Recent developments in liposomal drug delivery systems for the treatment of retinal diseases

Andrew J. Urquhart[1,2] and Anne Z. Eriksen[1]

[1] Department of Health Technology, Technical University of Denmark, 2800 Kongens Lyngby, Denmark
[2] Center for Nanomedicine and Theranostics, Technical University of Denmark, 2800 Kongens Lyngby, Denmark

Diseases of the retina cause vision loss and blindness, which have a profound impact on an individual's quality of life. The number of therapies available to treat retinal diseases is limited. Nanoparticle (NP)-based medicines represent one strategy to expand both the number of available therapies and the range of retinal diseases treated. Liposomes, phospholipid vesicles that frequently contain cholesterol and/or modified surface chemistries, have already had minor success in retinal disease treatment and hold significant promise. Here, we provide a snapshot of recent research developments in liposomal drug delivery systems for retinal diseases and discuss the challenges associated with liposomal systems in the context of recent developments.

Introduction

Diseases of the retina remain challenging to effectively treat. These challenges can be simplified into two points: (i) multiple disease factors contribute to the development of many retina diseases (e.g., genetic, mitochondrial dysfunction, activation of apoptotic signals, oxidative stress, growth and neurotrophic factor deprivation, etc. as an example please see REF [1]) and (ii) the eye comprises many barriers (e.g., tear film, conjunctiva, vitreous, inner blood–retina barrier, etc.) and clearance mechanisms (diffusion, advection, and active transport) for therapeutics [2]. NP-based strategies to treat diseases have attracted considerable attention in drug delivery research. Liposomes are phospholipid vesicles that represent one chemical branch of NP-based strategies, as reviewed elsewhere [3,4]. Briefly, the structure of liposomes enables the loading of both hydrophilic and hydrophobic therapeutics, whereas the aqueous core permits the loading of hydrophilic molecules by passive and remote loading methods. The phospholipid bilayer allows the loading of hydrophobic molecules by passive or postinsertion methods. Surface-modified liposomes with diameters <150 nm have shown enhanced residency/circulation time *in vivo*. Liposomes with diameters <200 nm can be readily endocytosed by cells, albeit depending on surface chemistry and cell type. As a general rule, liposomes show limited immunogenicity and low toxicity, with phospholipid constituents being readily metabolized once the liposome has disintegrated.

The ability to load multiple therapeutics into liposomes while exploiting both intracellular drug release and the ability to bypass barriers is a potential solution to the two main challenges facing the treatment of retinal diseases. Low toxicity, limited immunogenicity, and processing of NP constituents is crucial for tissues that rely on the fine control of homeostasis to function effectively (i.e., the retina and other central nervous system tissues). Liposomes with nanoscale diameters (<200 nm) have an established track record for clinical approval, particularly for the delivery of chemotherapeutics [5]. For the treatment of retinal diseases, only Visudyne, a nanoscale liposomal formulation of verteporfin, for wet age-related macular degeneration (AMD) is US Food and Drug Administration (FDA) approved [5]. The nanoscale liposomal formulation of amphotericin B, Ambisome, is used as an off-label treatment for fungal endophthalmitis [6].

Given that the liposome structure is driven by noncovalent intermolecular interactions, the chemical constituents and manufacturing methods used to produce liposomes substantially impact liposome properties [3,4]. For example: (i) the hydrophobic tail length of the phospholipid influences bilayer thickness, rigidity, and phase dynamics; (ii) tail stereochemistry influences pack-

Corresponding author: Urquhart, A.J. (anur@nanotech.dtu.dk)

https://doi.org/10.1016/j.drudis.2019.04.004

ing density, bilayer rigidity, and phase dynamics; (iii) incorporating nonphospholipid hydrophobic small molecules into the bilayer will alter phospholipid packing, bilayer rigidity, and phase dynamics; (iv) liposome diameter influences bilayer rigidity; (v) liposome lamellarity changes the volume:volume ratios of the aqueous core to lipid bilayer(s); and (vi) liposome lamellarity influences diameter [7]. Given these features, liposomes represent a complex NP where thermodynamics, spatial and mechanical aspects have a significant effect on both bench top and *in vivo* properties. Here, we present recent research developments in the field of liposomes for the treatment of retina diseases, covering a range of liposome systems, relevant results, and current challenges in the field.

Delivery of small-molecule therapeutics

Liposomal delivery of small-molecule therapeutics remains of major interest because of both the wealth of molecules available and the range of therapeutic targets. Liposomal therapeutics are administered via several different routes. Intravitreal administration is frequently used in research and the clinic to bypass the anterior eye barriers. This maximizes the dose of drug reaching the posterior segment of the eye, particularly the inner retina (Fig. 1) [8]. The role that liposomal properties have in liposome biodistribution after intravitreal administration remains a point of discussion, particularly in terms of which properties improve the passive targeting of the retina. Recently, Lee *et al.* reported that cationic liposomes with low zeta potentials ($< +20$ mV) and polyethylene glycol (PEG) surface modification (i.e., PEGylated) readily diffused to the murine retina, whereas PEGylated or nonPEGylated cationic liposomes with large zeta potentials ($> +20$ mV) were trapped in the vitreous [9]. The vitreous is hydrogel comprising water and a network of collagen fibers separated by glycosaminoglycans [10]. It has been proposed as a diffusion barrier to NPs depending on the size and surface charge of the NPs [11]. Eriksen *et al.* also reported the diffusion of PEGylated low-charge cationic liposomes through the porcine vitreous, albeit with cationic liposomes showing slower rates of diffusion than neutral or anionic liposomes [10]. Lee *et al.* reported that only PEGylated liposomes with a low cationic charge distributed throughout the murine vitreous, but these data were recorded 60 h post injection. Eriksen *et al.* reported that PEGylated liposome distribution across the porcine vitreous was independent of liposome zeta potential, although data were taken 2 h post injection [9,10]. Lee *et al.* did not explore negative zeta potential liposomes in their study. However, in both studies, the liposomes had similar phospholipid compositions and sizes. The liposomes developed by Eriksen *et al.* were loaded with two fluorophores (one in the bilayer and one in the core) to allow colocalization of fluorescence, whereas Lee *et al.* used a single bilayer-loaded fluorophore. The differences between species (e.g., intraocular volume, network density, etc.) and timeframes present challenges to drawing conclusions between the works.

Lee *et al.* also reported that hydrophilic fluorophores loaded into the aqueous core of liposomes did not penetrate the retina, whereas lipid-conjugated fluorophores loaded into the bilayer penetrated the retina [9]. Lipid-conjugated fluorophores with C_{16} lipid chains showed higher concentrations in the outer retina than either C_{14}- or C_{18}-conjugated chains [9]. This observation is challenging to explain. It could be speculated using molecular weight that C_{14} lipids would diffuse faster through the retina but it may be that proteins such as albumin preferentially carry C_{16} lipids [12]. In all these cases, lipid-conjugated fluorophores were loaded

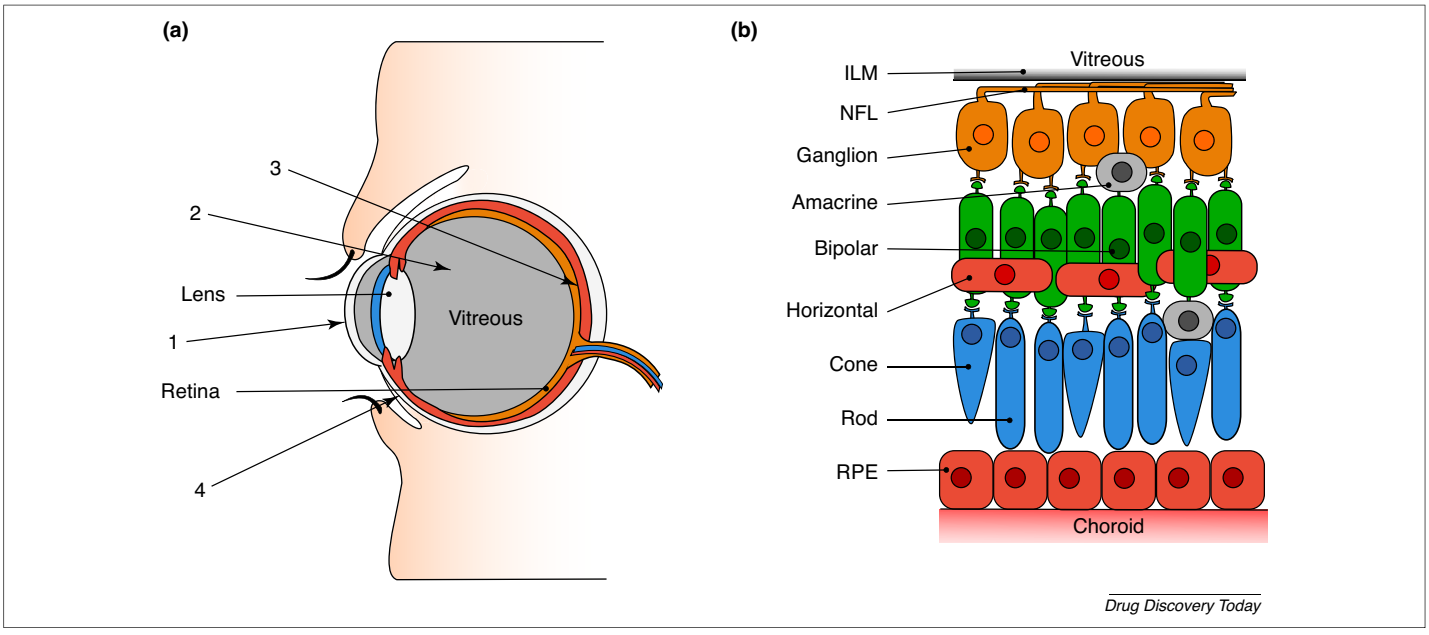

FIGURE 1

Simplified schematic diagram of the structure of the eye. (a) Schematic of the whole eye showing four routes of drug administration,:(1) topical, (2) intravitreal, (3) subretinal, and (4) subconjunctival; (b) Schematic of the retina showing cell layers, choroid, nerve fiber layer (NFL), and vitreous. The inner limiting membrane (ILM) is located between the vitreous and NFL, whereas the basement membrane (BM) is located between the choroid and retinal pigment epithelium (RPE). The inner retina is the region between the ILM and horizontal cells. The outer retina covers photoreceptors to the BM. Subretinal and systemic administration of liposomes is used to target the outer retina, whereas intravitreal administration is used to target the inner retina.

into liposomes comprising C_{14} lipid chains. To explain the fluorophore observations, the authors suggested that lipid transport was facilitated by extracellular vesicles secreted by retinal cells. Blocking extracellular vesicle formation through inhibitors greatly reduced the depth of lipid-conjugated fluorophore penetration into the retina [9]. However, these experiments did not determine whether the liposomes were intact or whether only the fluorophore was transported. Lipid-conjugated fluorophores can partition out of the liposome bilayer and into biological hydrophobic domains (e.g., cell membranes, carrier protein cores, etc.) if the thermodynamics are favorable. When designing liposomes, care has to be taken that lipid chain lengths and chain length stereochemistry are similar to avoid packing defects that might facilitate lipid partitioning. Equally, the bilayer phase and phase transition temperatures also affect partitioning. Liposomes in the liquid disordered phase at physiological temperature (e.g., C_{14} lipids) have less-stable bilayers and this can increase the probability of bilayer constituents escaping into biological hydrophobic domains (Fig. 2). The observation of little to no colocalization of hydrophilic versus hydrophobic fluorophore retina penetration would imply that intact liposomes either did not penetrate the retina or disintegrated into their constituents. The inner limiting membrane (ILM) is a proteinaceous border between the vitreous and retina with a proposed pore size of ~10 nm in humans [13]. There is no clear consensus about whether NPs can diffuse intact through the ILM, although small and macromolecules are able to do so. A liposome trapped at the ILM can disintegrate, allowing its constituents to diffuse towards and through the retina. There is a strong argument when fluorophore labeling liposomes to use the colocalization of two fluorophores, one membrane bound and one core loaded, to ensure liposome structure remains intact during transport [10]. However, further work is required to determine whether biological vesicles transport intact liposomes or liposome constituents. Interestingly, the intercellular space between neuroretinal cells has been reported to be <20 nm [14], but both exosomes (diameters >40 nm) [15] and liposomes are larger than these spaces.

Phospholipid structure has also been reported to influence the therapeutic efficacy of liposomes after intravitreal administration. Hironaka *et al.* showed that edaravone-loaded egg L--phosphatidylcholine (EPC) liposomes significantly protected retinal ganglion cells (RGCs) in an excitotoxin murine model of RGC apoptosis. However, edaravone-loaded distearoyl phosphatidylcholine (DSPC) liposomes showed only minor RGC protection [16]. It was suggested that liposomes with a fluidic rather than a rigid bilayer were more favorable for the treatment of retinal diseases with oxidative stress as a pathophysiological factor. This work highlights the challenges associated with using liposomes as NPs. Edaravone was remote loaded into liposomes but with low encapsulation efficiencies regardless of the phospholipid used. Remote loading is adopted as a method to ensure the high encapsulation efficiencies of hydrophilic molecules [3,4]. Low encapsulation

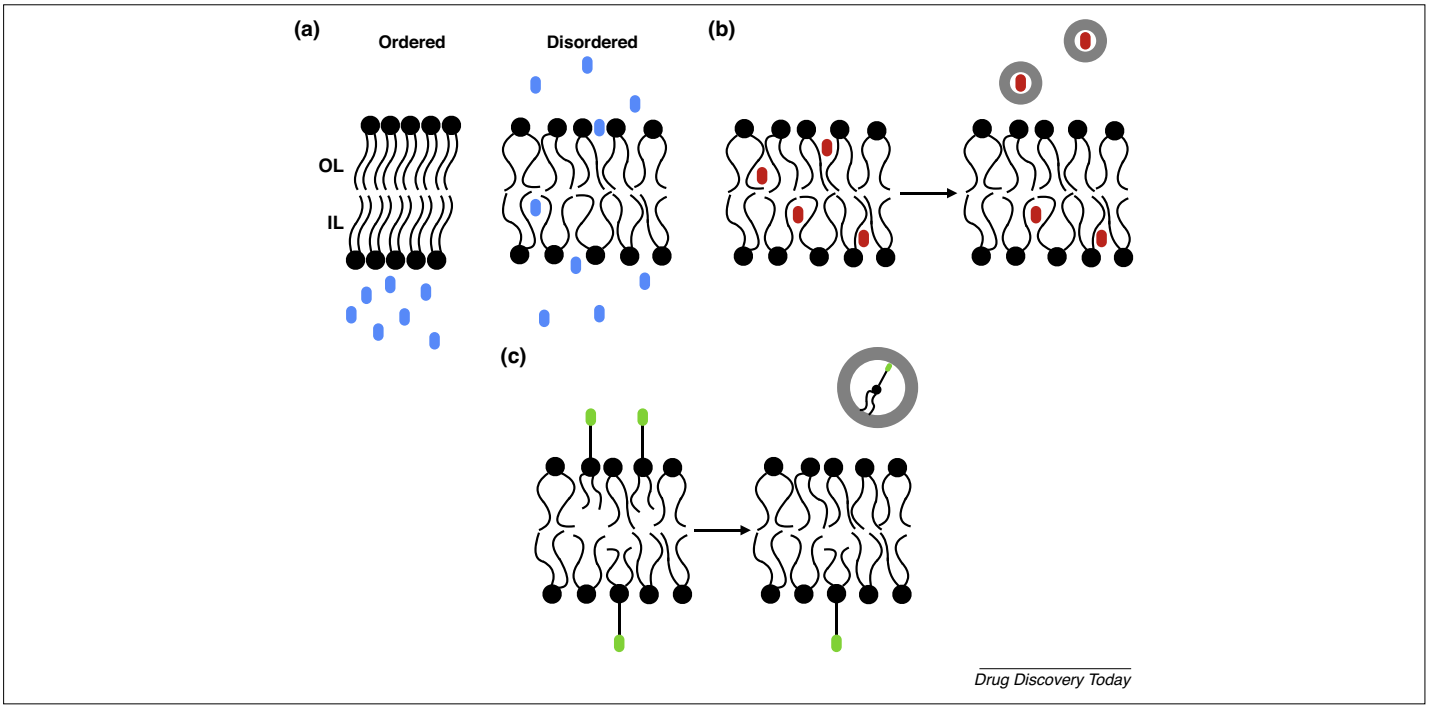

FIGURE 2

Simplified schematic diagram of liposome bilayer stability issues. (a) Phospholipids in bilayers can be densely (ordered) or loosely (disordered) packed. Packing governs bilayer rigidity and stability. Small-molecule therapeutics (in blue) loaded in the liposome core show reduced leakage through ordered bilayers than disordered bilayers [outer leaflet (OL) and inner leaflet (IL) are shown]; (b) disordered bilayers create a range of challenges. In an example of small-molecule therapeutics (in red) loaded into the bilayer, poor packing increases the probability of therapeutics partitioning into biological components (gray spheres); (c) Anchoring molecules with lipid tails (in green) of different length to the bulk phospholipid changes packing, which can also increase the probability of molecules partitioning into biological components (gray spheres). Liposome bilayers can rearrange after partitioning to reduce energetics associated with pore and/or hole formation (i.e., anisotropy).

efficiencies can have several causes, but can be generalized as being the result of an unstable load (i.e., drug is not precipitating in the core because the drug concentration is not high enough as a result of drug leaking back across the bilayer) [3]. Edaravone release kinetics for the DSPC liposome systems were faster than for EPC liposomes, which implies that DSPC liposomes had leaked a large amount of drug in the vitreous before reaching the retina. Water-soluble small molecules are rapidly cleared from the vitreous; therefore, it is difficult to determine whether the positive effects observed were simply changes in edaravone pharmacokinetics rather than the result of bilayer rigidity. Intravitreal administration is an invasive procedure that cannot be done with high frequency because of the associated risks. NP studies proposing therapeutic efficacy but where drug half-life is not extended are not clinically viable, which undermines arguments supported by the benefits of the system.

Intravitreal administration has potential risks (e.g., scleral scaring, infection, etc.), which have led to a growing interest in front of the eye administration via eye drops (topical) or subconjunctival injection. However, topically administered therapeutics must overcome many barrier mechanisms [2]. This results in very low drug concentrations at the retina. Topical administration of hydrophobic fluorophore coumarin 6-containing nonPEGylated liposomes to mice showed liposome size-dependent fluorescent intensity in the retina [17]. Liposomes with increasing diameters (105–600 nm) showed penetration of the inner retina but with fluorescent intensity decreasing with increasing liposome size. The topical administration of large multilamellar liposomes or free coumarin 6 resulted in little fluorescent intensity in the inner retina. DSPC liposomes were reported to deliver higher concentrations of coumarin 6 to the retina compared with EPC liposomes, with liposome bilayer rigidity (i.e., DSPC > EPC) being argued as a key parameter to facilitate retina uptake. This study was semiquantitative in that relative fluorescence intensity was used, with a similar emission wavelength to tissue autofluorescence. In a further study by the same group, Inokuchi *et al.* showed that nonPEGylated liposomal coumarin 6 liposomes with a diameters of 100 nm could reach the retina of the larger eyes of rabbits and monkeys after topical administration [18]. However, Lajunen *et al.* reported that PEGylated liposomes (hydrogenated soy L--phosphatidylcholine based) with diameters of 100 nm or more could not escape the choroidal endothelium in rats after topical administration even with transferrin-modified surface chemistries for enhanced retinal pigment epithelium (RPE) uptake. Only transferrin-labeled liposomes <80 nm in diameter were able to penetrate the RPE [19]. This was attributed to the small pores (80 nm) of the choroidal capillary network below the RPE [20]. Single-fluorophore studies create challenges associated with the interpretation of tissue sections. Significant data are available that show that small molecules can reach the retina after topical administration. Fluorophores in liposomes can leak or partition out of the liposome. It is not clear whether liposomes can readily reach the retina after topical administration, whether the concentration of liposomes is high enough for a therapeutic dose, and what transport mechanisms are involved. It is hard to imagine a large number of liposomes bypassing tear film, epithelial, and endothelial barriers without substantial loss. There is an argument that this might not be an issue if the loaded therapeutics have low effective concentrations (i.e., low nM).

Exploring the route of administration and whether intact liposomes reach the rat retina, Kaiser *et al.* performed a series of classic fluorescence and Förster resonance energy transfer (FRET) experiments [21]. NonPEGylated liposomes were either loaded with a single fluorophore or a FRET pair and administered via topical, subconjunctival, or intravitreal routes. After 15 min, subconjunctival administration resulted in the highest fluorescent intensity in the retina followed by topical then intravitreal administration. For subconjunctival administration, FRET experiments showed colocalization of the FRET pair, which the authors argued as providing evidence of intact liposomes. However, the FRET experiments only confirmed that the FRET pair is in the location of the liposome. The fluorescence microscopy data also challenge the suggestion that the FRET pair emission wavelength overlaps with the emission spectrum of DAPI. The group showed that minocycline-loaded liposomes administered via subconjunctival injection in a diabetic rat model reduced proinflammatory markers compared to untreated rats [21]. However, the concentration of minocycline at the retina after 30 min, 6 h, and 24 h was very low (i.e., ≤2% of the administered dose, <2 μg/ml) and significantly below the reported EC_{50} value of minocycline as an inhibitor of inflammation (≈15 μg/ml) [22]. Non-PEGylated unilamellar EPC liposomes loaded with latanoprost were reported to show significant reduction in intraocular pressure (IOP) values in rabbits after a single subconjunctival injection. In this study, liposomal latanoprost sizes reduced IOP for 90 days after a single injection [23]. Interestingly, nonloaded EPC liposomes showed progressive agglomeration (size instability) in PBS over 6 months, whereas loaded EPC liposomes did not agglomerate over this period. In a follow-up study, in which latanoprost-loaded EPC liposomes 60 nm in diameter were administrated to monkeys, IOP was reduced for 120 days after a single subconjunctival injection [24]. It could be argued that the instability of EPC liposomes was a key beneficial factor in the prolonged efficacy. Latanoprost leaving the EPC liposomes would increase liposome instability, resulting in agglomeration. In turn, agglomerated material would slow latanoprost release kinetics (i.e., a greater barrier to diffuse through), reducing the EPC clearance rate as the surface area decreased, thereby sustaining therapeutic effect. The infiltration of immune cells into the subconjunctival space or the conjunctiva was not reported [23,24] and further work is required to understand whether an immune response occurs across these long timeframes. Subconjunctival administration has been proposed as a depot site for liposomes [25]. There is a clear need to further confirm whether the low concentrations of therapeutics at the retina after subconjunctival administration reflect either liposomes held at a depot site or intact liposomes at the retina.

Visudyne is administered by intravenous injection and nonocular administrative routes have also attracted attention in delivering therapeutics to the outer retina. Hashida *et al.* functionalized ~100-nm diameter nonPEGylated liposomes with tetrasaccharide sialyl-Lewis X (sLe^X), a carbohydrate sequence that can recognize specific selectins on leukocytes and endothelial cells [26,27]. In an experimental autoimmune uveoretinitis (EAU) murine model, sLe^X liposomes accumulated throughout the retina over 30 min after intravenous administration, but did not accumulate in the

retina if selectins had been blocked with antibodies before liposome administration. Similar results were observed when dexamethasone was loaded into sLe^X liposomes [27]. Correspondingly, PEGylated liposomes loaded with methylprednisolone and where PEG chains were terminated with a glutathione (GSH) moiety showed reduced infiltration of inflammatory cells into the retina and improved retina protection in an EAU murine model [28,29]. Introducing glutathione to the PEG chain terminal allows the targeting of glutathione receptors on cells. Following GSH-functionalized liposomes, Vighi *et al.* recently reported an extensive study on liposomal cyclic GMP (cGMP) analogs for the treatment of photoreceptor cell loss [30]. The cGMP analogs were extensively screened *in vitro* before the most efficacious cGMP analog was remote loaded into unilamellar PEGylated liposomes (diameter ~110 nm) with glutathione-conjugated end groups (GSH-PEG liposomes). The liposomal cGMP analog showed improved efficacy *in vitro* compared with free analog and had a prolonged half-life (>24 h compared with 10 min) in the blood stream of rats after intravenous administration. Four days after a single intraperitoneal administration of carboxyfluorescein-loaded GSH-PEG liposomes, fluorescence was clearly measurable in the outer retina. In a transgenic mouse model of photoreceptor apoptosis-induced retinal degeneration, the liposomal cGMP analog (administered by intraperitoneal injection) showed significant photoreceptor protection and increased both a-wave (photoreceptors) and b-wave (interneuron cells) electrophysiological responses compared with untreated mice. Given that both sets of liposomes are larger than choroidal capillary pores, the results indicate either targeting facilitating liposome uptake and transcytosis through the retina layers, liposomes trapped in outer retina layers but releasing cargos to diffuse throughout the retina, and/or that the disease model has disrupted the outer retina–blood barrier, allowing liposomes to accumulate in the retina.

Delivery of gene therapeutics

Liposomes and lipoplexes have attracted considerable attention as nonviral vectors for gene editing. Lipoplexes are cationic lipid–DNA/RNA complexes, where the lipid is frequently a cationic phospholipid or cationic cholesterol derivative that can form lamellar and nonlamellar structures. The packing of cationic lipids and nucleic acids in lipoplexes has been well studied. There are fewer structural data available for complex component mixtures in the literature, containing a range of phospholipids (cationic in combination with neutral or anionic), DNA/RNA, and stabilizing macromolecules (e.g., protamine) compared with traditional lipoplexes. This raises questions when comparing the properties of the two systems. PEGylated lipid NPs, where small interfering (si)RNA was complexed with protamine and hyaluronic acid (HA), were reported to both transfect the RPE cell line ARPE-19 and reduce angiogenesis in a murine choroidal neovascularization (CNV) modal after intravitreal administration [31]. The concept was reversed where HA was used to surface modify lipoplexes for improved transfection [32]. Different HA-coating approaches (electrostatic and covalent), the changes in lipoplex properties with surface modification, and the resulting changes to *in vitro* transfection efficacy in ARPE-19 cells and *ex vivo* diffusion were explored. Covalently modified HA lipoplexes showed significantly improved transfection over both electrostatically modified HA lipoplexes and PEGylated lipoplexes. PEGylated lipoplexes showed limited transfection *in vivo* but the fastest diffusion rates through the vitreous of the *ex vivo* bovine eye. Diffusion rates then decreased in the order of electrostatically modified HA lipoplexes followed by covalently modified HA lipoplexes and then uncoated lipoplexes. These observations add further weight to the concept that PEGylated lipidic NPs (liposomes, lipoplexes, and lipid NPs) with low cationic zeta potentials can readily diffuse through the vitreous albeit depending on particle size. This adds subtlety to the concept of the vitreous being a barrier to cationic NPs. Instead, the vitreous is likely to only be a barrier to NPs with high cationic surface charge where nanoparticle sizes are small enough to diffuse through pores in the collagen-glycosaminoglycan network of the vitreous [10]. Evidently, there is a balance to the benefits of shielding lipoplex/lipid NP cationic charge with PEG to facilitate diffusion and improve *in vivo* stability with the reduction in transfection efficacy.

Viral vectors used for the gene editing of outer retina cells are frequently administered by subretinal injection (e.g., the recently FDA-approved Luxtrana) because this has shown better efficacy [33]. Subretinal injection has also been used to administer nonviral vectors, although this route of administration remains underutilized. Rajala *et al.* developed a lipid NP where the surface was functionalized with both a cell-penetrating peptide (TAT) and a nuclear localization signaling peptide (NLS) [34,35]. GFP expression was successfully transfected into the murine retina after lipid NPs were administered by subretinal injection, and GFP expression was maintained for 3 months. Moreover, RPE65 protein expression could be established in an *RPE65*-knockout murine model of blindness using RPE65 cDNA lipid NPs. Electroretinography (ERG) recordings of treated mice showed improvements in scotopic and photopic b-wave amplitudes compared with untreated mice, indicating improved cone photoreceptor and interneuron (bipolar and Müller glial cells) cell function. No significant improvement was observed for scotopic a-wave response, which has contributions from both rods and cones. The lack of scotopic a-wave response might indicate that rod photoreceptor function was not improved. TAT-NLS lipid NPs were also shown to deliver gene promoters to specific cell types *in vivo* [36]. RPE, rod, cone, and ganglion cells were all successfully transfected to express GFP when under the control of cell-specific promoters (e.g., thymocyte antigen promoter for RGCs). Intravitreal administration was used to treat RGCs, whereas subretinal injection was used to treat RPE and photoreceptor cells. Subretinal administration requires further exploration, but the benefit of delivering therapeutic payloads directly to diseased tissue while minimizing challenges associated with other routes of administration (e.g., circulation time, diffusion through the vitreous, carrier stability, etc.) is clear. Nevertheless, there are challenges to implementing subretinal injections in the clinic (e.g., large-scale use, risks, etc.) that have to be acknowledged.

Delivery of proteins

The vesicle structure of liposomes allows the loading of proteins and peptides into the aqueous core with minimal risk of denaturation, misfolding, and agglomeration, thus mimicking cellular vesicles. There is also the option to conjugate proteins and peptides to the surface of liposomes for therapy, although active

targeting and cell-penetrating sequences are more commonly used. The protein annexin A5, which binds to phospholipids and is an anticoagulant, has recently attracted attention as a mechanism to facilitate liposome transport across epithelial and endothelial barriers. This is partially attributed to annexin A5 promoting endosome formation and, by extension, transcytosis. Davis *et al.* developed a range of liposomes with surfaces functionalized with Annexin A5 where functionalization was achieved through intermolecular interaction rather than covalent attachment [37]. Annexin A5 liposomes loaded with hydrophilic fluorophore showed both enhanced corneal epithelial cell uptake and transcytosis in a Transwell corneal epithelial cell barrier model compared with nonfunctionalized liposomes. The topical administration of annexin A5 liposomes loaded with bevacizumab showed increased levels of bevacizumab in the retinas of both rats and rabbits compared with controls. Although liposome delivery increased bevacizumab concentration in the retina by nearly threefold in rats compared to free drug this was only ~0.003% of the administered dose to the eye. Bevacizumab concentration in rabbit vitreous was significantly lower than in values observed in rat retina (~3 ng/g compared to ~130 ng/g). Given that bevacizumab has an EC_{50} value of 22 ng/ml [37], rats would have shown efficacy whereas rabbits would have been unlikely to have shown an effect of the antibody. Using a similar concept but with different phospholipid compositions, Platania *et al.* produced Annexin A5 nonPEGylated unilamellar liposomes with similar size to those developed by Davis *et al.* [37,38] to deliver transforming growth factor (TGF)-ß1 to the vitreous of rabbits by topical administration. TGF-ß1 levels in the vitreous were significantly higher for liposome-treated rabbits than those treated with empty liposomes. However, TGF-ß1 concentrations were <150 pg/ml, which is very low and would not induce a therapeutic effect. Annexin A5 is a marker for cell apoptosis and necrosis [39]. Liposomes functionalized with Annexin A5 could trigger unwanted immune responses if repeated dosing is used to treat animal models.

The aqueous core and lipid bilayer of liposomes creates the opportunity to incorporate multiple components into the two environments. This is a potential strategy to treat multiple therapeutic targets in multifactorial diseases. Recently, liposome structure was exploited to load four different biologics into a single liposome [40]. These multifarious biologic-loaded liposomes (MBLs) contained both insulin-like growth factor (IGF)-1 and ciliary neurotrophic factor (CNTF) in an aqueous core, whereas the bilayer contained a lipid-conjugated PTEN peptide inhibitor (PAP) and a lipid-conjugated osteopontin peptide mimic (OPP). All biologics were loaded at concentrations above their corresponding EC_{50} values. These biologic payloads all stimulated the PI3K/AKT/mTOR signaling pathway, which has been shown to protect neurons and stimulate axon regrowth. In an excitotoxin murine model of retina and optic nerve damage, a single intravitreal administration of MBLs showed significant RGC protection, and improved scotopic b-wave and scotopic threshold response (STR, reflects inner retina function predominantly associated with RGCs) compared with empty liposome-treated mice. Furthermore, the combination of MBLs with transplant RGCs (both administered by intravitreal injection but on separate days, with MBLs being administered first), although not improving RGC protection or b-wave response, showed a significant improvement of STR. This loading approach is limited to therapeutics with high efficacy (i.e., low EC_{50} values) because the loading of multiple therapeutics in a limited space results in lower loading efficiencies for each therapeutic. Furthermore, these systems represent significant pharmacokinetic challenges associated with modeling release rates *in vivo*, which could limit their clinical applicability.

Discussion

There are clearly unresolved challenges in the interplay between biological responses and liposome properties. As shown in Table 1 (which includes example references not discussed), there is both inconsistency in the manufacturing of liposomes (i.e., size polydispersity) and a lack of basic characterization. In the context of clinically approved unilamellar liposomes, liposomes contain cholesterol in the bilayer and have surfaces modified with extended macromolecular chains (e.g., PEG) [3,4]. Cholesterol is added to increase bilayer rigidity through packing density effects. Improving phospholipid packing results in an increase in bilayer rigidity and a decrease in bilayer permeability to small molecules (e.g., water, ions, small molecule therapeutics, and fluorophores). This decreases drug leakage and increases liposome stability (e.g., less susceptible to osmotic pressure). Introducing extended macromolecular chains to the surface of liposomes (e.g., with PEG) improves colloidal stability (i.e., minimizes interactions between liposome–liposome, liposome–protein etc.) through nonelectrostatic mechanisms, slows protein corona formation, and thereby increases the circulation and/or residency time before clearance. However, many studies use liposomes without cholesterol and/or no macromolecular surface functionality. Lipid-conjugated constituents might exit bilayers for other hydrophobic environments (e.g., cell membranes, colloidal proteins, etc.) if inappropriately packed in the bilayer. Aqueous core constituents will leak if bilayer stability is poor. If these constituents are fluorophores then fluorophore location in tissues does not necessarily represent intact liposome biodistribution.

Paracellular transport is viewed as not applicable to liposomes because of the spaces between cells being too small (e.g., <20 nm for retina cells [14] or <2 nm for endothelial tight junctions [41]). Transcytosis of NPs *in vivo* remains a point of debate [42,43]. It is not clear whether the numbers of NPs that are transported across endothelial barriers is sufficient to be a realistic therapeutic option. However, NPs broken down in barrier cells can still release therapeutics (or fluorophores) that can readily diffuse to tissue beyond the barrier. Whether liposomes remain intact or breakdown to constituents is a moot point if a short distance between the liposome and target cells is involved (e.g., choroid to RPE, vitreous to inner retina, etc.). Challenges arise when multiple cell layers over significant distances are involved (e.g., in topical administration). It is difficult to envisage liposomes being readily transported intact across multiple cell layers comprising different cell types without a substantial loss of liposomes to cellular breakdown and clearance mechanisms. It is not difficult envisaging a single molecule constituent diffusing across these length scales and preferentially binding to specific tissues and cellular structures. Capillary pore size is frequently smaller than liposome diameter (e.g., choroidal capillary pores) and it remains unclear whether liposomes have the ability to deform through smaller pores. Liposome flexibility is influenced by size, with larger-diameter liposomes being

TABLE 1

Examples of reported liposome properties in cell, *ex vivo* and *in vivo* studies[a,b]

Size (nm)	Zeta potential	Size PDI	Lamellarity	Cholesterol	PEGylated	Surface	Cell and/or animal model	Refs
118–135	–1 to 47	0.04–0.17	N.S.	No	Yes	No	Healthy and CNV mice	[9]
121–137	–20 to 12	0.04–0.05	N.S.	Yes	Yes	No	*Ex vivo* porcine	[10]
90–125	–2 to 8	N.S.	N.S.	Yes	No	No	Excitotoxin mouse	[16]
105–125	–63 to –66	N.S.	N.S.	Yes	No	No	Healthy mouse	[17]
109–561	–77 to 26	N.S.	N.S.	Yes	No	No	Healthy mouse	[18]
68–100	36	N.S.	N.S.	Yes	Yes	Yes	Healthy rat	[19]
80	–21	N.S.	N.S.	Yes	No	No	Diabetic rat	[21]
83	N.S.	0.14	N.S.	No	No	No	Healthy rabbit	[23]
60	N.S.	N.S.	Unilamellar	No	No	No	Healthy monkey	[24]
100	–40	N.S.	N.S.	Yes	No	Yes	Healthy mouse	[27] ([26])
100	N.S.	0.04–0.09	Unilamellar	Yes	Yes	Yes	EAU mouse	[29] ([28])
100	N.S.	N.S.	Unilamellar	Yes	Yes	Yes	RD mouse	[30]
132	20	N.S.	N.A.	Yes	Yes	No	ARPE-19 cells, CNV rat	[31]
113–1091	–20 to 54	0.11 to 0.46	N.A.	No	Yes	Yes	ARPE-19 cells; *ex vivo* bovine eye	[32]
245	20	N.S.	N.A.	No	No	Yes	RPE65-knockout mouse	[34] ([35])
245	20	N.S.	N.A.	No	No	Yes	Healthy mouse	[36] ([35])
110–163	–5 to –7	0.07–0.2	Unilamellar	Yes	No	Yes	CE cells, healthy rat and rabbit	[37]
156	–29 to –17	0.09–0.18	N.S.	Yes	No	Yes	Healthy rabbit	[38]
97–109	–19 to –13	0.04–0.06	Unilamellar	Yes	Yes	Yes	Murine retina organoids, excitotoxin	[40]
N.S.	N.S.	N.S.	N.S.	No	No	No	CNV mouse	[46]
250–370	N.S.	N.S.	Multilamellar	No	No	No	ARPE-19 cells	[47]
N.S.	N.S.	N.S.	N.S.	Yes	No	No	HD mouse	[48]
140–220	N.S.	N.S.	N.S.	No	Yes	No	ARPE-19 cells	[49]
93–97	19–41	N.S.	N.S.	Yes	No	No	Light-induced retinal damage mouse	[50]
108–438	–1 to 20	0.16–0.54	N.S.	Yes	No	Yes	Healthy rabbit	[51]
N.S.	N.S.	N.S.	N.S.	No	No	No	Ischemia mouse	[52]
93	N.S.	0.13	N.S.	Yes	No	No	CNV rat	[53]
351	–21	0.39	N.S.	Yes	Yes	No	EAU rat	[54]
202	N.S.	N.S.	N.S.	Yes	No	No	EAU rat	[55]
130–608	N.S.	0.13–0.40	N.S.	Yes	No	No	*Ex vivo* murine retina, Healthy mouse	[56]
124–234	17–32	N.S.	N.A.	Yes	Yes	Yes	ARPE-19 cells	[57]
101–163	–18 to –1 mV	0.02–0.07	Unilamellar	Yes	Yes	Yes	HRE cells	[58]

[a] It is clear from a small sample of the literature that basic liposome properties are frequently not reported and stabilizing chemistries (i.e., cholesterol and PEG) are frequently not used. However, these systems are used in animals and the lack of basic liposome data does not allow correlations to be made between liposome properties and biological response. This is further compounded by the wide choice of liposome constituents and the molar ratios of constituents. The numerical values presented for size and zeta potential are rounded to the nearest whole integer. Standard deviations for size, zeta potential, and size polydispersity index those that hold the reported values for references discussed in the main text. Lamellarity is not applicable for lipoplex and lipid nanoparticle systems and is designated as 'N.A.'. Surface modification reflects additional surface moieties (e.g., glutathione) beyond PEG or with similar properties to PEG.
[b] Abbreviations: ARPE, adult retinal pigment epithelial cells; CE, corneal epithelial cells; HD, Huntington disease; HRE, human retinal endothelial cells; RD, retinal degeneration.

considerably more flexible than small-diameter liposomes [44,45]. PEGylation has also been shown to increase the rigidity of liposomes [45]. It might be that nonPEGylated liposomes in the liquid disordered phase are flexible enough to deform through pores. This has to be balanced with the concept of constituents (e.g., fluorophores) being able to escape the liposome and diffuse or be carried (i.e., by colloidal proteins, low-density lipoproteins, etc.) through the pore. Equally, it is not clear whether all retinopathy animal models have disrupted blood–retina barriers associated with the proinflammatory environment, which could explain liposome–capillary pore discrepancies. Further work is required on the fundamentals of biology–liposome dynamics (e.g., cellular transport mechanisms, routes of transport through organs, partitioning of constituents, etc.).

Furthermore, the route of administration needs to be more aggressively questioned. It is easy to present the argument as to why topical or subconjunctival administration is preferable over intravitreal administration, but there are substantial challenges in obtaining effective concentrations at the retina while minimizing drug loss through barrier and/or clearance mechanisms. Therapeutics with low EC_{50} values could have the potential to be administered by these routes but this is balanced by the costs associated with drug loss. Low EC_{50} therapeutics (e.g., monoclonal antibodies) often have high commercial costs. Although intravitreal administration has risks, it has repeatedly been shown to maximize drug concentrations at the retina and is widely used in the clinic. Similar to the discussion around endothelial barriers, NPs that do not bypass the ILM can still release drug close to the retina, thereby enhancing delivery. More work is required to assess whether subretinal injections are beneficial to NP systems. The limit to this route of administration is likely to be a combination of risk, single administration versus multiple administrations and the skills required to perform the operation. In the context of the number of administrations, systems that require only a single administration (e.g. gene therapy) will likely benefit the most from subretinal injections. Multiple administrations are likely to result in significant risks to retina health.

Concluding remarks

Liposomes represent promising drug delivery systems for the treatment of retinal diseases, but have yet to reach their full potential. The ability to distribute to the retina, deliver a range of therapeutics and disintegrate into nontoxic easily metabolized constituents are clear advantages. Yet. challenges remain, from liposome design to the chosen route of administration. The literature highlights a lack of consistency in the manufacturing of liposomes and an inadequate characterization of basic liposome properties. These two factors have a significant influence over *in vivo* outcomes and the potential to correlate outcomes with liposome properties for improved liposome engineering. The drug delivery community would benefit from presenting clear arguments based on fundamental properties as to the choice of liposome constituents, liposome properties, and resulting pharmacokinetics. Critical reflections of the data obtained, the limitations of analytical techniques used, and the increased use of quantitative techniques would also help the field develop.

References

1 (2013) Optic neuropathies: characteristic features and mechanisms of retinal ganglion cell loss. *Rev. Neurosci.* 24, 301–321
2 del Amo, E.M. *et al.* (2017) Pharmacokinetic aspects of retinal drug delivery. *Prog. Retinal Eye Res.* 57, 134–185
3 Allen, T.M. and Cullis, P.R. (2013) Liposomal drug delivery systems: from concept to clinical applications. *Adv. Drug Deliv. Rev.* 65, 36–48
4 Pattni, B.S. *et al.* (2015) New developments in liposomal drug delivery. *Chem. Rev.* 115, 10938–10966
5 Bulbake, U. *et al.* (2017) Liposomal formulations in clinical use: an updated review. *Pharmaceutics* 9, 12
6 Christoforidis, J.B. *et al.* (2012) Intravitreal devices for the treatment of vitreous inflammation. *Mediat. Inflam.* 2012, 126463
7 Alipour, E., Halverson, D., McWhirter, S. and Walker, G.C. (2017) Phospholipid bilayers: stability and encapsulation of nanoparticles. *Annu. Rev. Phys. Chem.* 68, 261–283
8 Kaur, I. and Kakkar, S. (2014) Nanotherapy for posterior eye diseases. *J. Control. Release* 193, 100–112
9 Lee, J. *et al.* (2016) Effective retinal penetration of lipophilic and lipid-conjugated hydrophilic agents delivered by engineered liposomes. *Mol. Pharm.* 14, 423–430
10 Eriksen, A.Z. *et al.* (2017) The diffusion dynamics of PEGylated liposomes in the intact vitreous of the ex vivo porcine eye: A fluorescence correlation spectroscopy and biodistribution study. *Int. J. Pharm.* 522, 90–97
11 Mains, J. and Wilson, C.G. (2013) The vitreous humor as a barrier to nanoparticle distribution. *J. Ocul. Pharmacol. Ther.* 29, 143150
12 Kamp, F. and Hamilton, J.A. (2006) How fatty acids of different chain length enter and leave cells by free diffusion. *Prostaglandins Leukot Essent Fatty Acids* 75, 149–159 Epub 2006 Jul 7
13 Jackson, T.L. *et al.* (2003) Human retinal molecular weight exclusion limit and estimate of species variation. *Invest. Ophthalmol. Vis. Sci.* 44, 2141–2146
14 Edelhauser, H.F. *et al.* (2010) Ophthalmic drug delivery systems for the treatment of retinal diseases: basic research to clinical applications. *Invest. Ophthalmol. Vis. Sci.* 51, 5403–5420
15 Raposo, G. and Stoorvogel, W. (2013) Extracellular vesicles: exosomes, microvesicles, and friends. *J. Cell Biol.* 200, 373–383
16 Hironaka, K. *et al.* (2011) Edaravone-loaded liposomes for retinal protection against oxidative stress-induced retinal damage. *Eur. J. Pharm. Biopharm.* 79, 119–125
17 Hironaka, K. *et al.* (2009) Design and evaluation of a liposomal delivery system targeting the posterior segment of the eye. *J. Control. Release* 136, 247–253
18 Inokuchi, Y. *et al.* (2010) Physicochemical properties affecting retinal drug/coumarin-6 delivery from nanocarrier systems via eyedrop administration. *Invest. Ophthalmol. Vis. Sci.* 51, 3162–3170
19 Lajunen, T. *et al.* (2014) Topical drug delivery to retinal pigment epithelium with microfluidizer produced small liposomes. *Eur. J. Pharm. Sci.* 62, 23–32
20 Guymer, R.H. *et al.* (2004) Cytoarchitecture of choroidal capillary endothelial cells. *Invest. Ophthalmol. Vis. Sci.* 45, 1660–1666
21 Kaiser, J.M. *et al.* (2013) Nanoliposomal minocycline for ocular drug delivery. *Nanomedicine* 9, 130–140
22 Dunston, C. *et al.* (2011) Proteomic analysis of the anti-inflammatory action of minocycline. *Proteomics* 11, 42–51
23 Natarajan, J.V. *et al.* (2012) Nanomedicine for glaucoma: liposomes provide sustained release of latanoprost in the eye. *Int. J. Nanomedicine* 7, 123–131
24 Natarajan, J.V. *et al.* (2014) Sustained drug release in nanomedicine: a long-acting nanocarrier-based formulation for glaucoma. *ACS Nano* 8, 419–429
25 Moon, J.-W. *et al.* (2006) Effect of subconjunctivally injected, liposome-bound, low-molecular-weight heparin on the absorption rate of subconjunctival hemorrhage in rabbits. *Invest. Ophthalmol. Vis. Sci.* 47, 3968–3974
26 Hirai, M. *et al.* (2007) Accumulation of liposome with Sialyl Lewis X to inflammation and tumor region: application to *in vivo* bio-imaging. *Biochem. Biophys. Res. Commun.* 353, 553–558
27 Hashida, N. *et al.* (2008) High-efficacy site-directed drug delivery system using sialyl-Lewis X conjugated liposome. *Exp. Eye Res.* 86, 138–149
28 Gaillard, P.J. *et al.* (2012) Enhanced brain delivery of liposomal methylprednisolone improved therapeutic efficacy in a model of neuroinflammation. *J. Control. Release* 164, 364–369
29 Reijerkerk, A. *et al.* (2014) Systemic treatment with glutathione PEGylated liposomal methylprednisolone (2B3-201) improves therapeutic efficacy in a model of ocular inflammation. *Invest. Ophthalmol. Vis. Sci.* 55, 2788–2794

30 Vighi, E. *et al.* (2018) Combination of cGMP analogue and drug delivery system provides functional protection in hereditary retinal degeneration. *Proc. Natl. Acad. Sci. U. S. A.* 115, 201718792

31 Liu, H. *et al.* (2011) A lipid nanoparticle system improves siRNA efficacy in RPE cells and a laser-induced murine CNV model. *Invest. Ophthalmol. Vis. Sci.* 52, 4789–4794

32 Martens, T.F. *et al.* (2017) Effect of hyaluronic acid-binding to lipoplexes on intravitreal drug delivery for retinal gene therapy. *Eur. J. Pharm. Sci.* 103, 27–35

33 Boye, S.E. *et al.* (2013) A comprehensive review of retinal gene therapy. *Mol. Ther.* 21, 509–519

34 Rajala, A. *et al.* (2014) Nanoparticle-assisted targeted delivery of eye-specific genes to eyes significantly improves the vision of blind mice *in vivo*. *Nano Lett.* 14, 5257–5263

35 Ma, K. *et al.* (2013) Synergetic targeted delivery of sleeping-beauty transposon system to mesenchymal stem cells using LPD nanoparticles modified with a phage-displayed targeting peptide. *Adv. Funct. Mater.* 23, 1172–1181

36 Wang, Y. *et al.* (2016) Cell-specific promoters enable lipid-based nanoparticles to deliver genes to specific cells of the retina in vivo. *Theranostics* 6, 1514–1527

37 Davis, B.M. *et al.* (2014) Topical delivery of avastin to the posterior segment of the eye *in vivo* using annexin A5-associated liposomes. *Small* 10, 1575–1584

38 Platania, C. *et al.* (2017) Topical ocular delivery of TGF-β1 to the back of the eye: implications in age-related neurodegenerative diseases. *Int. J. Mol. Sci.* 18, 2076

39 van Genderen, H.O. *et al.* (2008) Extracellular annexin A5: functions of phosphatidylserine-binding and two-dimensional crystallization. *Biochim. Biophys. Acta* 1783, 953–963

40 Eriksen, A.Z. *et al.* (2018) Multifarious biologic loaded liposomes that stimulate the mammalian target of rapamycin signaling pathway show retina neuroprotection after retina damage. *ACS Nano* 12, 7497–7508

41 Curry, F.E. (2005) Microvascular solute and water transport. *Microcirculation* 12, 17–31

42 Johnsen, K. *et al.* (2017) Targeting transferrin receptors at the blood-brain barrier improves the uptake of immunoliposomes and subsequent cargo transport into the brain parenchyma. *Sci. Rep.* 7, 10396

43 Fullstone, G. *et al.* (2016) From the blood to the central nervous system: a nanoparticle's journey through the blood-brain barrier by transcytosis. *Int. Rev. Neurobiol.* 130, 41–72

44 Seifert, U. and Lipowsky, R. (1990) Adhesion of vesicles. *Phys. Rev. A* 42, 4768–4771

45 Bendix, P. *et al.* (2009) Quantification of nano-scale intermembrane contact areas by using fluorescence resonance energy transfer. *Proc. Natl. Acad. Sci. U. S. A.* 106, 12341–12346

46 Gross, N. *et al.* (2013) Choroidal neovascularization reduced by targeted drug delivery with cationic liposome-encapsulated paclitaxel or targeted photodynamic therapy with verteporfin encapsulated in cationic liposomes. *Mol. Vis.* 19, 54–61

47 Paasonen, L. *et al.* (2010) Gold-embedded photosensitive liposomes for drug delivery: triggering mechanism and intracellular release. *J. Control. Release* 147, 136–143

48 Li, M. *et al.* (2013) Intravitreal administration of HA-1077, a ROCK inhibitor, improves retinal function in a mouse model of Huntington disease. *PLoS One* 8, e56026

49 Lajunen, T. *et al.* (2015) Light induced cytosolic drug delivery from liposomes with gold nanoparticles. *J. Control. Release* 203, 85–98

50 Shimazaki, H. *et al.* (2011) Edaravone-loaded liposome eyedrops protect against light-induced retinal damage in mice. *Invest. Ophthalmol. Vis. Sci.* 52, 7289–7297

51 Tan, G. *et al.* (2017) Bioadhesive chitosan-loaded liposomes: a more efficient and higher permeable ocular delivery platform for timolol maleate. *Int. J. Biol. Macromol.* 94, 355–363

52 Dvoriantchikova, G. *et al.* (2010) Liposome-delivered ATP effectively protects the retina against ischemia-reperfusion injury. *Mol. Vis.* 16, 2882–2890

53 Li, T. *et al.* (2015) Liposomal hypocrellin B as a potential photosensitizer for age-related macular degeneration: pharmacokinetics, photodynamic efficacy, and skin phototoxicity *in vivo*. *Photochem. Photobiol. Sci.* 14, 972–981

54 Zhang, R. *et al.* (2017) Treatment of experimental autoimmune uveoretinitis with intravitreal injection of infliximab encapsulated in liposomes. *Br. J. Ophthalmol.* 101, 1731–1738

55 Zhang, R. *et al.* (2010) Treatment of experimental autoimmune uveoretinitis with intravitreal injection of tacrolimus (FK506) encapsulated in liposomes. *Invest. Ophthalmol. Vis. Sci.* 51, 3575–3582

56 Asteriti, S. *et al.* (2015) Effective delivery of recombinant proteins to rod photoreceptors via lipid nanovesicles. *Biochem. Biophys. Res. Commun.* 461, 665–670

57 Chen, C.-W. *et al.* (2013) Efficient downregulation of VEGF in retinal pigment epithelial cells by integrin ligand-labeled liposome-mediated siRNA delivery. *Int. J. Nanomedicine* 8, 2613–2627

58 Arta, A. *et al.* (2018) Endothelial protein C-targeting liposomes show enhanced uptake and improved therapeutic efficacy in human retinal endothelial cells. *Invest. Ophthalmol. Vis. Sci.* 59, 2119–2132