagen and laminin produced by the basal cells [41][43], is located between the corneal epithelium and the stroma and regulates the levels of cytokines and growth factors in both of them. In addition is in charge of the adhesion of epithelial cells to the stroma and is involved in the migration, proliferation, and differentiation of epithelial cells [42] [45].

Bowman's layer is an acellular structure formed by collagen which has no regenerative capacity. It represents the superficial layer of the stroma, enclosed by the basement membrane and the anterior stroma. This layer appears to form as a result of the interaction between the corneal epithelial cells and the stromal keratocytes [39][46]. It provides protection to subepithelial nerve plexus [47].

The stroma represents 90% of the corneal thickness [39]. It is a highly innervated layer [47] composed of collagen (the major component disposed in regular lamellae), keratocytes and proteoglycans such as lumican and keratocan. The characteristic distribution allows light to pass through the collagen and prevents its dispersion [39][48]. This process is possible because proteoglycans interact with collagen (type I, IV and XII), allowing collagen fibrils to maintain their position [49]. Keratocytes, arranged among the lamellae, are in charge of synthesizing the components of the stroma. Moreover, they can respond to signals from corneal epithelial cells, going into apoptosis or activate into reparative phenotypes in the presence of damage [50].

Descemet's membrane is composed of an anterior layer formed by collagen and a posterior layer secreted by the endothelium, which thickens over time [39]. The presence of type IV and VIII collagen is characteristic in this membrane, forming a hexagonal grid. Its function is to maintain corneal hydration and protect the endothelium. Furthermore, it seems to have resistance capacity against intraocular pressure [51][52][53].

The deepest corneal layer is the endothelium, a single layer of hexagonal cells [39]. Although endothelial cells have proliferative capacity, it is too slow to replace cell loss, so the number of these cells decreases with age [54][55]. Its main function is to regulate the hydration of the stroma, allowing the transparency of the cornea to be maintained. The underlying mechanisms by which it is regulated involves the presence ionic pumps [56]. Also, the aquaporin-1 channels present in the endothelium have been suggested as responsible for regulating the transport of water through the endothelium and as a key to preventing corneal edema [44].

With regard to conjunctiva, it is a thin transparent mucous layer. Unlike the cornea, the conjunctiva is highly vascularized. It covers the sclera, which is made up of collagen fibrils and proteoglycans as well as the stroma, and the inner part of the eyelids. According to its location, it is divided into two areas: bulbar and palpebral conjunctiva. Bulbar conjunctiva covers the anterior part of the eye and surrounds the cornea. Palpebral conjunctiva is in charge of covering the back of the eyelids [40][57]. Conjunctival structure is formed by the epithelium with 3–5 cell layers resting on the basal membrane and the lamina propria. The lamina propria is composed of connective tissue and is highly vascularized. The conjunctival epithelium is separated from the corneal epithelium by the limbal epithelium and contains two main types of cells: stratified squamous cells and goblet cells. Both types of cells appear to be regulated by growth factors, and while the stratified squamous cells secrete water and electrolytes, the goblet cells, as mentioned above, are responsible for the secretion of mucins present in the tear film. This function is essential to maintain a correct lubrication of the eye, and its reduction may be responsible for pathologies such as dry eye disease [58][59][60][61]. Moreover, it has been shown the presence of Langerhans cells involved in the immune response, capable of migrating to the cornea when inflammation of the conjunctiva occurs [62]. Another important feature of the conjunctiva is the presence of the conjunctival associated lymphatic system (CALT) attached to the immune protection of the ocular surface. It is composed of lymphoid follicles as accumulations of B lymphocytes and follicular dendritic cells, specialized vessels, intraepithelial lymphocytes and a lymphoid layer located in the lamina propria which contain lymphocytes, mainly T cells, and plasma cells which mostly produce IgA [63][64][65].

2.3. Drug delivery across the ocular surface

As previously mentioned, topical administration of drugs, whose site of action is usually the anterior segment of the eye, including the ocular surface presents low drug bioavailability. The passage of drug through the cornea allows it to reach internal tissues such as the iris, the ciliary body and the lens. Otherwise, conjunctival penetration allows the drug to enter tissues such as the sclera, the choroid, and even the retina.

For the ocular topical administration of drugs, the physiological role of the tear film must be taken first into account. The tear film has a volume of 7 µl and a restoration time of 2 to 3 minutes. The maximum volume of eye drops that the eye can contain is 30 µl, which means that a limited volume of the ophthalmic formulation can be deposited in the eye. In addition, most of the eye drops are eliminated rapidly from the human eye surface due to blinking and tear turnover: 16% of the tear will be replenished in one minute. This means that less than 5% of the drug reaches the intraocular tissues due to the short time retention, which supposes a great loss of drug. [66][67][68]. The mucin layer attached to the corneal surface presents hydrophilic properties, and also present a negative charge due to its composition. Thus, the use of positive charged delivery systems implies an increase in the residence time on the surface, and therefore in its permeability [69].

Regarding the passage through the cornea, its low permeability and small surface area also becomes a challenge. Drugs can pass through the cornea via the transcellular or paracellular routes. The former involves dealing with the different layers of the tissue that act as a barrier. The corneal epithelium is a lipophilic layer, which supposes a resistance to the penetration of hydrophilic molecules. The corneal stroma composed of collagen fibrils has hydrophilic properties, making it difficult for lipophilic molecules to pass through. Endothelium, the barrier among the stroma and aqueous humor, as well as epithelium, is a lipophilic layer. With regards to the paracellular route, in the epithelium the superficial cells have a small junction space that hinders the paracellular penetration of the drug. Nevertheless, in the endothelium the leaky junctions between the cells are easier for macromolecules to traverse between stroma and aqueous humor, being less limiting than the epithelium. Accordingly, the main barriers for hydrophilic and lipophilic substances are the epithelium and stroma respectively [66][70].

The ability of drugs to cross the cornea is conditioned by the size and the distribution coefficient of the active substance. The higher the diffusion coefficient, the greater the importance of the transcellular pathway. For values of distribution coefficient between 0,01-10, the pass through the lipophilic epithelium and endothelium becomes more viable. When the value is higher than 10, almost all the passage occurs through the transcellular route and the stroma becomes the limiting barrier. This is the reason why when the distribution coefficient is too large the permeability stops increasing. However, in the case of solutes with a low distribution coefficient, that is, substances with a hydrophilic nature, the main impediment is the epithelium and the main passage through the cornea is the paracellular route. In this sense, the passage of hydrophilic substances depends on their size or molecular weight, being this process easier for small solutes with a molecular weight less than 500 Da, and especially difficult for macromolecules [71][72][73][74]. After penetration through the cornea, the drug will reach the intraocular tissues. First, the drug reaches the aqueous humor, from where it will pass to the intraocular tissues of the anterior segment. By this way, the drug will have to go through the anterior segment to reach the posterior segment [75].

Absorption through the conjunctiva is less productive due to the presence of blood and lymphatic vessels that cause a loss of the drug through the systemic circulation. Blood and lymphatic clearance are important dynamic barriers for the administration of drugs through the eye. It has been observed that the clearing produced by the blood and lymphatic vessels is related to the size of the drugs, being easier the elimination of small molecules [66]. When the drug enters the palpebral conjunctiva, a systemic absorption occurs. However, when it is absorbed through the bulbar conjunctiva which covers the sclera, the majority is lost in the systemic circulation but a small part of it passes to the intraocular tissues, being postulated as a possible via for the topical posterior segment treatment [70][76][77]. The sclera, with a similar composition to the corneal stroma, owns hydrophilic properties. In addition, because the negatively charged proteoglycans, the passage of positively charged molecules thought this layer is hindered by their binding to them [78][79].

Apart from passive diffusion, the presence of efflux and influx membrane transporters in the corneal and conjunctiva cells also plays an important role in drug delivery. The efflux transporters are responsible for decreasing bioavailability expelling the molecules out of the cells. Examples of efflux transporters are P-glycoprotein (P-gp), Breast Cancer Resistance Protein (BCRP) and Multidrug resistance protein (MRP). P-gp is a transporter of lipophilic molecules, which reduce the absorption of lipophilic drugs. Otherwise, MRP transporter effluxes organic anions and conjugated substances and BCRP transporter is also related to drug resistance [66][68]. On the other hand, the role of influx transporters is related to the transport through the membrane of nutrients and xenobiotics, so they are capable of transporting drugs with targeted modifications [70]. There are many types of influx transporters identified in ocular tissues, such as vitamins, glucose, nucleoside and monocarboxylate transporters. Among those, peptide and amino acid transporters are widely applied in ocular drug delivery. Transporter knowledge enables the development of targeted prodrugs capable of being recognized by carriers as substrates increasing ocular absorption [70][80][81][82].

3. Development and technological aspects of liposomes

3.1. Components and structure

As previously mentioned, liposomes are defined as spherical vesicles composed of lipid bilayer membranes dispersed in an aqueous solution or buffer [83]. The composition of such membranes can be tailored depending on the different physicochemical properties or characteristics that are required for the system. Normally, one type of phospholipid or a combination is chosen to engineer the liposome basic structure. All these constitutes the basic scaffold for adding the rest of the components including excipients, drugs or other substances.

Regarding ocular topical administration, soy phosphatidylcholine [84] and other phospholipids such as dioleoylphosphatidylglycerol (DOPG) have been employed due to their low immunoreactivity and benefits to corneal regeneration [85]. Besides, it is worth mentioning that soybean phosphatidylcholine is one of the most commonly used and interesting phospholipids, since contains phosphatidylcholine, the most common phospholipid present in cell membranes and incorporates a remarkably wide and rich profile of fatty acids, such as palmitic (C16:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2), and linolenic (C18:3). Some of them are unsaturated, that means that might provide an antioxidant effect for the ocular surface and the formulation itself [86].

Another essential component that stabilize liposomal membranes and provides bilayer rigidity is cholesterol [87]. In fact, cholesterol was previously described as an stabilizer of intermolecular forces between phospholipids improving stability and avoiding dispersion in liposomes [88]. According to the total amount or number of bilayers present as well as their size distribution, liposomes can be classified in multilamellar vesicles (MLVs) and unilamellar vesicles (ULVs). ULVs are also subdivided into small unilamellar vesicles (SUVs) and large unilamellar vesicles (LUVs) [83]. MLVs are commonly obtained in the first steps of liposome fabrication, being reduced up to LUVs or SUVs by mechanical procedures. Despite that, MLVs are composed of different superposed bilayers with diameters between 1-50 μm.

On the contrary, SUVs and LUVs only contain a single lipid bilayer but differing in the vesicle size. Furthermore, while SUVs tend to have 20-100 nm sizes, LUVs are in the range of 100 nm - 1 μm of diameter. Besides, a fourth type of liposomes has been proposed, giants unilamellar vesicles (GUVs). GUVs like MLVs, approaches to 1-50 μm but unlike them they are composed of a single lipid bilayer (Figure 2). Therefore, it could be said that GUVs share properties according to size of the different types of the above-mentioned vesicles, particularly MLVs and LUVs [89].

3.2. Methods for liposome preparation

Several manufacturing procedures can be used for liposome preparation. Some of them have been widely used for decades, and others that have recently become of great interest. Before getting into any method, it is important to note that in any selected method the phase transition temperature (Tc) of the phospholipids is critical in order to successfully prepare the liposomal dispersion. Working conditions below Tc and in particular while re-hydration and extrusion, could hamper the process and avoid the lipid mixture to go from gel state into the preferred ‘fluid’ or crystalline state [90]. For example, Tc of DOPG is -18 ºC and soy phosphatidylcholine -20ºC to -30ºC, but DMPC (dimyristoyl phosphatidylcholine) or DPPC (dipalmitoyl phosphatidylcholine) have 23ºC and 41ºC Tc respectively [91]. In this section, different methods are described as well as their applications, optimization, advantages and disadvantages.

Lipid film rehydration

Perhaps, one of the most famous methods for liposome preparation is the film rehydration method, first described by Bangham et al. where a lipid mixture is dissolved in an organic solvent to be later evaporated under vacuum ('Bangosomes'). Afterwards, the dry lipid film is rehydrated in a buffer solution forming the typical and well-known multilayer structures called liposomes [92].

Cholesterol is commonly added to provide rigidity to the membranes [84]. Normally, film rehydration methods tend to yield MLVs and that is why extrusion, freeze thawing and sonication methods are needed to homogenize sizes and stabilize the dispersion [93].

On one hand, the main benefits of using lipid film rehydration are the simplicity of the process and its capability of being used with different types of lipid mixtures. On the other hand, the main difficulties associated are poor encapsulation ratios of drugs associated to its chemical properties, low vesicle size homogeneity and the need of other techniques to tackle the issue and problems in industrial up-scaling [94].

Reverse-phase evaporation method

The reverse-phase evaporation technique first intends to form a two-phase system composed of inverted micelles in an aqueous phase or a water in oil (W/O) emulsion, and an organic phase such as chloroform, ethanol, methanol or a combination of those. Sometimes can be hard to distinguish this method from the lipid film rehydration since the first steps are usually the same. In the reverse-phase evaporation method, when the lipid film is formed in the rotary evaporator, an organic solvent and a buffer are added. Then the organic solvent is again removed by the rotary evaporator. Finally, the liposomal sample can undergo other processes discussed in the rest of the section, such as sonication, extrusion or freeze-thawing to obtain the desired liposomal dispersion [95]. Currently there are improved versions of this technique that have been further optimized through supercritical fluid technology. If a supercritical fluid is use, it dissolves the lipid film and while the aqueous buffer is added the solvent is completely removed. Supercritical CO2 is one of the best supercritical fluids that could be chosen for this method due to its environmentally friendly properties [96].

Dehydration-rehydration method

This method aims to develop new liposomes by fusion of already made liposomes. It uses dehydration and controlled rehydration in order to obtain MLVs and SUVs. With this technique large molecules such as DNA could be entrapped achieving high loading ratios. It was also described that encapsulation of small molecules is unstable [97]. Furthermore, liposomes are normally centrifuged, freeze-dried and slowly undergo a very controlled rehydration. The loss of lipids and materials during the different cycles can alter the osmotic conditions of the dispersion, thus changing concentrations and activity of entrapped compounds [98].

Freeze-thaw method

Freeze-thawing is a widely known technique that is generally is utilized in MLVs in order to increase their encapsulation efficiency or drug loading. This process occurs because in every freeze-thawing step MLVs are destroyed and reassembled again, thus decreasing the number of layers in every step. Normally the liposomal dispersions are immersed in a cooling bath or a freezer with a temperature range from -20 to -70ºC overnight. Finally, they can be introduced in a water bath at the desired optimized temperature or at room temperature [99].

Sonication

Ultrasounds have also been used to considerably homogenize and reduce the size of MLVs to form SUVs [100]. Normally the lipid mixture is achieved by means of an ultrasound bath or a sonication probe in order to achieve higher homogeneity ratios as well as smaller vesicles. The high pressures created by the ultrasounds violently breaks the vesicles that are spontaneously reassembled into small ones, forming SUVs. Furthermore, there is the inconvenience that, some metallic traces from the sonicator probe can stay in the sample, being difficult to be completely removed [101].

Ether and ethanol injection

Ether injection has been previously used to achieve single and homogeneous SUVs suspensions ranging from 100-300 nm [102]. Ether and ethanol injection consist of firstly prepare a lipid solution in ether, diethyl ether or ethanol and then slowly add it into an aqueous solution, normally containing a buffer that will finally form the liposomal dispersion [103] [104]. Ethanol injection together with microfluidics and micro emulsification are the chosen methods for scaling-up [105][106].

Calcium-induced liposome fusion method

This preparation method aims to obtain LUVs or even GUVs liposomes. The procedure is based on the fact that when SUVs interact with calcium, ‘cochleate cylinders’ structures are created by fusion of vesicles. Then, a planar sheet like figure is rolled in order to create circular structures [107]. Subsequently, ethylenediaminetetraacetic acid (EDTA) is added in order to create LUVs liposomal dispersions. As the main disadvantage, it is important to remark that this procedure can be only achieved with phospholipids that enclose an acidic nature.

Microfluidics

Microfluidics is a novel technology that as the name states, aims to manipulate fluids, such as lipid mixtures and aqueous solutions at micro or nano scale. This technique allows to monitor every parameter and therefore being able to control and adjust size distribution, polidispersity index and multi or mono-layered structures [108].

In comparison with other above-mentioned methods, microfluidics in general can be considered a novel technique that has provided significant advantages over other conventional methods. Thus, that allows to control sizes in a much more precise way and certain parameters such as flow rate as well as the ratios between injections of lipid mixtures and aqueous buffers. All these controlled features lead to obtain superior quality formulations and enhance drug loadings.

3.3. Physicochemical properties of liposomes, liposomal formulations and purification methods

As previously mentioned, it is worth noting that liposomes are thermodynamically unstable systems. Therefore, physicochemical characterization is a critical step in order to ensure that drug loading, stability and biocompatibility of the developed formulation. All these physicochemical properties can be tailored in order to make them acceptable for ocular surface drug delivery.

3.3.1. Physicochemical properties

Size distribution and zeta potential measurements

Normally, size depends on the type of liposomes that have been developed (GUVs, MLVs, LUVs or SUVs) according to the different procedures shown before. The ideal method for measuring size distribution is DLS (Dynamic Light Scattering), although cryo-TEM (cryo-Electron Transmission Microscopy) can be also used. The size and number of layers will affect drug loading as well as entrapment efficiency, depending on the nature of the loaded drug [109]. As mentioned in previous sections, extrusion, sonication or freeze thawing are some of the most effective methods to reduce size and increase homogeneity in size distribution [99].

Zeta potential determine the overall charge of the particles; therefore, it is going to play an important function when topical liposomes are in contact with the epithelial barrier and interact with cell membranes. Cells membranes are negatively charged, so in order to improve the pass of liposomes through membranes some cationic lipids or surfactants can be used. However, special care should be taken when adding cationic substances since they have been described as potentially toxic for the ocular surface [110].

According to some studies SUVs present the highest permeation ratio through the corneal epithelial barrier while MLVs the lowest [14]. Besides, some studies with the lipophile fluorophore cumarin-6 have demonstrated that liposomes around 190-200 nm pass through every corneal epithelial layer and are able to reach the stroma [111]. Regarding topical ophthalmic administration, liposomes close to 200 nm are normally desired to deliver drugs to the ocular surface [112]. Besides sizes of liposomes carrying hypotensive drugs are between 100 - 200 nm [113]. Furthermore, liposomes with sizes between 100 and 200 nm have been studied for avoiding the mononuclear phagocytic system uptake [114].

Morphology

Changes in liposome morphology can result in alteration of layers, liposome types (GUVs, MLVs, LUVs or SUVs) or even the drug loading efficiency. [115].

Optical microscopy is a good option when micrometric liposomes are developed. Fluorescence can be a proper tool to evaluate the presence of labelled proteins internalization in the inner aqueous compartment, particularly in GUVs [116]. However, a wide variety of them (SUVs and LUVs) are in the range of nanometers. Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) are not suitable techniques since freeze-dried samples and negative staining normally produce significant changes in structure and morphology of the vesicles. Besides, placing the sample in TEM grids dehydrate the sample and the high vacuum that experiment before taking the images break in many cases the liposomes and hamper the visualization of the structures [117]. Therefore, the most ideal technique and widely used for studying the morphology of liposomes is cryo-TEM which allows observation of the inner architecture and structure of liposomes [118].

When using Environmental scanning electron microscopy (ESEM) vesicles remains rehydrated during image acquisition. However, a main disadvantage entails the lack of information that can be achieved since only external structure can be analyzed [119]. Last but not least, atomic force microscopy (AFM) is presented as an interesting and useful method to study liposomes. AFM provides with information about the surface of liposomes and nanoparticles. In some studies AFM has been used to study in detail the attachment of certain antibodies, pegylated phospholipids or even polymers to the surface of the liposomes [120]. It entails a very useful method to find out whether specific bioadhesive polymers or potential therapeutic substances are fixed to the surface of the liposome [121].

Viscosity

Generally, liposomal dispersions for ocular topical administration present low viscosity values that are close to those of the natural tears (1 to 8.3 mPa·s), since higher viscosity values might cause blurry vision and discomfort [35]. In some cases, the use of viscosity enhancers in liposomal ophthalmic formulations increased their retention on the ocular surface for longer periods of time. For this purpose, also bioadhesive polymers can be included in the formulations. These compounds can also interact with the mucins on the preocular tear film increasing, by a complementary mechanism, the contact time of formulations on the ocular surface [122].

Surface Tension

Ophthalmic formulations with surface tension values similar to those of the natural tears (43.6 +/- 2,7mN/m) show a proper spreadability when blinking [123]. Surface tension values must be close to the one of the precorneal tear film to ensure proper spreadability. Caution must be taken with low surface tension values because the inner structure of the preocular tear film and the epithelium can be damaged [84]. One of the most important features that should be taken into account is that drugs and auxiliar substances may change surface tension properties of the liposomal formulation resulting in incompatibilities with the ocular surface [124].

It is well known that some components present in liposomal formulations, such as cholesterol and phospholipids (i.e., soy phosphatidylcholine) have relatively low surface tension values which result to be highly compatible with rather adhesive surfaces such as the ocular surface [125].

Osmolarity

Isotonicity of formulations is a rather important feature to adjust, but particularly in topical ocular liposomal dispersions. Tear osmolarity is minimum at night when the lids are closed and show its higher value during the day. Besides, alteration in osmolarity can make cellular tight junctions weaker and decrease the number of mucus secreting globet cells [126]. Generally, in healthy individuals the average osmolarity of tears and ocular surface is 300 mOsm/L [127]. In fact, tear osmolarity values higher than 308 mOsm/L are indicators of instability of the preocular tear film and are related to initial dry eye disease and over 316 mOsm/L moderate or severe dry eye disease [128]. That is the reason why some liposomal formulations developed for ocular surface diseases like keratoconjunctivitis sicca present hypotonic osmolar values between 200-290 mOsm/L in order to tackle hypertonic environment [129].

pH

pH of the ophthalmic formulations must be compatible with natural tears (pH 6.6 - 7.6) [130]. According to some of the established requirements for administering topical ophthalmic formulations the acceptable range of pH for topical ophthalmic drug delivery systems is between pH 6 – 9 [111].

The acidic pHs on the ocular surface may result in discomfort, inflammation and reduced wound healing capacity. Besides, a decrease in cell viability has been associated to pHs below 6 or above 8, so this parameter is an important feature to control when optimizing a novel liposomal formulation for topical ocular drug delivery [131].

3.3.2. Purification methods

A common inconvenient when developing liposomal formulations with hydrophilic or partially water-soluble drugs, is that a portion of the drug is free in the aqueous buffer or inside the aqueous core, and concentrations are balanced depending on the gradient. Normally, partially water-soluble drugs try to keep balance between the outer and the inner core of the liposome. However, when it comes to full or almost full water-soluble drugs it is important to purify the liposomal dispersion and discard the excess of the drug that has not been encapsulated [132]. That could be the case of potentially toxic drugs dissolved in the aqueous media. For this reason, purifying methods are of vital importance, because they can help also to remove lipidic debris that are not forming the liposomes and could generate toxic degradation products. The common purifying methods are dialysis, gel filtration column chromatography, ion exchange chromatography, centrifugation, ultrafiltration, protamine aggregation, liposome extruder purification and microfluidic [133].

3.3.3. Freeze drying of liposomes

Freeze drying has been widely used in the industry and research facilities to increase the stability, long storage capability of formulations such as nanoparticles as well as decreasing the risk of potential contaminations [134]. Regarding liposomal formulations, freeze-drying has been used for some authors to prepare lipidic materials in order to create liposomal transfection agents [135]. However, the freezing process and undergoing vacuum make liposomal dispersions unstable thus disrupting the vesicles leading to drug leakage as the potential disadvantage. However, recent research points out that when appropriate amounts of cryo-protectants such as trehalose are added to liposomal dispersions these problems could be avoided. Besides, coating liposomes with smart polymers could resolve the stability and leakage issues [106], therefore allowing researchers and industries to storage them as a powder. Another study has visualized liposomes through confocal and transmission electron microscopy techniques in order to demonstrate that liposomes that underwent freeze drying exhibited similar sizes and polidispersity indexes than those that were not freeze dried [117].

3.3.4. Sterilization

It seems that sterilization of liposomal dispersions is still unclear. The methods that can be used to sterilizing liposomal formulations are particularly challenging since, due to their nature, many of the lipid substances that create the system are rather unstable at high temperatures or susceptible to denaturation. Filtration through a sterilizing membrane is one of the best options because no heat is produced and as far as the liposomes size is below 200 nm, they can pass through 0.2 μm sterilizing filters. However, some difficulties that are related to viscosity and surface tension of the formulation can lead to a quick blockage of the filtration membrane, and an increment in permeation of bacteria and pathogens may occur. Therefore, it is important to choose the most appropriate membrane depending on the conditions for the liposomal sample [136]. For that reason, although these conventional methods might be enough, a final sterilizing process is still required by some manufacturers.

The most common used procedures for the industry involve the use of irradiation (γ or UV), which links to a direct damage of the DNA through the formation of free radicals that make DNA strand unstable. Furthermore, lipid peroxidation is the main problem that occurs when γ-irradiation or UV-irradiation hit phospholipids and cholesterol present in the liposomal dispersions, thus creating O_2^- and •OH radicals respectively [137].

Another well-known sterilization method is steam sterilization with the use of an autoclave (121ºC or 134ºC for 15-20 minutes). Although lipid peroxidation is avoided in this process due to the lack of oxygen and generation of free radicals, hydrolysis of the lipidic materials might occur. These could lead to an alteration in drug loading efficiency as well as a variation in size distribution [137]. However, when selecting an ideal aqueous buffer these issues could be minimized [138]. Regarding the use of dry heat sterilization, it has been described as an unsuitable method for liposomal formulations because of the constant heating ratios leads to the evaporation of the aqueous phase and the alteration of every property of the mixture [137].

Finally, sterilization through ethylene oxide is discussed as an alternative 'cold' method commonly used for thermosensitive preparations. According to previous published works this method does not alter neither vesicle size nor structure and liposomes are reconstituted upon lyophilization without any apparent changes [139]. However, one of the main drawbacks of this technique when used for industry manufacturing is the potential risk that encompass the presence of ethylene oxide vapors residues such as mutagenicity, flammability or being a potential carcinogen [140].

Therefore, according the above-mentioned methods, sterilizing filtration can be considered as the best option combined with cold methods if scalability or an industry approach is desired.

4. Liposomes as drug delivery systems in anterior and posterior segment diseases

Liposomes have been extensively studied for topical ocular administration due to their properties of biodegradability and biocompatibility and their ability to act as drug carriers. Furthermore, as previously mentioned, both hydrophilic and lipophilic drugs can be entrapped. Liposomes facilitate drug penetration in intraocular tissues by coming into intimate contact with the corneal and conjunctival surface, which resulted in special relevance for high molecular weight drugs, poorly soluble drugs or those with low distribution coefficients [141] [142].

Liposomes are able to interact with cells (Fig.3) and release the entrapped active substance, facilitating the entry of drugs by various mechanisms. Among them, specific or unspecific adsorption on the surface of cells, fusion with the membrane, lipid exchange by the transfer-protein-mediated exchange or endocytosis are the most employed. When endocytosis of liposomes occurs, the endosome can break and release the content in the cell cytoplasm, or reaching lysosomes where they are degraded (Figure 3) [14] [143] [144] [145].

Many authors have studied the advantages of liposomal formulations for ophthalmic application of active substances, reducing their potential toxicity and increasing their penetration and bioavailability compared to the free drug.

4.1. **Antimicrobial agents**

Liposomes are extremely versatile systems able to entrap a wide variety of substances such as hydrophilic, hydrophobic or biotechnological products like antibodies, genetic material or proteins. It is worth mentioning that entrapping substances of different nature into liposomes could entail an interesting strategy to increase the stability of potential therapeutic drugs that in solution could suffer hydrolytic or proteolytic processes as well as enzymatic degradation [113].

Although encapsulation is the common term to explain drug internalization or uptake in liposomes, entrapment efficiency is the most suitable one, since it may refer to adhered drug to the surface, entrapment in the bilayers or inclusion in the aqueous core of the liposomes [146]. For instance, these features have been used to entrap antibiotics, reduce their toxicity and increase their effectivity. Water soluble and moderate soluble antibiotics vancomycin, teicoplanin and rifampin were successfully encapsulated in liposomes, achieving high encapsulation efficiencies for teicoplanin and rifampin, 82.7% and 84.1% respectively through the reverse phase evaporation method [147]. For example, tobramycin is one of the most well-known and used topical antibiotics that has been successfully entrapped in liposomes. In fact, a volume of 0,4 mL of a 'mega' liposomal dispersion (10-100 µm) containing entrapped tobramycin (35 mg/mL) administered in a single dose to rabbits demonstrated higher or comparable efficacies compared to rabbits that received repeated instillations every hour [112] [148].

Liposomes have been studied as transporters in ocular infections due to their properties, being a vehicle that requires less dosage, increases its effectiveness, avoid systemic exposure and decreases antibiotic resistance [143][149]. Ciprofloxacin is a fluoroquinolone effective against gram-positive and gram-negative bacteria. Besides, 1 mg/mL of ciprofloxacin formulated in liposomes allows the *in vitro* controlled release of the drug for 24 hours in contrast to the drug solution at same concentration, which showed a 92,62% of the released drug in only 2-hour [150], This ability was previously reported in other studies [151]. In another *in vivo* study, after topical application of 50 µl of different formulations in rabbits, at least 3-folds greater bioavailability was obtained for liposomal formulations with doses ranging 107.63-114.52 µg ciprofloxacin compared to the dose contained in commercial eye drops (150 µg ciprofloxacin). Besides, higher concentrations of ciprofloxacin were found in the aqueous humor. In this sense, the liposomal formulation which reached the highest concentration in the aqueous humor obtained 3.87 µg/ml, compared to 2.68 µg/ml obtained with the commercial aqueous formulation [152]. Azithromycin liposomes also showed an increase in corneal permeability, increasing the permeability coefficient from 4.43 ± 0.27 cm/s in solution to 8.92 ± 0.56 cm/s for the liposomal formulation. On the other hand, in a dry eye rat model, after topical instillation for 7 days, 3 times a day of 20 µL of different eye drops, an improvement in symptoms were observed when using azithromycin liposomes, compared to the drug in solution (10 mg/mL of azithromycin in both of them), with a significantly greater improvement in tear break up time and fluorescein staining score ($P < 0.01$) [153].

4.2. Antiviral therapy

Topical treatment of viral eye infections like herpes simplex virus (HSV) or secondary herpes simplex keratitis has also been improved with the use of liposomes [154] [155]. A study in rabbits compared ganciclovir liposomes (1 mg/mL) with a ganciclovir solution (1 mg/mL) after the topical instillation of 50µL. The results shown a greater corneal permeability, resulting in an apparent coefficient of permeability 3.9 times higher than the drug solution, and an increase in absorption, obtaining an area under the curve of the concentration in aqueous humor 1.7 times higher [156]. Also, distamycin A liposomes used for acyclovir-resistant HSVs were reported to have the same antiviral capacity as distamycin in solution and to be less cytotoxic on rabbit corneal epithelial cells (the 48-hour viability of the liposomal formulation resulted 80% versus 60% of the drug solution).

Moreover, the amount of drug detected in the corneal tissues 30 minutes after 50 µL instillation of eye drops (0.05 mg of distamycin) in rabbits was greater for the liposomal formulation than for the solution, being 2,028 ± 0.063 ng / mg and 1,579 ± 0.087 ng / mg respectively [157].

4.3. Antifungal agents

Regarding to fungal keratitis, several authors have carried out studies with liposomal antifungal formulations [158][159]. A study in 40 rabbits with Candida albicans showed that the administration of 50 µl of topical liposomes loaded with fluconazole (2 mg/mL) significantly improved the healing compared to the same concentration of fluconazole solution, obtaining a whole healing at 3 weeks in 86.4% of cases, compared to 50% obtained in rabbits to which the fluconazole solution had been administered. The drops were administered during the first 3 days with a frequency of 4 times a day, and subsequently with a frequency of 3 times a day [160]. On the other hand, a clinical study was carried out with 11 patients with keratitis caused by Candida albicans. Patients were administered a 2 mg/mL fluconazole liposomal formulation 3 times a day. The mean diameter of the ulcers presented by the patients decreased from 5.5 mm to 1.3 mm after one month of treatment, obtained an amelioration in the rate of recovery and a decrease in the frequency of administration for fluconazole liposomes. [161]. It is worth mentioning that a liposomal collyrium of Amphotericin B 0.5% is developed and used for fungal infections of the ocular surface in hospitals [162].

4.4. Hypotensive agents as glaucoma treatment

Numerous studies have been conducted with drugs capable of reducing intraocular pressure carried with liposomes for topical administration. Brinzolamide, a carbonic anhydrase inhibitor, was characterized and tested *in vitro* and *in vivo* compared to a drug suspension with ten times more concentration (0.1% and 1% respectively). The transcorneal permeability study with the Franz diffusion chamber showed an increase in permeability compared to a commercial brinzolamide suspension, resulting 6 times higher for liposomes (2.58±0.04 in liposomes versus 0.35±0.01 in suspension). Furthermore, *in vivo* studies in rabbits showed that one topical instillation of 50 µL of the liposomal formulation was more effective in reducing long-term intraocular pressure, so that for liposomes the sustained effect in the reduction of intraocular pressure lasted 12 hours, while the suspension was no longer effective 30 minutes later [163].

LUVs have reported to be an interesting way of achieving a sustained release of lipophilic drugs into the eye. A good example of that, is a liposomal formulation of LUVs made out of egg phosphatidylcholine at a concentration of 18 mM (109 ± 18 nm average size) that was able to release latanoprost through a single subconjunctival injection for up to 90 days in rabbits. The animals were instilled with 1.5 µg of latanoprost per drop daily. The liposomal formulation presented high ratios of drug loading (94% ± 5%) and was able to lower the IOP in rabbits in more than 2.8 folds with residual effect of 50 days [113]. In addition, the incorporation of viscous polymers such as hypromellose (HPMC) 0.3% or hyaluronic acid (HA) 1.2% [11] has been previously reported for increasing drug uptake and efficacy of topical hypotensive liposomal formulations. Apart from increasing the time the formulation was in contact with the eye, therefore increasing permeation, these two polymers were hypothesized to contribute to the ocular surface protection [84].

4.5. Anti-inflammatory agents

Triamcinolone acetonide, usually employed in intravitreal injections for vitreoretinal diseases, was administered in a topical liposomal formulation in rabbits: 50 µL every 2 hours, 6 times a day for 14 days. The formulation (2 mg/mL) was able to reach the vitreous and the retina. Drug concentrations at 12 hours were 252.10 ± 90.00 ng / g in the retina, and 32.6±10.27 ng / g in the vitreous humor [164]. In a recent pilot study, 2 mg/mL triamcinolone acetonide liposomal formulation was tested in 12 patients suffering from refractory pseudophakic cystoid macular edema. In this study, a drop of the topical liposomal formulation was applied every 2 hours for 90 days, providing an adequate tolerability and therapeutic activity. Results showed an improvement of 20.08 ± 10.35 letters in the best corrected visual acuity (BVCA) and a reduction of 206.75 ± 135.72 µm in the central foveal thickness (CFT) at 20 weeks after the beginning of the study [165].

A liposome-based formulation also had the capacity of encapsulate the corticoid medroxyprogesterone acetate (MPA) (0,2 mg/mL) to treat inflammatory eye processes. The anti-inflammatory effect was tested in vitro after a 60-minute exposure, showing a further improvement of the effect in Human corneal epithelial cells than reference non-liposomal formulation (Medrivas®). The cytokine production after TNFα stimulation was determined by an enzyme-linked immunosorbent assay (ELISA). While the MPA solution only showed a reduction in the IL-6 cytokine, the liposomal formulation reduced both IL-6 and IL-8 production. The uptake of the liposomes by the cells was also evaluated in rabbits using coumarin-6 (C6) liposomes. After 5 minutes, the corneal epithelium showed fluorescence and after a 60-minute exposure, also the corneal stroma. [111]. Similar liposomal formulation was employed to encapsulate the thrombospondin-1-derived peptide KRFK. The liposomal formulation was tested in an in vitro model of corneal and conjunctival epithelium, obtaining an apparent enhancement in corneal permeability [166].

The use of antiangiogenic and anti-inflammatory-loaded liposomes entails a potential effective therapy for the treatment of diseases that affect the posterior segment of the eye. However, precipitation and crystallization are one the main drawbacks when co-encapsulating these compounds. Some of the strategies to solve these difficulties involves the use of different cholesterol/lecithin ratios or the adjustment of the pH, that help increase the active drug loading. With respect to this issue, Lai S et al studied the difficulty of encapsulating drugs of different polarities in liposomal formulations since two drugs that differ too much between their polarities could result in precipitation or decrease in encapsulation [167].

4.6. Antitumoral substances

Other strategies to increase drug entrapment or encapsulation include the attachment of PEG-like polymers to the surface of the liposomes that may increase interactions between the drug and polymers. In reference to this, liposomes have been used as useful and specific tools to encapsulate immunoreactive substances such as anti-tumoral products targeting HER2/ neu and CD20 receptors [168].

4.7. Gene delivery

Furthermore, over the last few years cationic liposomes containing DOTAP or DOTMA [169] loaded with genetic material have gained much attention since they constitute a useful approach to tackle genetic diseases, cancer, or even deliver vaccines [170]. For example, an interesting strategy to increase the DNA plasmid loading capacity of cationic liposomes is to combine it in appropriate ratios that allows DNA condensation and to include it together with cyclodextrins [114], as well as adding a spacer, based on amino acids, introduced between the polar head and the hydrocarbon tail. This strategy aims to increment the cellular uptake of liposomes containing a plasmid and to decrease lysosomal degradation. According to this study, liposomes containing a tailored lysine group as a spacer achieved much higher encapsulation ratios (22%) and transfection efficiencies than others. Furthermore, the combination of cationic liposomes and these poly-lysine spacer present encapsulation efficiencies similar to transfections reagents but showing a decrease in toxicity [135].

With respect to the use of liposomes that contain genetic material for topical ophthalmic administration, surface engineered liposomes have been developed in order to carry siRNA molecules to the retinal pigmented epithelium or regulate VEGF expression in age-related macular degeneration (AMD). This strategy could entail a less invasive and more effective strategy as an alternative for intravitreal injections [171]. SiRNA liposomes tackling heat shock protein 47 (HSP47) combined with vitamin A are presented as an attractive strategy for dry eye disease [172]. It is important to remark that liposomes are very interesting and attractive systems that allow to encapsulate and deliver topical ophthalmic substances that administered systemically or via intravitreal injections could entail risks or generate side effects [173].

4.8. Immunosuppressants

Immunosuppressive drugs such as cyclosporine, tacrolimus or everolimus have been successfully employed for treating mild to severe symptoms of dry eye disease or avoiding graft versus host disease after allogeneic transplants [174][175][176]. For example, a study published by Y. Dai demonstrated that liposomes carrying bile salts such as sodium deoxycholate, sodium taurocholate and sodium glycocholate as an alternative to cholesterol, and together with tacrolimus, experimented higher transcorneal permeation ratios (29.50 ± 5.78, 36.24 ± 3.51 and 29.73 ± 4.03 cm/sec respectively) than conventional liposomes loaded with the single drug (8.00 ± 2.05 cm/sec) [177].

Another comparative study between cyclosporine liposomes and the commercial emulsion Restasis® demonstrated enhanced corneal permeation and uptake by immunosuppressive liposomes. It seems that stabilization of the tear film by liposomes may seem an interesting and effective strategy to increase drug permeation through corneal layers [178]. Besides, latanoprost has been also included in liposomal systems for subconjunctival administration to treat ocular hypertension in glaucoma. Liposomes containing latanoprost 0.005% demonstrated enhaced drug permeation in clinical trials (NCT02466399) [179][164].

5. Strategies to increase ocular retention time of topical liposomal formulations

Different technological approaches have been developed to increase the retention time of liposomal ophthalmic formulations applied topically. To this, the use of positively charged liposomes or the addition of polymers with different properties to liposomal formulations have been assayed.

Positively charged liposomes have shown a more prolonged interaction with the negatively charged ocular surface allowing the formation of a layer that completely covers the eye surface. Electrostatic interactions between positively charged liposomes and the negative charges of the mucin layer increase the retention time of the ophthalmic formulation [112]. On this subject, stearylamine is a lipid that included in liposomes confers a positive charge on the lipid bilayer and has been studied to encapsulate different drugs. In a study conducted with acyclovir-loaded liposomes, stearylamine and dicetylphosphate were used to confer positive and negative charge to the vesicles respectively. Researchers reported an increase in the absorption of the drug into the cornea of rabbits when using positively charged liposomes. The charge of the liposomes influenced the amount of the drug in the cornea. Corneal concentration 2.5 hours after topical administration of 50 µL (1.24 mg/mL) resulted 1093.3 ± 279.7 ng/g and 571.7 ± 105.3 ng/g after the use of positively and negatively charged liposomes respectively, and 253.3±72.0 ng/g after the administration of the solution. In addition, the positively charged liposomes seemed to bind more intensely to the corneal surface [155].

The use of liposomes with different charges to encapsulate a acetazolamide, has also been studied. In this case, liposomes were formulated with phosphatidylcholine and cholesterol in different molar ratios and stearylamine or dicetylphosphate as charge inducing agents. The effect of both formulations was compared *in vivo*. Results showed that positively charged liposomes provided a more effective decrease in intraocular pressure in rabbits: The best results were obtained using multilamellar liposomes prepared with phosphatidylcholine: cholesterol: stearylamine in a molar ratio of 7: 4: 1, so that 3 hours after topical administration of positively charged liposomes (50 µL) with a 1% concentration of acetazolamide provided an IOP reduction of 7.8 mmHg versus 5.5 mmHg when neutral liposomes were used. [180]. A different investigation with 1.25 mg/mL prednisolone acetate positively charged liposomes composed of 1,2 dipalmitoyl-sn-glycerol-3-phosphocholine, cholesterol and stearylamine yielded similar results, observing a 2-times slower release rate than the solution (1.25 mg/mL) and an increase in the concentration of the drug in the aqueous humour of rabbits about 27-40% with respect to the drug solution after a topical administration of 50 µL. Furthermore, the AUC of positively charged liposomes was higher than that of neutral liposomes [181].

Another lipid component used to confer a positive charge on liposomes is dioleoyl-3-trimethyleammonium propane chloride (DOTAP). A study with a voriconazole liposomal formulation with DOTAP concluded that liposomes were capable of adhering to mucins *in vitro,* so that when adding a suspension of mucins with 500 nm size to the liposomal formulation, the size of liposomes increased from 96.5 ± 2.2 nm to 2441.3 ± 164.5 nm, showing the formation of aggregates, which was not observed with vesicles without positive charge. In addition, tolerance measured using the HET-CAM test (Hen´s Egg *Test* corioallantoic membrane) showed a weak irritation. In an *ex-vivo* permeation test performed with porcine corneas, the DOTAP-liposomal formulation with 2.5 mg/mL of voriconazole also managed to reach a voriconazole concentration of 45.31 ± 2.02 and 62.14 ± 7.84 μg / cm^2 after exposures times of 30 and 60 minutes respectively. [158]. In a recent study, researchers developed positively charged liposomes using DOTAP to encapsulate the antioxidant astaxanthin. The formulation was tested in a dry eye disease rat model. They observed a higher corneal affinity when using positively charged liposomes in comparison with a neutral liposomal formulation. The antioxidant positive liposomal formulation also appeared to be more effective in suppressing the up-regulated expression of age-related markers presented in the DED rat model [182].

Different polymers have been used together with liposomes to increase the retention time and mucoadhesion of ophthalmic formulations. One of the most studied polymers is chitosan, a biodegradable cationic heteropolysaccharide with great biocompatibility and low toxicity capable of interacting with the negatively charged corneal surface, increasing the retention time and drug penetration. Ciprofloxacin HCl loaded-liposomes (composed of phospholipid, cholesterol, and dicetylphosphate as negatively charged agent) were coated with 1% medium-molecular-weight chitosan. In this study a reduction in encapsulation efficiency was observed for the chitosan coated liposomes, being 60.280% ± 0.642 compared to 71.400% ± 0.247 in the non-coated liposomes. The apparent permeability coefficient was higher than that of the non-coated liposomes and the free drug in *ex vivo* permeability studies (8,632 ± 0.354, 4.412 ± 0.113 and 5,188 ± 0.228 respectively). Moreover, the chitosan-coated liposomes were able to inhibit the growth of *Pseudomonas aeruginosa* 24 hours after a single dose administration of 50 μL in rabbits with induced bacterial conjunctivitis [183]. In a different study, chitosan coating of flurbiprofen deformable liposomes improved transcorneal permeation *in vitro*, with an apparent permeability coefficient 1.29 folds higher than the uncoated ones and 4.59-fold higher than the 0.03% flurbiprofen solution. The study of the residence time *in vivo* also showed a significant improvement, being more than 2 times greater than the results of deformable liposomes without chitosan [184]. Chitosan coated timolol maleate liposomes (50 μL) resulted also more effective in reducing intraocular pressure than eye drops. In addition, the chitosan coated liposomes showed the ability to bind to mucins in a mucoadhesive study, showing an increase in the size of the liposomes from 151.2 ± 20.3 nm to 1013 ± 81.2 nm due to the formation of aggregates when adding mucins and an increase in the apparent diffusion coefficient in 3 times with respect to eye drops [185].

Recently, several studies have been conducted with triamcinolone acetonide encapsulated in chitosan-coated liposomes. *In vitro* and *in vivo* studies of chitosan 0.5% coated liposomes containing 1.5 mg/mL triamcinolone acetonide (TA) were performed. The authors reported a more sustained release profile for the coated liposomes than the ones observed for a suspension or non-coated liposomes of the drug. Moreover, the results of a histological study after the administration of the formulation in C57BL/6 mice showed an absence of corneal and conjunctive cell toxicity**.** In addition, they assessed the ability of chitosan-coated liposomes to reach the anterior and posterior segment eye tissues after topical application using Coumarin-6 (C6) as a fluorescent marker. C6 was carried in both liposomes with and without chitosan and in solution. The results suggested a higher up-take in corneal epithelial cells (HCEC) and retinal pigment epithelial cells (ARPE-19) for chitosan-coated liposomes, showing greater fluorescence. 5 µl of these formulations were also administered topically to C57BL/6 mice. The results showed higher fluorescence intensity in the anterior and posterior segment for C6 encapsulated in liposomes coated with chitosan [186]. After these results, the same researchers studied the application of TA in chitosomal liposomes for the treatment of macular edema (ME) in a rat model with induced retinal edema. The results after the administration of 20 µL with a TA concentration of 160 mg/L showed a remission of the edema after 10 days, similar to that produced by intravitreal injection of a suspension of triamcinolone acetate, being suggested as an alternative to intraocular injections, reducing the resulting complications [187]. Mehanna et al. also studied a triamcinolone acetate chitosan coated liposomal formulation in a rat model with induced Choroidal neovascularization (CNV), obtaining as a result a sufficient level of drug in the vitreous humour after topical administration of 2.5 mg/mL (0.5 mL) TA chitosan liposomes 3 times per day for 15 days [188].

One of the problems with chitosan is its low solubility in water. However, its aqueous solubility can be improved by using chitosan derivatives, such as low molecular weight chitosan or trimethyl chitosan. Low molecular weight chitosan coated liposomes loaded with diclofenac showed greater stability at 25ºC for 30 days than the conventional vesicles and the drug in solution. Furthermore, coated liposomes showed an extended release of the drug (23.8% at 6 hours compared to 38.9% with conventional liposomes) and an increase in the apparent permeability coefficient (1.174 ± 0.080 versus 0.789 ± 0.069 in the non-coated liposomes). *In vivo* studies in rabbits showed a significant increase in the retention time compared to non-coated liposomes and the free drug (0.1% diclofenac in all cases). Also, *in vivo* tolerance studies showed no irritation in either the short or long term after the instillation of 150 µL (3 times each 10 minutes and 5 times per day during 7 days respectively) [189]. The same authors prepared liposomes carried cyclosporine A (CsA) coated with low molecular weight chitosan. The formulation showed no cytotoxicity in conjunctival epithelial cells (cell viability greater than 90% after 2 h of exposure). *In vivo* studies were conducted in rabbits, administering 100 µL (1 mg/mL CsA) of the formulation topically and subsequently measuring the concentration of drug in the eye tissues. This study showed an increase in concentration compared to conventional liposomes at 2, 6, 12 and 24h in the sclera, conjunctiva and cornea [190]

Coenzyme Q10, an antioxidant used to reduce cataract formation, was loaded in N-trimethyl chitosan (TMC) coated liposomes. Rabbit assays reported decreased liposome drainage and therefore a longer retention time

on the corneal surface for TMC-coated liposomes compared to ^{99m}Tc-DTPA solution (1.5-fold higher). The effectiveness of Coenzyme Q10 loaded in TMC-coated liposomes was evaluated in rats with induced selenite cataracts being the opacity of the lens at 8 days 52% after the administration of the formulation (5µl/20 g) three times per day for 8 days, compared to 95% of the untreated group [191]. Similar results were found for the antioxidant cyanidin-3-glycoside (C3G), with a residence time in the cornea 3.3 times greater than the drug solution and 1.7 times higher than the conventional liposomes. The authors also reported a decrease in lens peroxidation with TMC-coated liposomes compared to non-coated liposomes in the rat animal model [192].

The low viscosity of liposomal formulation in an aqueous vehicle does not allow the retention time on the corneal surface to be sufficiently high, so the use of polymers with viscosizing and gelling properties allows the formation of a viscous layer covering the entire corneal surface protecting the drug from tear drainage. Also, mucoadhesive polymers improve the retention time thanks to the interaction with mucins of the preocular tear film.

Polysaccharide-derived polymers have been extensively employed in the preparation of ophthalmic formulations. Hyaluronic acid (HA) is an anionic polymer present in the extracellular matrix of animal tissues, being one of the main components of these in our body. HA is biocompatible and biodegradable with low toxicity. HA is able to retain water forming a hydrogel with mucoadhesive properties. Liposomes loaded with Doxorubicin and coated with HA showed a longer *in vitro* release, which continues at 24 hours. In addition, fluorescence cellular uptake studies in corneal epithelial cells also showed that liposomes in HA solution reached the cell nucleus the most compared to liposomes in an aqueous solution and the free drug. The authors also carried out *in vivo* studies in rabbits, with topical instillation of different formulations (50 µL with 0,8 mg/mL Doxorubicin). In addition, samples of tear fluid or samples of aqueous humour were collected to measure the retention time and the pharmacokinetic profile respectively. The results showed an increase in the retention time of the formulation with respect to the formulation without HA or the free drug: The mean retention time was 527.11 ± 604.89 min compared to 211.45 ± 52.04 and 152.73 ± 3.72 for the non-modified liposomes and free drug respectively. Furthermore, the liposomal formulation with hyaluronic acid also had the highest bioavailability, being 1.7 times greater than the free drug [193]. Another study combined the use of HA to form a hydrogel and liposomes, integrating HA into and out of fluconazole-loaded liposomal vesicles. This formulation was compared with a conventional liposomal formulation and a fluconazole suspension in studies *ex vivo* and *in vivo* (rabbits). *Ex vivo* corneal permeation studies showed promising results in relation to increased corneal permeation in liposomes with 0.7% HA. The cumulative concentration of fluconazole in corneal tissues resulted much higher compared to conventional liposomes and drug suspension (0.9% fluconazole), being 1.86 and 2.6 folds higher respectively after 6 hours. Moreover, *in vivo* studies in which drug concentrations in aqueous humour were measured over time after a topical administration of 50 µL of the formulations showed a more sustained permeation profile and a higher value of area under the curve (AUC) after 24 hours in the case of the liposomal formulation with 0.7% HA and 0.9% fluconazole compared to fluconazole suspension (0.9% fluconazole). The AUC measured by the linear trapezoidal method was 530.62 ± 44.94 and 204.34 ± 7.46 respectively [194].

Gellan gum is another biocompatible and biodegradable polymer widely used in the pharmaceutical industry. In a study with liposomes loaded with timolol maleate, the derivative deacetylated gellan gum (DGG) was employed. DGG is an anionic polymer that forms a gel in the presence of a positive charge. In this study, liposomes were incorporated into the DGG to form an ion-sensitive gel *in situ* and the formulation was compared to commercial drops of timolol maleate. The researchers reported a 1.93-fold increase in the apparent partition coefficient when compared to conventional eye drops. Furthermore, *in vitro* release studies comparing formulations with DGG and conventional liposomes showed a longer release profiles due to DGG. *In vivo* studies in rabbits after 50 µL of topical administration showed an absence of eye irritation and a longer corneal retention time of the DGG liposomal formulation relative to eye drops, timolol maleate liposomes, and gel formulations view by fluorescence imaging. Furthermore, intraocular pressure measurements in rabbits after the topical administration of 50 µL eye drops or DGG liposomes (0.25% timolol maleate) showed greater long-term efficacy for the liposomal gel, obtaining a minimum IOP of 11.96 ± 0.74 mm Hg (1 hour after instillation) and an effect duration of 300 minutes for the liposomal formulation with DGG, and a minimum of 13.61 ± 0.95 mm (2 hours after instillation) and an effect duration of 180 minutes for eye drops [195].

Cellulose-derivative polymers such as carboxymethyl cellulose (CMC) or hydroxypropyl methyl cellulose (HMPC) have also been extensively studied in ocular topical administration. A study carried out with 5-methoxycarbonylamino-N-acetyltryptamine (5-MCA-NAT) a hypotensive melatonin analogue (100 µM 5-MCA-NAT) formulated in solution, in liposomes and in liposomes combined with different polymers showed a more sustained *in vitro* release profile with the use of liposomes, being even slower if used together with polymers. The formulation with a slower release profile was the liposomal formulation dispersed in 0.5% CMC. The reduction of intraocular pressure in normotensive rabbits *in vivo* also after the instillation of 25 µL (0.7 µg 5-MCA-NAT) eye drops showed greater effectiveness in the case of liposomal formulations combined with polymers. The liposomal formulations that provided the significantly greatest reduction were the ones prepared with 0.2% sodium hyaluronate (SH) and in 0.5% CMC, with intraocular pressure reduction values of 39.13 ± 2.21% and 36.72 ± 2.77%, respectively. Furthermore, these formulations did not cause discomfort or eye irritation in 24-hour *in vivo* tolerance studies [196]. Tolerance and efficacy studies of acetazolamide formulations were carried out by comparing a liposomal formulation loaded with acetazolamide versus solution of the drug (0.7 mg/mL) and the vehicle, and subsequently the liposomal formulation of acetazolamide (0.7 mg/mL) with and without 0.3% HPMC. The reduction in intraocular pressure after topical administration of 25 µL of the formulations was measured every hour for 8 hours. The results first showed a significant decrease in the IOP values when acetazolamide was formulated in liposomes. The maximum intraocular pressure reduction was 16.6% in the case of liposomes compared to 10.1% in the acetazolamide solution. Furthermore, the 8-hour AUC was more than 2 times higher. Secondly, the addition of 0.3 % HPMC to the liposomal formulation showed a significant improvement in the reduction of intraocular pressure, increasing the maximum reduction in intraocular pressure by 1.4 times and providing an 8-hours AUC 1.5-fold higher compared to liposomal formulation without HPMC. Furthermore, none of the liposomal formulations

showed signs of ocular toxicity in rabbits after being administered topically every 30 minutes for 6 hours [84].

Researchers used different concentrations of poly (vinyl alcohol) (PVA) and polymethacrylic acid (PMA) derivatives to increase the viscosity and enhance release profile of ciprofloxacin. The results showed that the release resulted extended when increasing the polymers concentration. Furthermore, when the polymers were used in combination with a liposomal formulation of ciprofloxacin the release was more prolonged. The obtained values of half-time of release were 4600 minutes when combining the encapsulation of ciprofloxacin (0.1% (m/m)) in α-l-dipalmithoyl-phosphatidylcholine (DPPC) liposomes and the use of 0.1% (m/m) PMA, compared to 85 minutes in the case of not using liposomes. In the case of PVA, the half-time of release increased from 72 minutes to 644 minutes when combining 0.14% (m/m) PVA with the liposomal formulation [151].

Another example is carbopol, a polymer of acrylic acid with a large number of carboxyl groups in its structure that allow it to form a gel in the presence of water. Carbopol 940 was used by researchers to prepare a liposomal hydrogel with ciprofloxacin. The results of the study showed that the use of carbopol allowed a more delayed and prolonged release of ciprofloxacin compared to the liposomal suspension and the drug solution (0.3% ciprofloxacin). In addition, the use of the liposomal hydrogel showed an increase in the permeation in a study performed with albino rabbit corneas, being 5-folds higher than for the aqueous solution of ciprofloxacin. In addition, liposomal hydrogel showed a percentage permeated of 30.6% in contrast with the 20.4% of the liposomal suspension after 6 hours [197]. Mostafa Feghhi et al carried out a recent study with Carbomer 934 for coating 0.3% ciprofloxacin liposomes. They reported an enhancement in permeability and bioavailability, which resulted 4 times higher than commercial formulation, and antimicrobial effect in an *in vivo* study in rabbits. However, the results showed no improvement over those obtained with non-coated liposomes [198].

Poloxamers are co-polymers formed by a polypropylene central chain and two polyethylene side chains. In this way, the central chain has hydrophobic properties and the lateral ones have hydrophilic properties. An example is Pluronic F-127, a temperature sensitive polymer capable of changing from a liquid to a gel state when in contact with body temperature. The use of Pluronic F-127 as a vehicle in liposomal formulations has been studied by several authors as a method to increase the residence time of the drug and to provide a sustained release. This co-polymer has been employed as a vehicle for a latanoprost liposomal formulation, observing a longer release of the drug when compared with conventional liposomes or the use of other polymers such as HPMC, so that after 24 hours approximately 30% of the drug had been released, compared to the 40% in other liposomes. This is probably due to the hydrophobic interactions of the central block with latanoprost, which allows a slower diffusion through the gel. Moreover, the use of latanoprost-loaded liposomes vehiculized with Pluronic F-127 showed more reduction of intraocular pressure in normotensive rabbits compared to commercialized eye drops (both 50 µg/mL latanoprost). In this sense, while the intraocular pressure returned to baseline values 24 hours after topical administration of 50 µL eye drops, in the case of latanoprost liposomal gels the values did not return to baseline until 72 hours [199]. This thermosensitive Pluronic F-127 gel was also used

by other authors to disperse ketorolac liposomes. The 24-hour *in vitro* release study showed that the liposomal gel formulation provided a more sustained release than the liposomal formulation without Pluronic F-127, proving to be able to maintain the release for around 24 hours. [200].

Protein polymers have also been used to coat liposomes. This is the case of silk fibroin (SF), a non-toxic natural mucoadhesive polymer that can be degraded by proteolysis suitable for drug delivery. SF is capable of binding to proteoglycans and glycoproteins of the mucous layer. Yixuan Dong et al. performed a study comparing SF-coated liposomes with conventional ibuprofen-loaded liposomes and a drug solution, showing more sustained release profile and a greater corneal permeability *in vitro*. The apparent permeability coefficient in the case of SF liposomes was 1.23 ± 0.24 cm/s compared to 1.16 ± 0.23 for normal liposomes. Also, cell viability was always above 85% after the addition of SF in a concentration range of 0.25-2% to human corneal epithelial cells for 2-8 hours. Furthermore, the toxicity and adhesion capacity of SF-coated liposomes was also tested on human corneal epithelial cells, resulting in an absence of toxicity and rapid cell adhesion and strong cellular up-take by observing the fluorescence [201].

Biomaterials with binding properties to glycan residues on the corneal surface have been also used to increase the retention time of drugs on the cornea as it is the case of succinyl- Concavalin A. Changyou Zhan et al. studied functionalized liposomes composed of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DOPG) and cholesterol loaded with 1 mg/mL of tetrodotoxin (TTX) and 100 µg/mL of dexmedetomidine (DMED) and functionalized with 25 µg/mL succinyl-Concanavalin A to increase the duration of the anaesthetic effect of TTX and DMED by ocular topical application. The size of the resulting liposomes was 508 nm, and the encapsulation efficiency was 43% for TTX and 62% for DMED. Cellular toxicity was tested in corneal limbal epithelial cells and corneal keratocytes by 24-hour exposure, showing an absence of toxicity (approximately 100% viability). The sConA-functionalized liposomal formulation was compared to a non-functionalized formulation and the drugs in solution. The study in rats showed a significant increase in duration of anaesthesia when 30 µL of sConA-Lip/TD was administered, being between 2 and 3.9 times greater than in the case of non-coated liposomes. Furthermore, corneal persistence tests performed with fluorescent dye rhodamine 6G reported a greater persistence in the cornea when liposomes were conjugated with sConA [202].

6. Recent applications of liposomes for tear film restoration/recovery

6.1. Ocular surface protection.

As mentioned previously, the tear film performs important functions in the hydration, homeostasis and protection of the ocular surface. Many ocular pathologies, such as DED, involve an alteration of the tear film and increase of tear evaporation. Dry eye disease is a pathology whose prevalence varies between 5% and 50% depending on the criteria, increasing with age and being more common in women than in men. This disease has a great impact on visual function and quality of life [203]. The integrity of the lipid layer plays an important role in preventing evaporation of the tear film. Furthermore, the thickness of the lipid layer has been related to the evaporation of the tear [203] [204]. For this reason, the inclusion of lipids in artificial tears intended to restore the lipid layer has attracted a lot of attention. In addition, bioadhesive polymers and components with osmoprotective or anti-inflammatory properties can also be included in the formulations to improve effectiveness against DED [205] [15].

Tear film dysfunction has also been linked to environmental factors or as a consequence of medication or systemic diseases. When there is an alteration in the tear film, damage to the ocular surface and symptoms of discomfort can occur. Furthermore, tear film dysfunction has been associated with an increase in hyperosmolarity due to a loss of the aqueous component of the tear [206] [207].

These circumstances have made it necessary to develop treatments based on artificial tears to restore the protection of the ocular surface. Artificial tears have been widely used to lubricate the ocular surface. Furthermore, as mentioned previously, bioadhesive compounds are widely used in eye drops because of their interaction with the negatively charged ocular surface. Polymers such as CMC, HPMC, HA and carbomers, have commonly been included in its composition, which increases the retention time on the ocular surface [206].

Lipid based eye drops have been shown to be well tolerated and to decrease the symptoms of DED. Among these we can find ointments, which do not have aqueous components, emulsions and liposomes [15]. The internal phase of emulsions is made up of oils that form small drops due to the presence of surfactants.

Depending on the components of the emulsions, they can be anionic or cationic emulsions. Cationic emulsions, composed of positively charged nanodroplets due to the presence of substances like stearylamine or DOTAP, are able to interact with the negatively charged ocular surface, resulting in a longer residence time and spreading of artificial tears in the eye and therefore a greater improvement in symptoms [15] [208].

In the market there are several lipid-based artificial tears, such as Neovis® (Horus Pharma, Saint-Laurent du Var, France) which contains hyaluronic acid, lipoic acid and phospholipids, Systane® Balance (Alcon, Fort Worth, Texas) which is composed of an emulsion and propylene glycol or Systane® Complete (Alcon, Fort Worth, Texas) formed by nanoparticles based on lipids and propylene glycol. Both Systane® Balance and Systane® Complete contain the phospholipid dimyristoylphosphatidylglycerol. Systane® Balance was administered in an investigator-masked controlled clinical trial in 49 dry eye patients with meibomian gland dysfunction. Patients were randomly administered Systane® Balance (n = 25) or saline (n = 24) as a control group, 4 times daily topically. After 4 weeks of treatment, patients treated with Systane® Balance experienced an increase in lipid film

stability, with a non-invasive tear film break-up (NITBUT) of 2.83 ± 0.74 seconds compared to 0.66 ± 0.55 in the control group ($p < 0.001$). In addition, in the treated patients there was an increase in the expression of the meibomian glands and the density of globet cells (NCT01718028) [209]. Systane® Complete has also been tested in a clinical trial in patients with symptomatic dry eye for the use of contact lenses. The investigator-masked clinical trial was conducted in 46 patients, of whom 22 received Systane® Complete and 24 were untreated. After two weeks of treatment, the treated patients showed better results on the Contact Lens Dry Eye Questionanire-8 (CLDEQ-8), showing an improvement in symptoms, the results were 12.86 ± 6.40 compared to 17.92 ± 5.30 in the untreated group [210].

Cationorm® (Santen) is a hypotonic cationic nanoemulsion (150-300 nm) free of preservatives used for dry eye treatment. Cationorm® is based on Novasorb®, a groundbreaking technology employing high pressure homogenization. That aims to use cationic nanoemulsions in specifically designed buffers able to bind the cornea and conjunctiva in order to tackle different ocular surface diseases [211]. Besides, there are electrostatic interactions between the positive charged nano system and the ocular surface epithelium negatively charged (cornea and conjunctiva [212]. Ikervis® (Cyclosporine 1mg/mL) also based on the previously described Novasorb® technology is a nanoemulsion system entrapping cyclosporine as an effective immunomodulator and anti-inflammatory drug able to reduce symptoms and control some level of inflammation caused by keratoconjunctivitis sicca or dry eye disease [213]. It constitutes a novel technology that increase the drug permeation and efficacy due to the enhanced permeation of nanoemulsions through corneal epithelium [214].

Liposomes results of great interest in the treatment of DED. Soy phosphatidylcholine is widely used in the manufacture of liposomes. The main advantage of using phosphatidylcholine is that it is the main component present in the lipid layer of the tear film. In these liposomes, other lipophilic components such as vitamin A and vitamin E can also be added to the lipid bilayer preventing the oxidation of unsaturated lipids due to their antioxidant's properties [15]. A liposomal spray formulation for the treatment of dry eye is currently marketed under the name of Tears Again®. This liposomal spray is made up of phospholipids (phosphatidylcholine) and vitamins A and E and is intended to be applied to the surface of the eyelid with the eye closed. A comparative study between Tears Again and a saline spray was reported. The controlled, double-blind, prospective and randomized study in design was carried out in 22 subjects with dry eye. The liposomal spray was applied once to the treated eye, while a saline spray was administered to the other eye (control). The results showed a significant increase in the thickness of the lipid layer ($p < 0.005$) at 30, 60 and 90 minutes after the application of the liposomal spray. Furthermore, there was an increase in tear film stability in the treated eyes ($p < 0.001$). Moreover, 70% patients reported greater comfort after 30 minutes of applying the liposomal spray [215].

The use of liposomes for the formulation of topical eye drops for the treatment of pathologies such as DED has been developed

There is a currently commercialized liposome-based artificial tears called Aquoral Lipo® (ESTEVE, Farmigea, Pisa, Italy) (EX3652-19-01). Aquoral Lipo is made up of liposomes, cross-linked hyaluronic acid and crocin. It is designed to be instilled in the eye topically for the treatment of dry eye [216]. Another commercialized formulation is Lacrisek® Ofta (BIOOS Italia, Italy), a product based on liposomes with vitamins A and E, intended for topical instillation for the treatment of dry eye. This topical formulation was tested on evaporative dry eye patients in a single instillation. Results showed that 60 minutes after instillation, improvements in tear film evaporation and tear break-up time (TBUT) continued, unlike Artelac Rebalance®, an aqueous formulation with polyethylene glycol and hyaluronic acid, whose protection only lasted 10 minutes [15] [217].

An interesting formulation with lipid components similar to the one present in the preocular tear film has been developed by Vicario-de-la-Torre M et al. to treat the DED. These authors designed a formulation based on phosphatidylcholine, cholesterol and vitamin E in an 8:1:0.8 ratio prepared by the lipid film hydration method. The formulations dispersed in water and 0.9% NaCl were characterized. In addition, a liposomal formulation dispersed in 0.9% NaCl diluted in proportions 1/2 with 0.2% sodium hyaluronate to increase the corneal surface adhesion was also studied. The *in vitro* tolerance of the formulations with 0.9% NaCl and 0.2% sodium hyaluronate were evaluated at 15 minutes, 1 hour and 4 hours in immortalized human corneal-limbal epithelial cells (HCLE) and normal human conjunctive cells (IOBA-NHC). The results showed a cell viability greater than 90% in the HCLE and IOBA-NHC cell lines at all times. Furthermore, *in vivo* tolerance studies in New Zealand rabbits after topical administration of 30 µL of the liposomal formulation with 0.9% NaCl (20 mg/mL PC) and 0.2% sodium hyaluronate (10 mg/mL PC) every 30 minutes and a total duration of 6 hours showed an absence of symptoms of discomfort and disturbances [218]. The same group dispersed the liposomes in a solution with trehalose, which protects cells from desiccation, and a borate buffer solution as a dispersion vehicle. The liposomes had 186.3 nm size. This liposomal formulation diluting with sodium hyaluronate (10 mg/mL PC and 0.2 % SH) gave *in vitro* cytotoxicity results greater than 80% in HCLE and IOBA-NHC cell lines and showing good tolerance *in vivo* after the topical administration of 30 µL each 30 minutes for 6 hours [129]. Also, the liposomal formulation composed of phosphatidylcholine and cholesterol was enriched using vitamin E and vitamin A to form the liposomes. Furthermore, in order to achieve in situ gelling artificial tears, gellan gum and hydroxypropyl methylcellulose were used, with a final concentration of 0.25% and 0.12% respectively. Other compounds such as levocarnitine, with osmoprotective activity, have been included in the formulation to attenuate the hyperosmolarity produced in DED. The resulting liposomes prepared by the lipid film hydration method had a size of 200.1 ± 4.4 nm. Cell viability in human carcinoma epithelial cells (HeLa) and J774 macrophages was greater than 90% after 2 hours of exposure. Furthermore, *in vivo* studies in rabbits showed good tolerance after administration of 30 µL of the formulation (0.5% PC) every 30 minutes for 6 hours [219].

6.2. Liposomal formulations as supplementation in dry eye treatment

In addition to phospholipids and other lipids such as cholesterol to replace the lipid layer of the tear film, other components can be added to the lipid bilayer in order to provide additional supplementation. An example of this is the use of vitamin E and vitamin A, mentioned in the previous section. Vitamin E can be incorporated into the liposomal lipid bilayer enabling the stabilization and preventing degradation of phospholipid chains. Vitamin E avoids the oxidation of unsaturated phospholipids, such as phosphatidylcholine, thus increasing the stability of liposomes. Furthermore, its antioxidant properties, making it capable of protecting cells from damage [218].

Regarding vitamin A, in addition to its antioxidant properties, a study in an animal model of dry eye in mice showed that vitamin A had the ability to reduce apoptosis of corneal epithelial cells. Moreover, it also showed an increase in the volume of the tear film, as well as its stability [220]. Vitamin A is capable of regulating the differentiation and proliferation of corneal epithelial cells, and its supplementation is important to maintain adequate vision. For this reason, the use of liposomes has been studied to increase retention in the cornea, and therefore the bioavailability of vitamin A [221].

Another interesting possibility for supplementation of the dry eye disease could be the use of fatty acids omega 3 and omega 6. These fatty acids have been shown to be effective in reducing symptoms as an oral supplement due to its anti-inflammatory properties. However, recently they have also been shown to be effective topically in eye drops [222]. A study with eye drops containing hyaluronic acid and omega 3 essential fatty acids showed a decrease in corneal irregularities, in addition to reducing oxidative stress and inflammation in a mouse model of dry eye disease, compared to eye drops containing only hyaluronic acid [223]. Another study used various types of fatty acids formulated in emulsion. The authors tested alpha-linolenic acid omega-3 (ALA) and linoleic acid omega 6 (LA). These fatty acids were tested alone and in combination, compared to the vehicle in a mouse animal model. The formulations were applied topically every 48 hours, up to a total of 3 doses. In the case of treatment with alpha-linolenic acid, the results showed a decrease in damage to the corneal epithelium. Furthermore, the use of ALA showed a decrease in proinflammatory cytokines TNF-alpha and IL-1. These results may mean a decrease in inflammation produced in dry eye pathology [224]. The introduction of these fatty acids in liposomal formulations could be of great interest.

Other compounds, such as squalene, that can be used as supplements in artificial tears. Squalene, which has been found in the tear, has numerous properties, including antioxidant, anti-inflammatory and hydrating capacity. Squalene appears to be placed on the thinnest regions of the lipid layer of the tear film, thus allowing the entire surface to be covered by this film, increasing protection [225].

7. Limitations and future prospects.

Liposomes represent a great advance in topical ocular administration, with the advantage of being well tolerated by the eye thanks to their biodegradable and biocompatible properties. Phosphatidylcholines, which are the main phospholipids that constitute the lipidic bilayer of liposomes, are present in the tear film, providing an adequate tolerability and allowing to improve the stability of the tear film. Furthermore, their capacity to act as drug carriers, allows to reduce the dose, and therefore the toxicity of the administered drugs [15].

Moreover, it is possible to manufacture them by simple methods. However, they also have limitations and there is much to improve in the field. As mentioned in this review, liposomes are capable of increasing the bioavailability of drugs, one of the main problems of ophthalmic topical administration. Despite this, there is still much to improve and research in this regard. Currently, to solve this problem, there are numerous resources to increase mucoadhesion and retention time of topical ophthalmic formulations and subsequently, ocular drug bioavailability. These strategies, mentioned in depth in this review, include the use of charged components or polymers with biodegradable and biocompatible properties, capable of increasing drug penetration.

One of the biggest limitations of liposomes is their stability. On the one hand, the unsaturated lipids present in the lipid bilayer are easily oxidizable and can also undergo hydrolysis processes, which makes liposomes less chemically stable. However, this problem can be mitigated by including antioxidant compounds, such as vitamin E, in the formulation. On the other hand, liposomes can also become physically unstable. For example, they can undergo aggregation, forming larger particles that will be more difficult to absorb and that produce a greater tendency to be phagocytized. Liposome aggregation can be prevented by the development of charged liposomes and phagocytosis can be prevented by using polymers that coat the liposomes, such as polyethylene glycol. Another stability problem related to liposomes is the loss of the encapsulated drug, or the leaked of lipid components of the bilayer, being released into the aqueous phase. To avoid the loss of encapsulated drug and favor the stability of the liposomes, it is possible to resort to the incorporation of appropriate amounts of cholesterol in the structure [112].

Regarding sterilization, there is still a need to develop robust methods that do not alter the composition of the formulation, since as previously mentioned the nature of the sample may be affected by the selected sterilization method. The big limitation for sterilizing liposomal dispersion would be testing their posterior efficacy and safety, ensuring that no toxic or degradation have occurred. Perhaps, studying and developing alternative ‘cold methods’ such as ethylene oxide could solve the problem out and provide with an established method to sterilize every single liposomal formulation on the market without any risks associated [137].

As above mentioned, lyophilization of liposomal formulations is still controversial since some have described that presents stability problems but others ensure to have developed optimized protocols that allow to sort these issues and achieve long periods of storage therefore avoiding alteration of the formulation or physicochemical changes [117]. Although this still needs to be further investigated, great advances are being developed and perhaps in the future the industry could create freeze drying protocols to storage liposomes for long times.

Regarding stability of liposomes, there are some important issues that are perhaps the limiting step when reaching a clinical translational approach. The shelf-life of liposome dispersions using phospholipids are related to oxidative and hydrolytic degradation pathways. Oxidation of phospholipids molecules takes place via a free radical chain mechanism in the absence of specific oxidants. Peroxidation of phospholipids in liposomes can be minimized thanks to the use of hydroperoxides purified raw materials, less unsaturated fatty acyl chain-containing phospholipids and antioxidants. Also, storage at low temperature with protection from light and oxygen and working under inert gas atmosphere reduces the oxidation of the phospholipids. Hydrolysis kinetic of phospholipids depends on pH, temperature, buffer concentration and ionic strength. For long-term stability, storage of liposomes in an aqueous dispersion at low temperatures (4-6ºC) and pH adjustment to values of maximum stability of liposomes is recommended [226].

Scalability is another delicate issue that is gaining importance since liposomal formulations are entering the market. Developing large amounts of liposomes in a fast, easy and not very expensive way is strongly associated to the type of liposomes and products that are going to be encapsulated. Nonetheless, novel techniques such as microfluidics chips and micro emulsification and optimization of other better-known methods, such as ethanol injection, are being investigated since they constitute a potential source of liposome making for industries [90].

Moreover, it bears mentioning the exosomes, innovative systems considered an evolution of liposomal formulations, have gained much interest over the last few years. Exosomes are sphere-like extracellular vesicles that are produced in endosomes of all eukaryotic cells. They constitute an effective and fast mechanism of communication between cells and their environment with different specialized functions depending on the cell type. A clear example are exosomes present in dry eye patients, which can modify the activity of matrix metalloproteinases and therefore play an important role in remodeling the extracellular matrix [227]. Exosomes from mesenchymal stem cells have shown anti-inflammatory activity, regenerative properties and being able to regulate the immune response in the eye. Although in many ocular therapies involving exosomes, these systems have been administered through intravitreal injections [228], a very recent study has shown that exosomes isolated from corneal mesenchymal stromal cells can be useful for wound-healing purposes [229]. Besides, a clinical trial is being conducted using exosomes from umbilical mesenchymal stem for relieving dry eye associated symptoms (NCT04213248).

Finally, some interesting liposomal formulations have been developed as a novel approach to treat DED [111]. These formulations contain natural phospholipids and lipidic components similar to those present in the tear film. In addition, they aim to restore the precorneal tear film by not only treating the dry eye symptoms but also restoring normality in the ocular surface and suppressing the inflammation cascade given in DED. A good example of these type of formulations is one containing soy phosphatidylcholine (20 mg/mL), cholesterol (2,5 mg/mL) and vitamin E (0,2 mg/mL). Besides, the formulation is made hypotonic regarding tears by containing trehalose and a borated-buffer solution [129]. These technological approaches aim, not only to restore the preocular tear film but also to tackle the hypertonic environment commonly given in DED and supply the ocular surface with osmoprotective properties.

8. Conclusion

Although very accessible, the ocular surface has been for many years and still is a rather complex and delicate structure to deliver drugs and formulations. Different mechanisms and physiological structures work together making permeation and delivery a very difficult task. Fortunately, liposomes are tremendously useful systems that were developed with the purpose of entering cells and tissues when other common substances could not. Due to their similarity with cell membranes, liposomes entrapping a wide variety of therapeutic products are an effective strategy to surpass the physiological barriers present in the ocular surface such as tear clearance, tight junctions of the corneal epithelium and even corneal stroma and endothelium. Despite their suitability and usefulness, there is still a need to study scalability and market adaption so everyone can benefit from their countless applications.

9. Expert opinion (500 words minimum)

Drug delivery in ophthalmology constitutes a particular challenge since many physiological and anatomical barriers work perfectly together in order to avoid alterations in tears balance, tear film stability, pH and osmolar changes. Besides, the ocular surface is widely prepared to fight and prevent invasion and permeation of bacteria and other pathogens.

Over the last few years ocular drug delivery formulations have experimented a dramatic growth and improvement. This is due to the increment in novel and interesting techniques that allow to design, and tailor new formulations targeting specific structures of the eye and increase drug effectivity. Among many of the systems employed with this purpose, liposomes are well studied lipid-based carriers, very similar to cell membranes and cell structures. They are in many cases nano-scaled and can be specifically design and tailored to interact with specific structures of the tissues which allow them to effectively deliver the drug. Furthermore, one of the many reasons why liposomes are ideal nanocarriers is the wide variety of designs that can be made depending on the target place and function that is desired. Moreover, they have gained much interest by its suitability of entrapping both hydrophilic and hydrophobic drugs, increasing their stability and lowering drug associated toxicity.

Despite their long background, liposomes continue being the alternative for many researchers to create novel and innovative formulations, particularly in ocular drug delivery. High hydrophobic drugs, almost no soluble in aqueous media, have been entrapped in liposomes and delivered successfully to the ocular surface. Also, liposomes have been assayed to treat posterior segment diseases such as glaucoma or AMD.

Some of the last advances in liposome technology highlights the recent use of bioactive molecules such as annexin V associated to liposomes to enhance bevacizumab topical delivery in AMD. Normally annexin V has been widely used for staining techniques in apoptosis detection, but researchers have discovered that helps liposomes to go through cell membranes by a trans-cytosis mechanism [230]. Furthermore, including agents and substances in liposomes that tackle oxidative strategies for ocular surface disease is becoming a different and innovative approach like including carotenoids in liposomes, regulating gene expression and tear volume balance [182].

Despite the great progress in this area, there is still much to improve and optimize but important advances are being made. Perhaps, immunogenicity can be tuned and modified in order to avoid a disproportionate reaction of the tissue. That is the reason why, soy phospholipids are eligible and are being optimized although they show a profile rather complex to characterize since their extraction may cause some changes in the fatty acid profile [86]. For instance, DOPG has very recently been discovered to promote tissue regeneration of the corneal epithelium, so this means that specific liposomal systems that inherently possess therapeutic properties can be designed [85]. SiRNA gene therapy is evolving in the field of liposomes and especially in topical administration for diseases previously mentioned such as DED or AMD. Apart from the containing siRNA molecules recent advances point out the importance of combining liposomes with some polymers such as HA and specific target molecules like CD44 that could enhance adhesion and cell permeation [173].

All these strategies demonstrate that liposomal field is constantly evolving and taking advantage of all the new discoveries and growing technology, highly specified systems with great tissue affinities can be created so low toxicities, doses and reduced administrations may be possible. Moreover, a combination between liposomal formulation and exosome technology could be used to specifically re-design these systems and fight these diseases from another different perspective. Ocular disease needs from these breakthroughs to discover and develop new treatments and strategies that focus not only in pathways and therapeutic substances but also in substances and materials that already possess beneficial characteristics such as antioxidative, anti-inflammatory or wound-healing properties.

Furthermore, the previously mentioned therapies containing natural components that resembles the preocular tear film has gained much interest and creates a new area of research that could be further investigated to develop new potential therapies that allows to treat more effectively ocular surface pathologies. To our view, this novel approach opens a new possibility to treat DED and ocular surface pathologies. It aims to be the next generation of liposomal formulations not necessarily containing active drugs in order to treat pathologies of the ocular surface presenting tear film instability, alteration of the physiological properties of ocular surface and ocular inflammation. Liposomal formulations with components resembling the preocular tear film could also be used as vehicles for active drugs in long term treatments avoiding the associate side effects related to chronic therapy.

Funding

Research Group UCM 920415, Innovation, Therapy and Pharmaceutical Development in Ophthalmology (InnOftal) and the ISCIII-FEDER RETICS (OFTARED) (RD16/0008/0009 and RD16/0008/0004). FEDER-CICYT FIS-PI17/00079 and PI17/00466.

Acknowledge

All the figures were created with BioRender (www.biorender.com)

M.G.C. thanks for a PhD fellowship from the Spanish MECD (FPU18/03445).

Declaration of interest

The authors declare no conflict of interest.

References

[1] Bucolo C, Drago F, Salomone S. Ocular drug delivery: A clue from nanotechnology. Front. Pharmacol. 2012;3 OCT:2002–2004.

[2] Shen J, Lu GW, Hughes P. Targeted ocular drug delivery with pharmacokinetic/pharmacodynamic considerations. Pharm. Res. 2018;35:217.

[3] Kaur IP, Kanwar M. Ocular preparations: The formulation approach. Drug Dev. Ind. Pharm. 2002;28:473–493.

[4] Bhanu M, Harsha K, Anupam P. Ocular drug delivery systems. Nat. Polym. drug Deliv. 2017;160–170.

[5] Bravo-Osuna I, Andrés-Guerrero V, Pastoriza Abal P, et al. Pharmaceutical microscale and nanoscale approaches for efficient treatment of ocular diseases. Drug Deliv. Transl. Res. [Internet]. 2016;6:686–707. Available from: http://dx.doi.org/10.1007/s13346-016-0336-5.

[6] Urtti A. Challenges and obstacles of ocular pharmacokinetics and drug delivery. Adv. Drug Deliv. Rev. 2006;58:1131–1135.

[7] Geroski DH, Edelhauser HF. Drug Delivery for Posterior Segment Eye Disease. Invest. Ophthalmol. Vis. Sci. 2000;41:39–42.

[8] Moisseiev E, Loewenstein A. Drug delivery to the posterior segment of the eye. Dev. Ophthalmol. 2017;58:87–101.

[9] Gote V, Sikder S, Sicotte J, et al. Ocular drug delivery: Present innovations and future challenges. J. Pharmacol. Exp. Ther. 2019;370:602–624.

[10] Gaudana R, Jwala J, Boddu SHS, et al. Recent perspectives in ocular drug delivery. Pharm. Res. 2009;26:1197–1216.

[11] Herrero-Vanrell R, Vicario De La Torre M, Andrés-Guerrero V, et al. Nano and microtechnologies for ophthalmic administration, an overview. J. Drug Deliv. Sci. Technol. [Internet]. 2013;23:75–102. Available from: http://dx.doi.org/10.1016/S1773-2247(13)50016-5.

[12] Amadio M, Pascale A, Cupri S, et al. Nanosystems based on siRNA silencing HuR

expression counteract diabetic retinopathy in rat. Pharmacol. Res. [Internet]. 2016;111:713–720. Available from: http://dx.doi.org/10.1016/j.phrs.2016.07.042.

[13] Wadhwa S, Paliwal R, Paliwal S, et al. Nanocarriers in Ocular Drug Delivery: An Update Review. Curr. Pharm. Des. 2009;15:2724–2750.

[14] Mishra GP, Bagui M, Tamboli V, et al. Recent Applications of Liposomes in Ophthalmic Drug Delivery. J. Drug Deliv. 2011;2011:1–14.

[15] Garrigue JS, Amrane M, Faure MO, et al. Relevance of Lipid-Based Products in the Management of Dry Eye Disease. J. Ocul. Pharmacol. Ther. 2017;33:647–661.

*** An excellent review on the treatment of dry eye using lipid-based products**

[16] Cher I. A new look at lubrication of the ocular surface: Fluid mechanics behind the blinking eyelids. Ocul. Surf. 2008;6:79–86.

[17] Tiffany J. The normal tear film. Dev. Ophthalmol. 2008;41:1–20.

[18] Davidson HJ, Kuonen VJ. The tear film and ocular mucins. Vet. Ophthalmol. 2004;7:71–77.

[19] Dilly PN. STRUCTURE AND FUNCTION OF THE TEAR FILM. Lacrimal Gland. Tear Film. Dry Eye Syndr. 1994;239–247.

[20] Lemp, M. A., Holly, F. J., Iwata, S., & Dohlman CH. The Precorneal Tear Film I. Factors in Spreading and Maintaining a Continuous Tear Film Over the Corneal Surface. Arch. Ophthalmol. 1970;83:89–94.

[21] Argüeso P, Gipson IK. Epithelial mucins of the ocular surface: Structure, biosynthesis and function. Exp. Eye Res. 2001;73:281–289.

[22] Willcox MDP, Argüeso P, Georgiev GA, et al. TFOS DEWS II Tear Film Report. Ocul. Surf. [Internet]. 2017;15:366–403. Available from: http://dx.doi.org/10.1016/j.jtos.2017.03.006.

**** An in-depth review of the involvement of tear film in dry eye syndrome.**

[23] Gipson IK. Distribution of mucins at the ocular surface. Exp. Eye Res. 2004;78:379–388.

[24] Bron AJ, Tiffany JM, Gouveia SM, et al. Functional aspects of the tear film lipid layer. Exp. Eye Res. 2004;78:347–360.

[25] Hodges RR, Dartt DA. Tear film mucins: Front line defenders of the ocular surface; comparison with airway and gastrointestinal tract mucins. Exp. Eye Res. 2013;117:62–78.

[26] Rolando M, Zierhut M. The Ocular Surface and Tear Film and Their Dysfunction in Dry Eye Disease. Surv. Ophthalmol. 2001;45:203–210.

[27] Butovich IA, Millar TJ, Ham BM. Understanding and Analyzing Meibomian Lipids — A Review. Curr. Eye Res. 2008;33:405–420.

[28] Dean AW, Glasgow BJ. Mass Spectrometric Identification of Phospholipids in Human Tears and Tear Lipocalin. Invest. Ophthalmol. Vis. Sci. 2012;53.

[29] Lam SM, Tong L, Duan X, et al. Extensive characterization of human tear fluid collected using different techniques unravels the presence of novel lipid amphiphiles. J. Lipid Res. 2014;55:289–298.

[30] Green-church KB, Butovich I, Willcox M, et al. The International Workshop on Meibomian Gland Dysfunction : Report of the Subcommittee on Tear Film Lipids and Lipid – Protein Interactions in Health and Disease OF THE. Invest. Ophthalmol. Vis. Sci. 2011;52:1922–1929.

[31] Kulovesi, P., Telenius, J., Koivuniemi, A., Brezesinski, G., Vattulainen, I., Holopainen JM. The impact of lipid composition on the stability of the tear fluid lipid layer. Soft Matter. 2012;8:5826–5834.

[32] Schuett BS, Millar TJ. Lipid Component Contributions to the Surface Activity of Meibomian Lipids. Invest. Ophthalmol. Vis. Sci. 2012;7208–7219.

[33] Glasgow BJ, Marshall G, Gasymov OK, et al. Tear Lipocalins : Potential Lipid Scavengers for the. Invest. Ophthalmol. Vis. Sci. 1999;40:3100–3107.

[34] Millar TJ, Mudgil P, Butovich IA, et al. Adsorption of Human Tear Lipocalin to Human Meibomian Lipid Films AND. Invest. Ophthalmol. Vis. Sci. 2009;50:140–151.

[35] Gouveia SM, Tiffany JM. Human tear viscosity : An interactive role for proteins and lipids. Biochim. Biophys. Acta. 2005;1753:155–163.

[36] Meek KM, Knupp C. Corneal structure and transparency. Prog. Retin. Eye Res. [Internet]. 2015;49:1–16. Available from: http://dx.doi.org/10.1016/j.preteyeres.2015.07.001.

[37] Girolamo N Di. Stem cells of the human cornea. Br. Med. Bull. 2011;100:191–207.

[38] Almubrad T, Akhtar S. Structure of corneal layers, collagen fibrils, and proteoglycans of tree shrew cornea. Mol. Vis. 2011;17:2283–2291.

[39] Eghrari AO, Riazuddin SA, Gottsch JD. Overview of the Cornea: Structure, Function, and Development [Internet]. 1st ed. Prog. Mol. Biol. Transl. Sci. Elsevier Inc.; 2015. Available from: http://dx.doi.org/10.1016/bs.pmbts.2015.04.001.

[40] Ramos T, Scott D, Ahmad S. An Update on Ocular Surface Epithelial Stem Cells: Cornea and Conjunctiva. Stem Cells Int. 2015;2015.

[41] DelMonte DW, Kim T. Anatomy and physiology of the cornea. J. Cataract Refract. Surg. [Internet]. 2011;37:588–598. Available from: http://dx.doi.org/10.1016/j.jcrs.2010.12.037.

[42] Khodadoust, A. A., Silverstein, A. M., Kenyon, K. R., & Dowling JE. Adhesion of regenerating corneal epithelium: the role of basement membrane. Am. J. Ophthalmol. 1968;65:339–348.

[43] Dua HS, Azuara-blanco A. Limbal Stem Cells of the Corneal Epithelium. Surv. Ophthalmol. 2000;44:415–425.

[44] Thiagarajah JR, Verkman AS. Aquaporin deletion in mice reduces corneal water permeability and delays restoration of transparency after swelling. J. Biol. Chem. 2002;277:19139–19144.

[45] Torricelli AAM, Singh V, Santhiago MR, et al. The corneal epithelial basement membrane: Structure, function, and disease. Investig. Ophthalmol. Vis. Sci. 2013;54:6390–6400.

[46] Wilson SE, Hong JW. Bowman's layer structure and function: Critical or dispensable to corneal function? A Hypothesis. Cornea. 2000;19:417–420.

[47] Müller LJ, Marfurt CF, Kruse F, et al. Corneal nerves: Structure, contents and function. Exp. Eye Res. 2003;76:521–542.

[48] Lewis PN, Pinali C, Young RD, et al. Structural Interactions between Collagen and Proteoglycans Are Elucidated by Three-Dimensional Electron Tomography of Bovine Cornea. Structure [Internet]. 2010;18:239–245. Available from: http://dx.doi.org/10.1016/j.str.2009.11.013.

[49] Meek KM, Boote C. The organization of collagen in the corneal stroma. Exp. Eye Res. 2004;78:503–512.

[50] West-mays JA, Dwivedi DJ. The keratocyte : Corneal stromal cell with variable repair phenotypes. Int. J. Biochem. Cell Biol. 2006;38:1625–1631.

[51] Danielsen CC. Tensile mechanical and creep properties of Descemet's membrane and lens capsule. Exp. Eye Res. 2004;79:343–350.

[52] Hayashi S, Osawa T, Tohyama K. Comparative observations on corneas, with special reference to Bowman's layer and Descemet's membrane in mammals and amphibians. J. Morphol. 2002;254:247–258.

[53] Leung EW, Rife L, Smith RE, et al. Extracellular matrix components in retrocorneal fibrous membrane in comparison to corneal endothelium and Descemet's membrane. Mol. Vis. 2000;6:15–23.

[54] Joyce NC. Proliferative capacity of the corneal endothelium. Prog. Retin. Eye Res. 2003;22:359–389.

[55] Joyce NC. Cell cycle status in human corneal endothelium. Exp. Eye Res. 2005;81:629–638.

[56] Bonanno JA. Molecular mechanisms underlying the corneal endothelial pump. Exp. Eye Res. [Internet]. 2012;95:2–7. Available from: http://dx.doi.org/10.1016/j.exer.2011.06.004.

[57] Messmer EM, Mackert MJ, Zapp DM, et al. In vivo confocal microscopy of normal conjunctiva and conjunctivitis. Cornea. 2006;25:781–788.

[58] Dartt DA. Regulation of mucin and fluid secretion by conjunctival epithelial cells. Prog. Retin. Eye Res. 2002;21:555–576.

[59] Jumblatt MM, McKenzie RW, Steele PS, et al. MUC7 expression in the human lacrimal gland and conjunctiva. Cornea. 2003;22:41–45.

[60] Doughty MJ. Goblet Cells of the Normal Human Bulbar Conjunctiva and Their Assessment by Impression Cytology Sampling. Ocul. Surf. [Internet]. 2012;10:149–169. Available from: http://dx.doi.org/10.1016/j.jtos.2012.05.001.

[61] Gipson IK. Goblet cells of the conjunctiva: A review of recent findings. Prog. Retin. Eye

Res. [Internet]. 2016;54:49–63. Available from: http://dx.doi.org/10.1016/j.preteyeres.2016.04.005.

[62] Kobayashi A, Yoshita T, Sugiyama K. In Vivo Findings of the Bulbar / Palpebral Conjunctiva and Presumed Meibomian Glands by Laser Scanning Confocal Microscopy. Cornea. 2005;24:985–988.

[63] Steven P, Andreas G. Conjunctiva-Associated Lymphoid Tissue – Current Knowledge, Animal Models and Experimental Prospects. Ophthalmic Res. 2009;42:2–8.

[64] Knop N, Knop E. Conjunctiva-associated lymphoid tissue in the human eye. Invest. Ophthalmol. Vis. Sci. 2000;41:1270–1279.

[65] Knop E, Knop N. The role of eye-associated lymphoid tissue in corneal immune protection. J. Anat. 2005;206:271–285.

[66] Gaudana R, Ananthula HK, Parenky A, et al. Ocular drug delivery. AAPS J. 2010;12:348–360.

[67] Davies NM. BIOPHARMACEUTICAL CONSIDERATIONS IN TOPICAL OCULAR DRUG DELIVERY. Drug Deliv. 2000;558–562.

[68] Bennett L. Drug Delivery to Specific Compartments of the Eye. Ocul. Drug Deliv. Adv. Challenges Appl. 2016;37–52.

[69] Rabinovich-guilatt L, Couvreur P, Lambert G, et al. Cationic Vectors in Ocular Drug Delivery. J. Drug Target. 2004;12:623–633.

[70] Barar J, Javadzadeh AR, Omidi Y. Ocular novel drug delivery: Impacts of membranes and barriers. Expert Opin. Drug Deliv. 2008;5:567–581.

[71] Prausnitz MR. Predicted Permeability of the Cornea. Pharm. Res. 2001;18:1497–1508.

[72] Zhang W, Prausnitz MR, Edwards A. Model of transient drug diffusion across cornea. J. Control. Release. 2004;99:241–258.

[73] Prausnitz MR. Permeability of cornea, sciera, and conjunctiva: A literature analysis for drug delivery to the eye. J. Pharm. Sci. 1998;87:1479–1488.

[74] Grass GM, Robinson JR. Mechanisms of corneal drug penetration I: In vivo and in vitro kinetics. J. Pharm. Sci. 1988;77:3–14.

[75] Rodrigues GA, Lutz D, Shen J, et al. Topical Drug Delivery to the Posterior Segment of the Eye: Addressing the Challenge of Preclinical to Clinical Translation. Pharm. Res. 2018;35.

[76] Ramsay E, Ruponen M, Picardat T, et al. Impact of Chemical Structure on Conjunctival Drug Permeability: Adopting Porcine Conjunctiva and Cassette Dosing for Construction of In Silico Model. J. Pharm. Sci. [Internet]. 2017;106:2463–2471. Available from: http://dx.doi.org/10.1016/j.xphs.2017.04.061.

[77] Andrés-Guerrero V, Herrero-Vanrell R. Ocular drug absorption by topical route. Role of conjunctiva. Arch. Soc. Esp. Oftalmol. 2008;83:683–686.

[78] Kim SH, Lutz J, Sun N, et al. Transport Barriers in Transscleral Drug Delivery for Retinal Diseases. Ophthalmic Res. 2007;39:244–254.

[79] Cheruvu NPS, Kompella UB. Bovine and Porcine Transscleral Solute Transport : Influence of Lipophilicity and the Choroid – Bruch ' s Layer. Invest. Ophthalmol. Vis. Sci. 2006;47:2–11.

[80] Dey S, Mitra AK. Transporters and receptors in ocular drug delivery : opportunities and challenges. Expert Opin. Drug Deliv. 2005;2:201–204.

[81] Saba P, Yang JJ, Lee VHL. Existence of a p-Glycoprotein Drug Efflux Pump in Cultured Rabbit Conjunctival Epithelial Cells. 1998;39:3–8.

[82] Karla PK, Earla R, Boddu SH, et al. Molecular Expression and Functional Evidence of a Drug Efflux Pump (BCRP) in. Curr. Eye Res. 2009;34:1–9.

[83] Akbarzadeh A, Rezaei-Sadabady R, Davaran S, et al. Liposome: Classification, preparation, and applications. Nanoscale Res. Lett. [Internet]. 2013;8:1. Available from: Nanoscale Research Letters.

[84] Gómez-Ballesteros M, López-Cano JJ, Bravo-Osuna I, et al. Osmoprotectants in hybrid liposome/HPMC systems as potential glaucoma treatment. Polymers (Basel). 2019;11.

[85] Wendy B. Bollag, Lawrence O. Olala, Ding Xie, Xiaowen Lu, Haixia Qin, Vivek Choudhary, Rachana Patel, David Bogorad, Amy Estes and MW. Dioleoylphosphatidylglycerol Accelerates Corneal Epithelial. 2020;3:1–4.

[86] Thomas AH, Catalá Á, Vignoni M. Soybean phosphatidylcholine liposomes as model membranes to study lipid peroxidation photoinduced by pterin. Biochim. Biophys. Acta - Biomembr. 2016;1858:139–145.

[87] Briuglia ML, Rotella C, McFarlane A, et al. Influence of cholesterol on liposome stability and on in vitro drug release. Drug Deliv. Transl. Res. 2015;5:231–242.

[88] Virden JW, Berg JC. NaCl-induced Aggregation of Dipalmitoylphosphatidylglycerol Small Unilamellar Vesicles with Varying Amounts of Incorporated Cholesterol. Langmuir. 1992;8:1532–1537.

[89] Kreutzberger MA, Tejada E, Wang Y, et al. GUVs Melt Like LUVs: The Large Heat Capacity of MLVs Is Not Due to Large Size or Small Curvature. Biophys. J. [Internet]. 2015;108:2619–2622. Available from: http://dx.doi.org/10.1016/j.bpj.2015.04.034.

[90] Ateeq R, Veikko U, Daniel L. Mini review on emerging methods of preparation of liposome and its application as Liposome drug delivery systems. Open J. Pharmacol. Pharmacother. 2018;3:005–021.

[91] Li Jing, Wang Xuling, Zhang Ting, Wang Chunling, Huang Zhenjun LX, Yihui D. A review on phospholipids and their main applications in drug delivery systems. Asian J. Pharm. Sci. [Internet]. 2014;10:81–98. Available from: http://dx.doi.org/10.1016/j.ajps.2014.09.004.

[92] Bangham AD, Horne RW. Negative staining of phospholipids and their structural modification by surface-active agents as observed in the electron microscope. J. Mol. Biol. [Internet]. 1964;8:660–668. Available from: http://dx.doi.org/10.1016/S0022-2836(64)80115-7.

**** First to discover self- assembly of a lipid mixure into liposome-forming bilayers**

[93] Angelini G, Campestre C, Boncompagni S, et al. Liposomes entrapping β-

cyclodextrin/ibuprofen inclusion complex: Role of the host and the guest on the bilayer integrity and microviscosity. Chem. Phys. Lipids [Internet]. 2017;209:61–65. Available from: https://doi.org/10.1016/j.chemphyslip.2017.09.004.

[94] Deepika Srivastava, Vaseem A. Ansari, Satya Prakash Singh SA and JA. Development of liposomal cosmeceuticals. 2016;8:834–838.

[95] Coatings O, Division P. Preparation of liposomes by reverse-phase evaporation using alternative organic solvents. 1999;16:251–256.

[96] Otake K, Shimomura T, Goto T, et al. Preparation of liposomes using an improved supercritical reverse phase evaporation method. Langmuir. 2006;22:2543–2550.

[97] Kirby C, Gregoriadis G. Dehydration-rehydration vesicles: A simple method for high yield drug entrapment in liposomes. Bio/Technology. 1984;2:979–984.

[98] Rewar S, Singh CJ, Bansal BK, Pareek R SA. A Vital Role of Liposome's on Controlled and Novel Drug Delivery. 2014;5:51–63.

[99] Strauss G, Ingenito EP. Stabilization of liposome bilayers to freezing and thawing: Effects of cryoprotective agents and membrane proteins. Cryobiology. 1980;17:508–515.

[100] Roberto Mendez SB. Sonication-Based Basic Protocol for Liposome Synthesis. Methods Mol. Biol. 1609 [Internet]. 2017. p. 255–260. Available from: http://www.springer.com/series/7651.

[101] Lapinski MM, Castro-Forero A, Greiner AJ, et al. Comparison of liposomes formed by sonication and extrusion: Rotational and translational diffusion of an embedded chromophore. Langmuir. 2007;23:11677–11683.

[102] Jamai-osmania N. Experimental Section Preparation of Large Uni-Lamellar Liposomes by the Ether Injection Method and Evaluation of the Physical Integrity by Osmometry JOHN C MATHAI and V SITARAMAM National Institute o f Nutrition Osmometry of liposomes Results and Discussi. Control. :147–149.

[103] Deamer DW. Preparation and Properties of Ether-Injection Liposomes. Ann. N. Y. Acad. Sci. 1978;308:250–258.

[104] Jaafar-Maalej C, Diab R, Andrieu V, et al. Ethanol injection method for hydrophilic and lipophilic drug-loaded liposome preparation. J. Liposome Res. 2010;20:228–243.

[105] Pons M, Foradada M, Estelrich J. Liposomes obtained by the ethanol injection method. Int. J. Pharm. 1993;95:51–56.

[106] J.S. Dua, Prof. A. C. Rana DAKB. LIPOSOME: METHODS OF PREPARATION AND APPLICATIONS. Int. J. Pharm. Stud. Res. 2012;3:14–20.

[107] Miller DC, Dahl GP. Early events in calcium-induced liposome fusion. BBA - Biomembr. 1982;689:165–169.

[108] Bo Y, Lee RJ, Lee LJ. Microfluidic Methods for Production of Liposomes. 2009;465:129–141.

[109] Fan M, Xu S, Xia S, et al. Effect of different preparation methods on physicochemical properties of salidroside liposomes. J. Agric. Food Chem. 2007;55:3089–3095.

[110] Schaeffer HE, Krohn DL. Liposomes in topical drug delivery. Investig. Ophthalmol. Vis. Sci. 1982;22:220–227.

[111] Soriano-Romaní L, Vicario-de-la-Torre M, Crespo-Moral M, et al. Novel anti-inflammatory liposomal formulation for the pre-ocular tear film: In vitro and ex vivo functionality studies in corneal epithelial cells. Exp. Eye Res. [Internet]. 2017;154:79–87. Available from: http://dx.doi.org/10.1016/j.exer.2016.11.010.

[112] Agarwal R, Iezhitsa I, Agarwal P, et al. Liposomes in topical ophthalmic drug delivery: an update. Drug Deliv. 2014;7544:1–17.

[113] Natarajan J V., Ang M, Darwitan A, et al. Nanomedicine for glaucoma: Liposomes provide sustained release of latanoprost in the eye. Int. J. Nanomedicine. 2012;7:123–131.

[114] Elsana H, Olusanya TOB, Carr-wilkinson J, et al. Evaluation of novel cationic gene based liposomes with cyclodextrin prepared by thin film hydration and microfluidic systems. Sci. Rep. [Internet]. 2019;9:1–17. Available from: http://dx.doi.org/10.1038/s41598-019-51065-4.

[115] Mui BL, Döbereiner HG, Madden TD, et al. Influence of transbilayer area asymmetry on the morphology of large unilamellar vesicles. Biophys. J. 1995;69:930–941.

[116] Klymchenko AS, Oncul S, Didier P, et al. Visualization of lipid domains in giant unilamellar vesicles using an environment-sensitive membrane probe based on 3-hydroxyflavone. Biochim. Biophys. Acta - Biomembr. [Internet]. 2009;1788:495–499. Available from: http://dx.doi.org/10.1016/j.bbamem.2008.10.019.

[117] Bibi S, Kaur R, Henriksen-Lacey M, et al. Microscopy imaging of liposomes: From coverslips to environmental SEM. Int. J. Pharm. [Internet]. 2011;417:138–150. Available from: http://dx.doi.org/10.1016/j.ijpharm.2010.12.021.

[118] Robson AL, Dastoor PC, Flynn J, et al. Advantages and limitations of current imaging techniques for characterizing liposome morphology. Front. Pharmacol. 2018;9:1–8.

[119] Ruozi B, Belletti D, Tombesi A, et al. AFM, ESEM, TEM, and CLSM in liposomal characterization: a comparative study. Int. J. Nanomedicine. 2011;6:557–563.

[120] Bendas G, Krause A, Bakowsky U, et al. Targetability of novel immunoliposomes prepared by a new antibody conjugation technique. Int. J. Pharm. 1999;181:79–93.

[121] Moutardier V, Tosini F, Vlieghe P, et al. Colloidal anticancer drugs bioavailabilities in oral administration models. Int. J. Pharm. 2003;260:23–38.

[122] Davies NM, Fair SJ, Hadgraft J, et al. Evaluation of Mucoadhesive Polymers in Ocular Drug Delivery. I. Viscous Solutions. Pharm. Res. An Off. J. Am. Assoc. Pharm. Sci. 1991. p. 1039–1043.

[123] Tiffany JM, Winter N, Bliss G. Tear film stability and tear surface tension. Curr. Eye Res. 1989;8:507–515.

[124] Hotujac Grgurević M, Juretić M, Hafner A, et al. Tear fluid-eye drops compatibility assessment using surface tension. Drug Dev. Ind. Pharm. [Internet]. 2017;43:275–282. Available from: http://dx.doi.org/10.1080/03639045.2016.1238924.

[125] Holly FJ, Lemp MA. Tear physiology and dry eyes. Surv. Ophthalmol. 1977;22:69–87.

[126] Stahl U, Willcox M, Stapleton F. Osmolality and tear film dynamics. Clin. Exp. Optom. 2012;95:3–11.

[127] Foster JB, Lee WB. The Tear Film: Anatomy, Structure and Function [Internet]. Ocul. Surf. Dis. Cornea, Conjunctiva Tear Film. Elsevier Inc.; 2013. Available from: http://dx.doi.org/10.1016/B978-1-4557-2876-3.00003-1.

[128] Merayo Lloves J, Benítez del Castillo Sanchez JM, Montero Iruzubieta J, et al. Guías Españolas para el tratamiento de la Enfermedad de Ojo Seco [Internet]. 2017. Available from: http://www.lasuperficieocular.com/resources/documents/guias_ojo_seco_SESOC_THEA.pdf.

[129] Vicario-de-la-Torre M, Caballo-González M, Vico E, et al. Novel Nano-liposome formulation for dry eyes with components similar to the preocular tear film. Polymers (Basel). 2018;10:1–13.

[130] Mark B. Abelson, MD; Ira J. Udell MJHW. Normal human Tear pH by Direct Measurement. Arch. Oftalmol. 1981;99:301–301.

[131] Coles WH, Jaros PA. Dynamics of ocular surface pH. Br. J. Ophthalmol. 1984;68:549–552.

[132] Dimov N, Kastner E, Hussain M, et al. Formation and purification of tailored liposomes for drug delivery using a module-based micro continuous-flow system. Sci. Rep. 2017;7:1–13.

[133] Lin M, Qi X-R. Purification Method of Drug-Loaded Liposome. 2019;1–11.

[134] Abdelwahed W, Degobert G, Stainmesse S, et al. Freeze-drying of nanoparticles: Formulation, process and storage considerations. Adv. Drug Deliv. Rev. 2006;58:1688–1713.

[135] Obata Y, Saito S, Takeda N, et al. Plasmid DNA-encapsulating liposomes: Effect of a spacer between the cationic head group and hydrophobic moieties of the lipids on gene expression efficiency. Biochim. Biophys. Acta - Biomembr. [Internet]. 2009;1788:1148–1158. Available from: http://dx.doi.org/10.1016/j.bbamem.2009.02.014.

[136] Singh B, Mundlamuri R, Friese T, et al. Benchmarking of sterilizing-grade filter membranes with liposome filtration. PDA J. Pharm. Sci. Technol. 2017;72:223–235.

[137] Toh M-R, Chiu GNC. Liposomes as sterile preparations and limitations of sterilisation techniques in liposomal manufacturing. Asian J. Pharm. Sci. [Internet]. 2013;8:88–95. Available from: http://dx.doi.org/10.1016/j.ajps.2013.07.011.

[138] Mozafari MR. Vesicular phospholipid gels. Liposomes, Methods Mol. Biol. 2010. p. 205–212.

[139] Ratz H, Freise J, Magerstedt P, et al. Sterilization of contrast media (isovist) containing liposomes by ethylene oxide. J. Microencapsul. 1989;6:485–492.

[140] Nicolas J. Zuidam, Stephen S. L. Lee DJAC. Sterilization of Liposomes by Heat Treatment. Pharm. Res. 1993;10:1591–1596.

[141] Çağdaş, M., Sezer, A. D., & Bucak S. Liposomes as Potential Drug Carrier Systems for Drug Delivery. Appl. Nanotechnol. Drug Deliv. 2014. p. 1–100.

[142] Kaur IP, Garg A, Singla AK, et al. Vesicular systems in ocular drug delivery : an overview. Int. J. Pharm. 2004;269:1–14.

[143] Ebrahim S, Peyman GA, Lee PJ. Applications of liposomes in ophthalmology. Surv. Ophthalmol. 2005;50:167–182.

[144] Torchilin VP. Recent advances with liposomes as pharmaceutical carriers. Nat. Rev. Drug Discov. 2005;4:145–160.

[145] Bhattacharjee A, Das PJ, Adhikari P, et al. Novel drug delivery systems for ocular therapy : With special reference to liposomal ocular delivery. Eur. J. Ophthalmol. 2019;29:113–126.

[146] Alavi M, Karimi N, Safaei M. Application of various types of liposomes in drug delivery systems. Adv. Pharm. Bull. 2017;7:3–9.

[147] Gonzalez Gomez A, Syed S, Marshall K, et al. Liposomal Nanovesicles for Efficient Encapsulation of Staphylococcal Antibiotics. ACS Omega. 2019;4:10866–10876.

[148] Assil KK, Frucht-Perry J, Ziegler E, et al. Tobramycin liposomes: Single subconjunctival therapy of pseudomonal keratitis. Investig. Ophthalmol. Vis. Sci. 1991;32:3216–3220.

[149] Dubald M, Bourgeois S, Andrieu V, et al. Ophthalmic drug delivery systems for antibiotherapy- A review. Pharmaceutics. 2018;10:10.

[150] Mehanna MM, Elmaradny HA, Samaha MW. Ciprofloxacin liposomes as vesicular reservoirs for ocular delivery: Formulation, optimization, and in vitro characterization. Drug Dev. Ind. Pharm. 2009;35:583–593.

[151] Budai L, Hajdú M, Budai M, et al. Gels and liposomes in optimized ocular drug delivery: Studies on ciprofloxacin formulations. Int. J. Pharm. 2007;343:34–40.

[152] Taha EI, El-Anazi MH, El-Bagory IM, et al. Design of liposomal colloidal systems for ocular delivery of ciprofloxacin. Saudi Pharm. J. [Internet]. 2014;22:231–239. Available from: http://dx.doi.org/10.1016/j.jsps.2013.07.003.

[153] Ren T, Lin X, Zhang Q, et al. Encapsulation of Azithromycin Ion Pair in Liposome for Enhancing Ocular Delivery and Therapeutic Efficacy on Dry Eye. Mol. Pharm. 2018;15:4862–4871.

[154] Fresta M, Panico AM, Bucolo C, et al. Characterization and In-vivo Ocular Absorption of Liposome-encapsulated Acyclovir. J. Pharm. Pharmacol. 1999;51:565–576.

[155] Law SL, Huang KJ, Chiang CH. Acyclovir-containing liposomes for potential ocular delivery Corneal penetration and absorption. J. Control. Release. 2000;63:135–140.

[156] Shen Y, Tu J. Preparation and Ocular Pharmacokinetics of Ganciclovir Liposomes. AAPS J. 2007;9:E371.

[157] Chetoni, P., Monti, D., Tampucci, S., Matteoli, B., Ceccherini-Nelli, L., Subissi, A., & Burgalassi S, Burgalassi S. Liposomes as a potential ocular delivery system of distamycin A. Int. J. Pharm. [Internet]. 2015;492:120–126. Available from: http://dx.doi.org/10.1016/j.ijpharm.2015.05.055.

[158] Augusto F, Sá P De, Fleury S, et al. Liposomal voriconazole (VOR) formulation for

improved ocular delivery. Colloids Surfaces B Biointerfaces [Internet]. 2015;133:331–338. Available from: http://dx.doi.org/10.1016/j.colsurfb.2015.06.036.

[159] Zhang Z, Teng F, Sun Q, et al. Rapamycin liposome gutta inhibiting fungal keratitis of rats. Int. J. Ophthalmol. 2019;12:536–541.

[160] Habib FS, Fouad EA, Abdel-Rhaman MS, et al. Liposomes as an ocular delivery system of fluconazole: In-vitro studies. Acta Ophthalmol. 2010;88:901–904.

[161] Abdel-Rhaman MS, Soliman W, Habib F, et al. A new long-acting liposomal topical antifungal formula: Human clinical study. Cornea. 2012;31:126–129.

[162] Morand K, Bartoletti AC, Bochot A, et al. Liposomal amphotericin B eye drops to treat fungal keratitis : Physico-chemical and formulation stability. Int. J. Pharm. 2007;344:150–153.

[163] Li H, Liu Y, Zhang Y, et al. Liposomes as a Novel Ocular Delivery System for Brinzolamide : In Vitro and In Vivo Studies. AAPS PharmSciTech [Internet]. 2016;17:710–717. Available from: http://dx.doi.org/10.1208/s12249-015-0382-1.

[164] Altamirano-vallejo JC, Navarro-partida J, Rosa AG, et al. Characterization and Pharmacokinetics of Triamcinolone Acetonide-Loaded Liposomes Topical Formulations. J. Ocul. Pharmacol. Ther. 2018;00:416–425.

[165] Rosa AG, Navarro-partida J, Altamirano-vallejo JC, et al. Novel Triamcinolone Acetonide-Loaded Liposomes Topical Formulation for the Treatment of Cystoid Macular Edema After Cataract Surgery : A Pilot Study. J. Ocul. Pharmacol. Ther. 2019;35:1–10.

[166] Soriano-Romaní L, Álvarez-Trabado J, López-García A, et al. Improved in vitro corneal delivery of a thrombospondin-1-derived peptide using a liposomal formulation. Exp. Eye Res. [Internet]. 2018;167:118–121. Available from: https://doi.org/10.1016/j.exer.2017.12.002.

[167] Lai S, Wei Y, Wu Q, et al. Liposomes for effective drug delivery to the ocular posterior chamber. J. Nanobiotechnology [Internet]. 2019;17:1–12. Available from: https://doi.org/10.1186/s12951-019-0498-7.

[168] Muthu MS, Singh S. Targeted nanomedicines: Effective treatment modalities for cancer, AIDS and brain disorders. Nanomedicine. 2009;4:105–118.

[169] Zylberberg C, Gaskill K, Pasley S, et al. Engineering liposomal nanoparticles for targeted gene therapy. Gene Ther. [Internet]. 2017;24:441–452. Available from: http://dx.doi.org/10.1038/gt.2017.41.

[170] Fotoran WL, Santangelo R, de Miranda BNM, et al. DNA-Loaded Cationic Liposomes Efficiently Function as a Vaccine against Malarial Proteins. Mol. Ther. - Methods Clin. Dev. [Internet]. 2017;7:1–10. Available from: https://doi.org/10.1016/j.omtm.2017.08.004.

[171] Du JD, Fong WK, Caliph S, et al. Lipid-based drug delivery systems in the treatment of wet age-related macular degeneration. Drug Deliv. Transl. Res. [Internet]. 2016;6:781–792. Available from: http://dx.doi.org/10.1007/s13346-016-0299-6.

[172] Ohigashi H, Hashimoto D, Hayase E, et al. Ocular instillation of Vitamin A–coupled liposomes containing HSP47 siRNA ameliorates dry eye syndrome in chronic GVHD.

Blood Adv. 2019;3:1003–1010.

[173] Jiang J, Zhang X, Tang Y, et al. Progress on ocular siRNA gene-silencing therapy and drug delivery systems. Fundam. Clin. Pharmacol. 2020;1–21.

[174] Malta JB, Soong HK, Shtein RM, et al. Treatment of ocular graft-versus-host disease with topical cyclosporine 0.05%. Cornea. 2010;29:1392–1396.

[175] Tulio B Abud, Francisco Amparo, Ujwala S Saboo, Antonio Di Zazzo Thomas H Dohlman, Joseph B Ciolino, Pedram Hamrah and RD. A Clinical Trial Comparing the Safety and Efficacy of Topical Tacrolimus versus Methylprednisolone in Ocular Graft-Versus- Host Disease. Physiol. Behav. 2016;123:1449–1457.

[176] Li XQ, Büch G, Otasevic L, et al. Prolongation of corneal allograft survival by topical application of everolimus in experimental keratoplasty. Ophthalmic Res. 2008;40:309–314.

[177] Dai Y, Zhou R, Liu L, et al. Liposomes containing bile salts as novel ocular delivery systems for tacrolimus (FK506): In vitro characterization and improved corneal permeation. Int. J. Nanomedicine. 2013;8:1921–1933.

[178] Karn PR, Do Kim H, Kang H, et al. Supercritical fluid-mediated liposomes containing cyclosporin A for the treatment of dry eye syndrome in a rabbit model: Comparative study with the conventional cyclosporin A emulsion. Int. J. Nanomedicine. 2014;9:3791–3800.

[179] Santos A, Altamirano-vallejo JC, Navarro-partida J, et al. Breaking down the Barrier: Topical Liposomes as Nanocarriers for Drug Delivery into the Posterior Segment of the Eyeball. Role Nov. Drug Deliv. Veh. Nanobiomedicine. IntechOpen. 2019.

[180] Hathout RM, Mansour S, Mortada ND, et al. Liposomes as an ocular delivery system for acetazolamide: In vitro and in vivo studies. AAPS PharmSciTech. 2007;8.

[181] Elbialy NS, Abdol-Azim BM, Shafaa MW, et al. Enhancement of the ocular therapeutic effect of prednisolone acetate by liposomal entrapment. J. Biomed. Nanotechnol. 2013;9:2105–2116.

[182] Shimokawa T, Fukuta T, Inagi T, et al. Protective effect of high affinity liposomes encapsulating astaxanthin against corneal disorder in the in vivo rat dry eye disease model. J. Clin. Biochem. Nutr. 2020;66:224–232.

[183] Mehanna MM, Elmaradny HA, Samaha MW. Mucoadhesive liposomes as ocular delivery system: Physical, microbiological, and in vivo assessment. Drug Dev. Ind. Pharm. 2010;36:108–118.

[184] Chen H, Pan H, Li P, et al. The potential use of novel chitosan-coated deformable liposomes in an ocular drug delivery system. Colloids Surfaces B Biointerfaces [Internet]. 2016;143:455–462. Available from: http://dx.doi.org/10.1016/j.colsurfb.2016.03.061.

[185] Tan G, Yu S, Pan H, et al. Bioadhesive chitosan-loaded liposomes: A more efficient and higher permeable ocular delivery platform for timolol maleate. Int. J. Biol. Macromol. [Internet]. 2017;94:355–363. Available from: http://dx.doi.org/10.1016/j.ijbiomac.2016.10.035.

[186] Li J, Cheng T, Tian Q, et al. A more efficient ocular delivery system of triamcinolone

acetonide as eye drop to the posterior segment of the eye. Drug Deliv. 2019;26:188–198.

[187] Cheng T, Li J, Cheng Y, et al. Triamcinolone acetonide-chitosan coated liposomes efficiently treated retinal edema as eye drops. Exp. Eye Res. 2019;188:107805.

[188] Khalil M, Hashmi U, Riaz R, et al. Chitosan coated liposomes (CCL) containing triamcinolone acetonide for sustained delivery: A potential topical treatment for posterior segment diseases. Int. J. Biol. Macromol. [Internet]. 2020;143:483–491. Available from: https://doi.org/10.1016/j.ijbiomac.2019.10.256.

[189] Li N, Zhuang C, Wang M, et al. Liposome coated with low molecular weight chitosan and its potential use in ocular drug delivery. Int. J. Pharm. 2009;379:131–138.

[190] Li N, Zhuang CY, Wang M, et al. Low molecular weight chitosan-coated liposomes for ocular drug delivery: In vitro and in vivo studies. Drug Deliv. 2012;19:28–35.

[191] Zhang J, Wang S. Topical use of Coenzyme Q10-loaded liposomes coated with trimethyl chitosan: Tolerance, precorneal retention and anti-cataract effect. Int. J. Pharm. 2009;372:66–75.

[192] Zhang J, Liang X, Li X, et al. Ocular delivery of cyanidin-3-glycoside in liposomes and its prevention of selenite-induced oxidative stress. Drug Dev. Ind. Pharm. 2015;42:546–553.

[193] Lin J, Wu H, Wang Y, et al. Preparation and ocular pharmacokinetics of hyaluronan acid-modified mucoadhesive liposomes. Drug Deliv. [Internet]. 2016;23:1144–1151. Available from: http://dx.doi.org/10.3109/10717544.2014.991952.

[194] Moustafa MA, Elnaggar YSR, El-Refaie WM, et al. Hyalugel-integrated liposomes as a novel ocular nanosized delivery system of fluconazole with promising prolonged effect. Int. J. Pharm. [Internet]. 2017;534:14–24. Available from: https://doi.org/10.1016/j.ijpharm.2017.10.007.

[195] Yu S, Wang QM, Wang X, et al. Liposome incorporated ion sensitive in situ gels for opthalmic delivery of timolol maleate. Int. J. Pharm. [Internet]. 2015;480:128–136. Available from: http://dx.doi.org/10.1016/j.ijpharm.2015.01.032.

[196] Quinteros D, Vicario-De-La-Torre M, Andrés-Guerrero V, et al. Hybrid formulations of liposomes and bioadhesive polymers improve the hypotensive effect of the melatonin analogue 5-MCA-NAT in rabbit eyes. PLoS One. 2014;9.

[197] Hosny KM. Ciprofloxacin as ocular liposomal hydrogel. AAPS PharmSciTech. 2010;11:241–246.

[198] Feghhi M, Sharif Makhmalzadeh B, Farrahi F, et al. Anti-microbial Effect and in Vivo Ocular Delivery of Ciprofloxacin-loaded Liposome through Rabbit's Eye. Curr. Eye Res. [Internet]. 2020;1–7. Available from: https://doi.org/10.1080/02713683.2020.1728777.

[199] Ghareb M DF. Development and in vitro/in vivo Evaluation of Liposomal Gels for the Sustained Ocular Delivery of Latanoprost. J. Clin. Exp. Ophthalmol. 2015;06.

[200] Mehanna MM, El-kader NA, Samaha MW. Liposomes as potential carriers for ketorolac ophthalmic delivery : formulation and stability issues. Brazilian J. Pharm. Sci. 2017;53:1–10.

[201] Dong Y, Dong P, Huang D, et al. Fabrication and characterization of silk fibroin-coated liposomes for ocular drug delivery. Eur. J. Pharm. Biopharm. 2015;91:82–90.

[202] Zhan C, Santamaria CM, Wang W, et al. Biomaterials Long-acting liposomal corneal anesthetics. Biomaterials [Internet]. 2018;181:372–377. Available from: https://doi.org/10.1016/j.biomaterials.2018.07.054.

[203] Stapleton F, Alves M, Bunya VY, et al. TFOS DEWS II Epidemiology Report. Ocul. Surf. [Internet]. 2017;15:334–365. Available from: http://dx.doi.org/10.1016/j.jtos.2017.05.003.

[204] Foulks GN. The Correlation Between the Tear Film Lipid Layer and Dry Eye Disease. Surv. Ophthalmol. 2007;52:369–374.

[205] Vicario-De-La-Torre M, Herrero-Vanrell R, Benítez-Del-Castillo JM, et al. New formulations for dry eye treatment. Arch. Soc. Esp. Oftalmol. 2007;82:395–396.

[206] Jones L, Downie LE, Korb D, et al. TFOS DEWS II Management and Therapy Report. Ocul. Surf. [Internet]. 2017;15:575–628. Available from: http://dx.doi.org/10.1016/j.jtos.2017.05.006.

[207] Moshirfar M, Pierson K, Hanamaikai K, et al. Artificial tears potpourri: A literature review. Clin. Ophthalmol. 2014;8:1419–1433.

[208] Weisenberger K, Fogt N, Swingle Fogt J. Comparison of nanoemulsion and non-emollient artificial tears on tear lipid layer thickness and symptoms. J. Optom. [Internet]. 2020; Available from: https://doi.org/10.1016/j.optom.2020.03.002.

[209] Aguilar AJ, Marquez MI, Albera PA, et al. Effects of systane® balance on noninvasive tear film Break-Up time in patients with Lipid-Deficient dry eye. Clin. Ophthalmol. 2014;8:2365–2372.

[210] Pucker AD, McGwin G, Franklin QX, et al. Evaluation of Systane Complete for the Treatment of Contact Lens Discomfort. Contact Lens Anterior Eye [Internet]. 2019;1–7. Available from: https://doi.org/10.1016/j.clae.2019.10.141.

[211] Lallemand F, Daull P, Benita S, et al. Successfully Improving Ocular Drug Delivery Using the Cationic Nanoemulsion, Novasorb. J. Drug Deliv. 2012;2012:1–16.

[212] Robert PY, Cochener B, Amrane M, et al. Efficacy and safety of a cationic emulsion in the treatment of moderate to severe dry eye disease: A randomized controlled study. Eur. J. Ophthalmol. 2016;26:546–555.

[213] Georgiev GA, Yokoi N, Nencheva Y, et al. Surface chemistry interactions of cationorm with films by human meibum and tear film compounds. Int. J. Mol. Sci. 2017;18.

[214] Baudouin C, De La Maza MS, Amrane M, et al. One-year efficacy and safety of 0.1% cyclosporine a cationic emulsion in the treatment of severe dry eye disease. Eur. J. Ophthalmol. 2017;27:678–685.

[215] Craig JP, Purslow C, Murphy PJ, et al. Effect of a liposomal spray on the pre-ocular tear film. Contact Lens Anterior Eye [Internet]. 2010;33:83–87. Available from: http://dx.doi.org/10.1016/j.clae.2009.12.007.

[216] https://www.esteve.com/es/areas-terapeuticas/productos-sin-receta [Internet]. Available from: https://www.esteve.com/es/areas-terapeuticas/productos-sin-receta.

[217] Meng T, Kulkarni V, Simmers R, et al. Therapeutic implications of nanomedicine for ocular drug delivery. Drug Discov. Today [Internet]. 2019;24:1524–1538. Available from: https://doi.org/10.1016/j.drudis.2019.05.006.

[218] Vicario-de-la-Torre M, Benítez-del-Castillo JM, Vico E, et al. Design and characterization of an ocular topical liposomal preparation to replenish the lipids of the tear film. Investig. Ophthalmol. Vis. Sci. 2014;55:7839–7847.

[219] Acar D, Molina-Martínez IT, Gómez-Ballesteros M, et al. Novel liposome-based and in situ gelling artificial tear formulation for dry eye disease treatment. Contact Lens Anterior Eye [Internet]. 2018;41:93–96. Available from: http://dx.doi.org/10.1016/j.clae.2017.11.004.

[220] Zhang W, Li W, Zhang C, et al. Effects of vitamin A on expressions of apoptosis genes bax and bcl-2 in epithelial cells of corneal tissues induced by benzalkonium chloride in mice with dry eye. Med. Sci. Monit. 2019;25:4583–4589.

[221] He W, Guo X, Feng M, et al. In vitro and in vivo studies on ocular vitamin A palmitate cationic liposomal in situ gels. Int. J. Pharm. [Internet]. 2013;458:305–314. Available from: http://dx.doi.org/10.1016/j.ijpharm.2013.10.033.

[222] Barabino S, Horwath-winter J, Messmer EM, et al. The role of systemic and topical fatty acids for dry eye treatment. Prog. Retin. Eye Res. [Internet]. 2017; Available from: http://dx.doi.org/10.1016/j.preteyeres.2017.05.003.

[223] Li Z, Choi J, Oh H, et al. Effects of Eye Drops Containing a Mixture of Omega-3 Essential Fatty Acids and Hyaluronic Acid on the Ocular Surface in Desiccating Stress-induced Murine Dry Eye Effects of Eye Drops Containing a Mixture of Omega-3 Essential Fatty Acids and Hyaluronic Ac. 2014;3683.

[224] Rashid S, Jin Y, Ecoiffier T, et al. Topical Omega-3 and Omega-6 Fatty Acids for Treatment of Dry Eye. 2015;126:219–225.

[225] Ivanova S, Tonchev V, Yokoi N, et al. Surface properties of squalene/meibum films and NMR confirmation of squalene in tears. Int. J. Mol. Sci. 2015;16:21813–21831.

[226] Grit M, Crommelin DJA. Chemical stability of liposomes" implications for their physical stability. Chem. Phys. Lipids. 1992;64:3–18.

[227] Klingeborn M, Dismuke WM, Bowes Rickman C, et al. Roles of exosomes in the normal and diseased eye. Prog. Retin. Eye Res. 2017;59:158–177.

[228] Yu B, Li X-R, Zhang X-M. Mesenchymal stem cell-derived extracellular vesicles as a new therapeutic strategy for ocular diseases. World J. Stem Cells. 2020;12:178–187.

[229] Samaeekia R, Rabiee B, Putra I, et al. Effect of human corneal mesenchymal stromal cell-derived exosomes on corneal epithelial wound healing. Investig. Ophthalmol. Vis. Sci. 2018;59:5194–5200.

***Highlights the potential use of mesenchymal derived exosomal vesicles to accelerate corneal regeneration**

[230] Urtti A. Comment on " Topical Delivery of Avastin to the Posterior Segment of the Eye In Vivo Using Annexin A5-Associated Liposomes ": Topical Liposomal Bevacizumab Results in Negligible Retinal Concentrations. Small. 2019;15:1805199.

.This article demonstrates that topical liposomal anti-VEGF therapy could replace repeated intravitreal administration presenting a comparable efficacy**