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The Future of Nanomaterials Tackling the Challenge of Delivering Nucleic Acids to the Retina

José Hurst, Friederike Adams, and Sven Schnichels**

Ocular gene therapy targets eye diseases at the genetic level. Systemic transport of nucleic acids to ocular tissues poses a significant challenge, as the effectiveness of crossing the blood-retina barrier limits nucleic acid penetration. Therefore, local administration, such as topical, periocular, or intraocular, can improve the outcome of in vivo gene therapy by bypassing the first-pass effect and minimizing the systemic toxic effects. The eye is an immune-privileged organ with limited local immune response, making it an ideal candidate for local gene therapy. Ocular gene therapy offers a promising solution for the treatment of a wide range of retinal diseases including age-related macular degeneration, diabetic retinopathy, retinitis pigmentosa, and Leber congenital amaurosis. Gene therapy enables replacement of mutated genes essential for visual function, delivery of genes expressing neurotrophic factors and anti-apoptosis factors for retinal degeneration, and delivery of genes expressing anti-angiogenic proteins for ocular neovascularization. This perspective discusses the potential of nanoparticles for nucleic acid delivery to the retina, explores challenges, and evaluates different delivery methods, including non-viral agents such as liposomes and polymers. These nonviral agents present advantages over traditional viral vectors, showing promise in overcoming limitations and offering a viable option for retinal gene therapy.

1. Introduction

In recent years, the use of nanoparticles (NPs) from non-viral vectors as carriers for delivering therapeutic substances (small molecules, peptides, oligonucleotides, etc.) to target tissues has gained popularity and shown significant potential for treating various retinal diseases. The discovery of RNAi has sparked interest in the use of DNA, siRNAs, and miRNAs for ophthalmic therapeutic purposes. To address the challenges related to the instability, charge, and molecular weight of nucleic acid molecules, researchers have explored NP formulations for in vivo applications. For a general overview of non-viral delivery agents, including liposomes and polymers, and their performance, refer to references.[1] In general, particles with diameters smaller than 1 µm are classified as NPs, but those found to be effective for gene delivery typically possess hydrodynamic diameters below 400 nm.

Smaller particles are generally considered more efficient for traversing biological barriers. In particular, very small particles (<25 nm) can traverse the pores of the nuclear membrane, which is a critical factor for the delivery of nucleic acids like plasmid DNA that require nuclear localization for gene expression. However, for nucleic acids such as mRNA or antisense oligonucleotides, which function in the cytoplasm, overcoming the nuclear membrane barrier is less relevant.[2]

Delivering drugs and genes to the posterior segment of the eye is a significant challenge for ophthalmologists and pharmacologists. This difficulty arises because of the presence of numerous biological barriers within the eye that hinder the penetration of foreign substances, including drugs and nucleic acids, and impede the effective permeation of these substances into ocular tissues.[3] Consequently, delivering nucleic acids to the posterior segment of the eye, particularly for gene therapy or other therapeutic purposes, is a complex task because of several anatomical and physiological barriers.

To address these challenges, researchers and pharmaceutical companies are actively exploring various delivery strategies, including viral and non-viral vectors (NPs, polyplexes, liposomes). Advances in nanotechnology, biotechnology, and targeted delivery systems are essential for the successful development of nucleic acid-based therapies for posterior eye diseases.

J. Hurst, F. Adams, S. Schnichels
Center for Ophthalmology
University Eye Hospital Tübingen, Section for Translational Research in Ophthalmology
Elfriede-Aulhorn-Strasse 7, 72076 Tübingen, Germany
E-mail: friederike.adams@ipoc.uni-stuttgart.de; sven.schnichels@med.uni-tuebingen.de

F. Adams
Chair of Macromolecular Materials and Fiber Chemistry
Institute of Polymer Chemistry
University of Stuttgart
Pfaffenwaldring 55, 70569 Stuttgart, Germany

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Most researchers consider adeno-associated viruses (AAV) when discussing nucleotide or plasmid delivery to the eye. AAVs represent the predominant vector platform used in gene therapy across various organ systems, including the eye.[4] Their widespread adoption stems from their ability to target diverse tissues, facilitate sustained gene expression without genomic integration, and maintain a favorable safety profile. More than 150 clinical trials (www.clinicaltrials.gov; search terms: gene therapy; ocular, eye, optic, visual function) focusing on ocular gene therapy have been completed or are currently underway to address a range of inherited retinal diseases, such as hereditary Leber optic neuropathy, choroideremia, Stargardt disease, X-linked retinoschisis, and X-linked retinitis pigmentosa.[5] The approval of Luxturna, a through AAV delivered complementary DNA (cDNA) for RPE65 by the U.S. Food and Drug Administration (FDA) in 2017 for the treatment of congenital Leber amaurosis marked a significant milestone in the use of ocular gene therapy in clinical practice. Currently, ongoing clinical trials are progressing through Phases I/II and III.[6] Despite the promise of ocular gene therapy, there are inherent risks associated with the invasive procedures required for vector delivery into the eye, with emerging evidence suggesting the potential development of uveitis following intravitreal or subretinal administration.[7] Furthermore, challenges persist in achieving optimal efficacy, prompting a growing demand for novel approaches that facilitate effective nucleotide delivery to the posterior segment of the eye.

Most companies rely on AAV or lentiviruses for gene therapy. Although the eye is immune-privileged and AAV is non-pathogenic, local and systemic immune responses have been observed in many studies despite the administration of corticosteroids (immunosuppressants). This triggered disease is now referred to as gene therapy-associated uveitis (GTAU) and can develop into a relevant chronic inflammation that (further) reduces visual acuity. In addition, a loss of efficacy has been reported after a longer period of time.[8] Attempts are now being made to reduce immunogenicity through various modifications.[9] Another problem is that although there are ways to improve viral gene therapy, only the extent of the side effects is reduced. In addition, many retinal diseases that do not yet have a (gene) therapy option cannot be addressed because, for example, only gene sequences of relatively small size (max. 4.8 kilobases) can be transferred with AAV.[10] Many genes that could protect patients from blindness are simply too large for these viral transport vehicles. Therefore, there is a strong need for novel non-viral gene therapy approaches. There are two main barriers to overcome: the limited uptake into the cell and the cell nucleus and the limited long-term stability or volatile gene expression.[11] Strategies that combine the safety profile of non-viral approaches with the efficacy of viral vectors are ideal and are the focus of several research groups.[12] There are various experimental developments with non-viral particles for ocular gene therapy on which we will elaborate in this article.

Non-viral delivery of exogenous nucleotides, usually DNA, offers an alternative method that theoretically allows transportation of large nucleotide fragments in a manner that is less likely to provoke an immune response. However, for the expression of these nucleotides, they must navigate a challenging path from the delivery site to the nucleus, evading extracellular degradation, immune reactions, cytoplasmic enzymatic breakdown, and traversing cell membranes and nuclear envelopes, which are typically impermeable outside of cell division.[13] Generally, non-viral gene transfer to non-dividing cells is notably less efficient than viral delivery, especially in the eye, where physical barriers such as the vitreous, inner/outer limiting membranes, and interphotoreceptor matrix significantly hinder cellular access. Moreover, high concentrations of glycosaminoglycans found throughout the eye, particularly in the vitreous, tend to sequester DNA, causing aggregation and further impeding cellular uptake.[14] Non-viral transfer methods typically rely on chemical or physical approaches. For any method to be clinically viable, it must be minimally invasive, ensuring that administration does not cause significant damage to the retinal structure or disrupt cellular function.[15]

1.1. Barriers and Delivery Routes

The posterior segment of the eye includes the retina, choroid, and vitreous body, which are spread throughout the entire interior of the eye. Ensuring an effective delivery to this region poses unique challenges. The human eyeball has a layered structure that encompasses the outer shell and internal components. The outer shell comprises the sclera and cornea, which are bordered by a vascular membrane containing the choroid, iris, and ciliary body. The innermost layer of the eyeball contains the retina, which is composed of both sensory and supporting cells, as well as retinal pigment epithelium. The internal components of the eyeball are the iris, ciliary body, crystalline lens, and vitreous body (**Figure 1**).[16]

The cornea and tear film are recognized as the first and significant barriers to ocular drug delivery. The cornea, with its trilaminate structure, exhibits selective permeability that impedes the passage of both highly hydrophobic and hydrophilic substances.[17] The tear film, in conjunction with the lids and lacrimal apparatus, not only maintains ocular health by protecting the cornea and conjunctiva, but also presents a challenge for drug absorption due to its continuous renewal and dilution effect on administered drugs.[18] Interestingly, although these barriers serve essential protective functions, they also limit the therapeutic efficacy of topical ophthalmic agents. The rapid clearance of drugs by tear turnover and nasolacrimal drainage can lead to suboptimal drug concentrations in target tissues, particularly for treatments aimed at the posterior segment of the eye.[19]

In addition to the cornea, the vitreous body functions as a significant barrier to the penetration of drugs into the retinal and choroidal tissues, particularly after intravitreal injection, though it can also be a challenge following topical administration. Consequently, the most frequently used approach for administering drugs directly into the eye is intravitreal injection. However, the bioavailability of the drug to the retina depends significantly on the characteristics of the drug itself; for instance, the number of nanoparticles reaching the retina may be more limited compared to small molecules. The vitreous consists of a framework of hyaluronic acid and collagen fibers, and up to 99% water. They exhibit gel-like properties that change with age, leading to phase separation.[20] The diffusion of drug molecules in the vitreous body is controlled by the net anionic charge. Specifically, negatively charged particles are free to diffuse, whereas

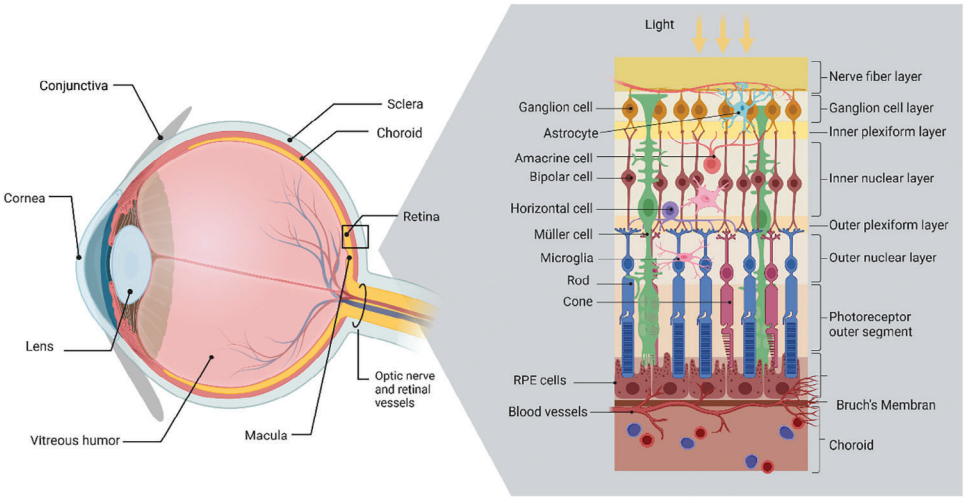

Figure 1. Schematic cross-section of a human eye: All relevant tissues of the anterior (left) and posterior (right) sections of the eye are labeled. The light travels through the cornea, iris, lens, and vitreous body and then reaches the retina, where the signals are received and processed and then transmitted to the brain via the optic nerve. Overview of the individual retinal layers: The retinal pigment epithelium lies above the choroid and the overlying Bruch's membrane, followed by the outer segments of the photoreceptors, the outer nuclear layer, the outer plexiform layer, the inner nuclear layer and the inner plexiform layer, the ganglion cell layer and finally the nerve fiber layer. The five neuronal cell types within the retina are (in the order of the visual signaling pathway) photoreceptors, horizontal cells, bipolar cells, and amacrine cells, and finally the retinal ganglion cells. Three types of glial cells can be found in the retina: microglia, astrocytes, and Müller cells – Müller cells are macroglia, which can only be found in the retina. Created with Biorender.com.

positively charged particles are trapped in the vitreous body.[21] The molecular weight and charge of the administered drug significantly affect its distribution in the vitreous and its retinal bioavailability. After the vitreous body, the inner limiting membrane (ILM) is the next structural barrier for molecular diffusion to the retina.[22] ILM, formed by the footplates of Müller glial cells, has an average pore size of 10–25 nm and acts as a strict physical barrier against most nanoparticles. However, uptake and transcellular permeation via Müller cells may provide an alternative route to enter the inner retina from the vitreous chamber (**Figure 2**).[23]

At the cellular level, the retina is a complex organ composed of specialized cells.[24] The retina is an intricate structure that consists of sensory and supporting cells. The five primary

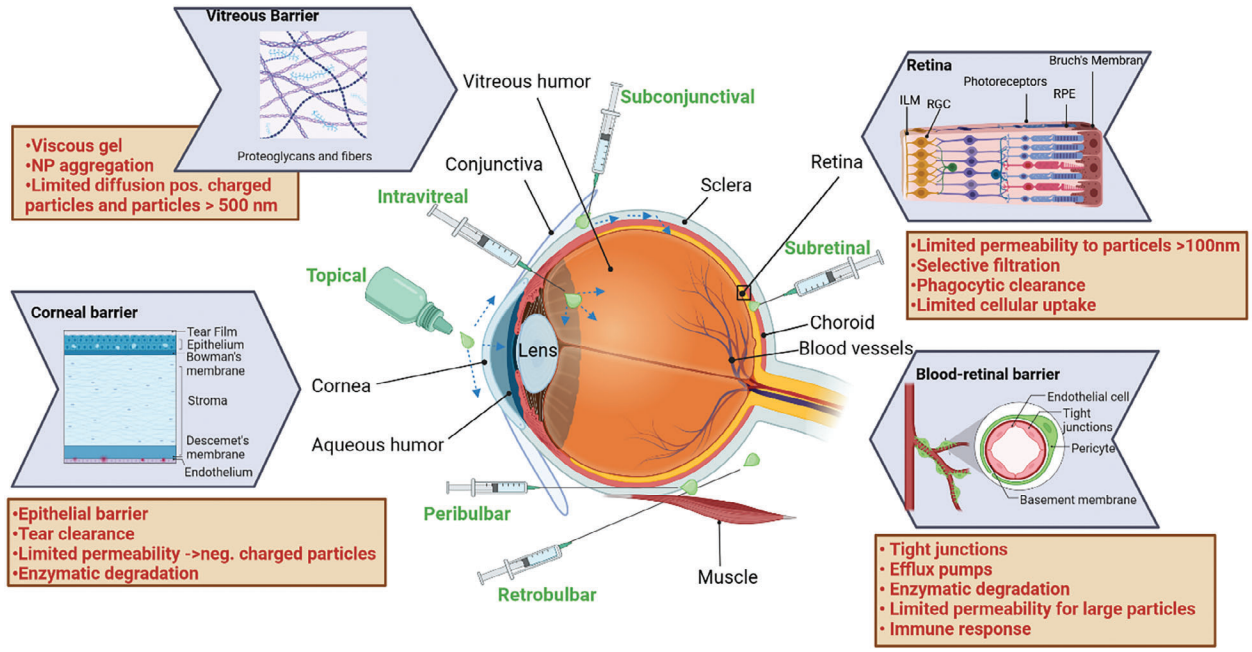

Figure 2. Anatomy of the human eye, barriers, and drug application methods. Ocular drug delivery can be accomplished via multiple pathways, including topical, systemic, intravitreal, and periocular administration. Barriers include the corneal barrier, the vitreous barrier, and the blood retina barrier. Abbreviations: ILM: inner limiting membrane; NP: Nanoparticles; RPE: retinal pigment epithelium; RGC: retinal ganglion cells. Created with Biorender.com.

neuronal cell types within the retina are photoreceptors, bipolar cells, amacrine cells, horizontal cells, and ganglion cells.[25] Additionally, three types of glial cells (microglia, astrocytes, and Müller cells) play essential roles in supporting and maintaining retinal function.[26]

The structure of the retina presents difficulties in delivering gene therapy, primarily due to cellular barriers, including the blood-retinal barrier. The blood-retinal barrier (BRB), similar to the blood-brain barrier (BBB), poses a significant challenge for foreign substances and pharmaceutical agents to reach the target tissue of the eye, making it difficult to administer certain drugs to treat retinal diseases.[27] For ocular therapeutics to reach their target site, various barriers must be overcome depending on the application form. The presence of BRB poses a significant challenge to the systemic administration of drugs for the treatment of retinal diseases. The inner blood-retinal barrier (iBRB) is a unique and highly restrictive physiological barrier that separates the retina from the bloodstream barrier and closely resembles the BBB. It consists of retinal capillary endothelial cells (RCECs) joined by tight junctions, covered with pericytes and glial cells (Müller cells or astrocytes) (Figure 2).[28] The iBRB is formed by two capillary beds: the inner capillary bed within the ganglion nerve cell layer, regulated by astrocytes, and the outer capillary bed within the inner and outer plexiform layers, where Müller cells control BRB function. The inner and outer components regulate the movement of ions, proteins, and water into and out of the retina. This barrier is crucial for maintaining the privileged status of the eye and for ensuring normal visual function.[27,28]

The outer blood-retinal barrier (oBRB) is composed of retinal pigment epithelium (RPE) cells connected by tight junctions. The RPE is a monolayer of pigmented cells situated between the neuroretina and choroid. Its apical membrane faces the outer segments of photoreceptor cells, while its basolateral membrane faces Bruch's membrane.[29]

In addition to the cornea, the sclera is another substantial barrier with limited permeability for drug delivery to the eye. It is a dense, hydrophilic, collagenous connective tissue that constitutes the outer shell of the major eyeball and consists of an intersecting stacked scleral collagen matrix with a negatively charged proteoglycan matrix in the inter-fibril space.[30] The thickness of the sclera appears to be a critical factor for transscleral drug delivery.[31]

The choroid, situated between the sclera and retina, plays a crucial role as an active barrier because of its extensive vascularization and innervation, which ensure blood supply to the retina. The choroidal blood flow in the eye exhibits one of the highest rates in the human body, with an estimated volume of ≈800–1200 mL per minute per 100 g of tissue when measured by volume per tissue mass. This elevated blood flow rate is essential for maintaining the temperature and oxygen supply in the retina, as well as for facilitating the removal of metabolic waste products.[32]

The choroid can be divided into five distinct layers, from the outermost layer to the innermost: the suprachoroidal cavity, two vascular layers, choroid capillary layer, and Bruch's membrane. The Bruch's membrane, is a thin collagenous membrane (2-4 µm) sandwiched between the choriocapillaris from the outside and the RPE from the inside. Because RPE expresses a wide range of enzymes (esterases, peptidases, dehydrogenases, and cytochrome enzymes) and efflux proteins, it serves as a metabolic barrier to drug permeability.[33] The Bruch's-choroid complex presents a more significant obstacle to drug delivery through the transscleral pathway than through the sclera itself. It can bind solutes, particularly positively lipophilic drugs, resulting in the formation of a slow-release drug depot within the Bruch's-choroid complex.[34]

1.2. Ocular Drug Administration

Various methods can be applied for administering ocular drugs to the eye.[35] The anterior eye segment can be treated using topical or subconjunctival routes, or by injecting drugs into the eye. Drugs can also be applied topically, systemically, or periocularly to treat the posterior eye segments (Figure 2). Additionally, drugs can be administered into the suprachoroidal space or directly into the eye via intravitreal injection. The effectiveness of drug administration depends on factors such as the specific site of administration, tissue barriers, and properties of the drugs being used.[36] One of the main objectives of drug delivery research is to enhance and prolong the topical penetration of drugs through the cornea. However, this process is inefficient. After topical application to the cornea, less than 3% of the administered drug can penetrate the anterior chamber of the eye, primarily because of the efficient drainage system in the anterior part of the eye.[37] Although topical delivery is less effective in reaching the posterior segment, it is preferred for its ease of use compared to invasive methods, such as intravitreal injection, which can result in serious complications.[38] Another method of transport to the posterior segment of the eye is systemic administration, mainly via intravenous and oral routes, which enters the eye through the choroidal capillaries. However, the blood-retinal and blood-aqueous barriers prevent the penetration of drug molecules, necessitating a higher dose to achieve drug efficacy, leading to increased drug toxicity.[39]

1.2.1. Topical Administration

Topical administration is the easiest and cheapest method of drug administration. However, the toughest barrier to overcome with topical application is the pre-corneal barrier, which consists of the tear film and blinking of the eyelid. These two factors combined can eliminate up to 99% of the applied medication.[40] Corneal uptake is hindered by the multilayered structure of the cornea, with a lipophilic epithelial layer, an endothelial layer, and a hydrophilic stroma between.[41] After topical application, absorption through the conjunctiva is also possible. The permeability of the conjunctiva is higher; however, efflux pumps and clearance through the vasculature hinder drug uptake through this pathway. A maximum bioavailability of 5% was achieved.[42]

If a topically applied drug aims to reach the posterior segment, further permeation through intraocular tissues is required, and bioavailability is very low. To reach the retina, drugs can either take the transcorneal route through the anterior chamber and vitreous body or the periocular route through the conjunctiva, sclera, and choroid.[19] Moreover, some studies have shown promising results for the delivery of substances to the retina via

topical application in animal models, suggesting their potential for future human applications.[43]

1.2.2. Intravitreal Injections

Intravitreal injections represent the most prevalent method of intraocular drug administration. Intravitreal injection of VEGF inhibitors is the standard treatment for age-related macular degeneration (AMD). The distribution and elimination of drugs in the vitreous body are highly dependent on injection location.[44] After injection into the vitreous, the drug has to diffuse through the vitreous. The ocular half-life of intravitreally administered VEGF inhibitors ranges from seven to ten days.[45]

Ocular injections are costly, unpleasant, and invasive but also lead to higher drug availability in the posterior segment, including the retina.[46] In recent years, intravitreal injections of drugs into the posterior segment of the eye have become more common.[47] This type of administration has the advantage that most of the barriers can be bypassed. The intravitreal retention times increased with increasing molecular weight of the drug. Therefore, large molecules (40– 70 kDa) tend to have longer retention times because of the dense barrier surrounding the vitreous.[48] To pass through the collagen network, a size smaller than 550 nm is necessary.[49] Also, polyplex dimensions have an influence at the cellular uptake mechanism. Research indicates that larger particles are internalized more slowly, primarily through caveolae-mediated endocytosis or macropinocytosis, while smaller particles are rapidly taken up, mainly via clathrin-mediated endocytosis. Consequently, larger polyplexes might progress more slowly within the cell.[12]

1.2.3. Subretinal Injection

Delivery via subretinal injections is a method of delivering therapeutic agents directly to the subretinal space located between the retinal pigment epithelium and the photoreceptor layer of the retina. This route is particularly relevant for the treatment of conditions that affect the outer layers of the retina, such as retinal dystrophies. The subretinal approach, which is mainly applied for AAV-based gene therapy, aims to provide high local concentrations of the drug or mainly the gene while minimizing systemic exposure and potential side effects.[50] However, subretinal injections are highly invasive and carry several risks, including retinal detachment and endophthalmitis, which may limit their use in severe cases.[51] Additionally, the procedure typically requires surgical intervention by a highly trained specialist.

1.2.4. Periocular Injection

The periocular route of drug administration is an alternative to topical and systemic delivery for the treatment of ocular diseases, with the aim of overcoming barriers to drug absorption encountered with conventional methods. Periocular refers to the administration of medication around the eye but outside the eyeball itself. The periocular route, which encompasses the peribulbar, posterior juxtascleral, retrobulbar, subtenon, and subconjunctival routes, is widely regarded as the most effective way to deliver drugs to the posterior eye segment.[52] This route involves positioning the drug solution close to the sclera, resulting in high drug concentrations in the retina and vitreous body.[53] However, periocular injections especially of steroids are associated with potential side effects, such as elevated intraocular pressure, cataract formation, and corneal decompensation, which limit their desirability.[51]

Further sclera, which is primarily composed of fibrous tissue, has low drug permeability and the retinal penetration through sclera is very limited.[54] Despite these drawbacks, periocular injections remain part of the therapeutic arsenal because of their ability to deliver higher drug concentrations to target tissues than topical applications.[55] Ongoing research into advanced drug delivery systems seeks to mitigate these risks and improve the therapeutic outcomes of periocular administration.[56]

1.2.5. Suprachoroidal Injections

Suprachoroidal injections represent an innovative approach to ocular drug delivery, specifically targeting the posterior segment of the eye. This method facilitates direct access to the choroid and retina, offering high bioavailability and minimizing systemic exposure.[57] The technique has been validated in animal models and is showing promise in clinical trials for a range of conditions, including macular edema, age-related macular degeneration, and choroidal melanoma.[58] The pharmacokinetic profiles of drugs administered via this route are favorable, with several small molecule suspensions and gene therapies currently under investigation.[57,58] Suprachoroidal injections have demonstrated potential advantages over traditional methods, such as intravitreal injections, by providing targeted therapy with fewer complications and a reduced treatment burden.[59] The technology is rapidly advancing, with ongoing research to optimize drug formulations and delivery devices to enhance the safety and efficacy of treatments for retinal and choroidal disorders.[60]

1.3. Retinal Diseases

Prominent diseases that disturb the posterior segment of the eye include AMD, diabetic retinopathy (DR), retinitis pigmentosa (RP), Usher syndrome, Stargardt disease (STGD), and Leber congenital amaurosis (LCA).[15,61]

1.3.1. Acquired Retinal Diseases

DR is the primary cause of blindness among working-age individuals and affects an estimated 93 million people worldwide.[62] This occurs because of insufficient oxygen and nutrient supply to the retina, leading to vision impairment. Chronic retinal ischemia and vascular endothelial growth factor upregulation contribute to vision loss by causing neovascularization and retinal hemorrhage. Prevention through blood sugar and blood pressure control is crucial because treatment options are limited. Laser photocoagulation, anti-VEGF therapy, and steroid supplementation are some of the available treatments.[63]

AMD is a leading cause of central vision loss in individuals over 65 years of age, with estimates projecting 288 million

affected individuals by 2040.[64] AMD involves the degeneration of the retinal pigment epithelium (RPE) and photoreceptor cells in the macula. Drusen accumulation, RPE dysfunction, and changes in Bruch's membrane permeability characterize its pathophysiology.[65] AMD manifests as two primary forms: dry or atrophic macular degeneration (dAMD) and wet or neovascular macular degeneration (nAMD). dAMD is characterized by the presence of small yellow deposits, known as drusen, beneath the retina, leading to a gradual breakdown of light-sensing cells and a corresponding loss of central vision.[66] In contrast, nAMD involves the growth of abnormal blood vessels under the macula, which can leak fluid or blood and cause rapid and severe vision loss.[64b] Current non-gene therapies for nAMD, including anti-VEGF agents, aim to inhibit angiogenesis mainly with antibodies (fragments). Although these therapies slow disease progression, they require regular intravitreal injections into the eye, which is stressful and can lead to various serious side effects.[67] The recent approval of the complement inhibitors Syfovre® (pegcetacoplan) and Izervay® (avacincaptad pegol) has given rise to great hope; however, the data from the corresponding phase III studies make it clear that the danger of occlusive vasculitis can occur here and that these therapies probably only have a positive risk-benefit ratio in a small proportion of patients with a subtype of dAMD (early extrafoveal geographic atrophy). Genetic factors such as complement factor H (CFH) play a significant role and are potential therapeutic targets.[64c,68]

1.3.2. Inherited Retinal Diseases

Inherited retinal diseases (IRDs) are a group of genetically and clinically diverse ocular disorders that can lead to vision impairment and blindness. They affect ≈1 in 1000 people worldwide, with over 300 causative genes identified.[53] These conditions are characterized by progressive degeneration of the retina, ranging from severe forms, such as Leber congenital amaurosis, to milder dysfunctions, such as night blindness, and are caused by mutations in various genes.[69] RP, STGD, Usher syndrome, and LCA are among the most common forms of IRDs.

Retinitis pigmentosa is the most prevalent form of IRDs, with pathogenic variants identified in over 80 different genes with mutations inherited in autosomal recessive, dominant, or X-linked patterns. RP is a rod-cone dystrophy, and its clinical phenotype can vary significantly, depending on the specific genetic mutation involved. It can lead to night blindness and tunnel vision, affecting approximately one in 3500 individuals.[61c,70] RP-associated genes have various functions including phototransduction, retinal metabolism, tissue development, cellular structure, and splicing. Despite extensive studies, the understanding of genotype–phenotype correlations remains incomplete and is often complicated by multiple clinical features resulting from mutations within the same gene.[53] For instance, *RHO*, a prominent RP-associated gene, encodes a 7-transmembrane receptor protein crucial for initiating the phototransduction cascade. Mutations in *RHO* typically follow an autosomal dominant inheritance pattern, leading to RP, often through gain-of-function or dominant-negative effects.[71] Another gene, *USH2A*, is associated with autosomal-recessive RP and Usher syndrome type 2, which include both vision and hearing loss.[72] Gene therapy, particularly using adeno-associated viral vectors (AAV), has shown promise for improving visual acuity in some patients with RP.[73]

Usher syndrome is part of a broader heterogenous category of genetic conditions that cause dual sensory impairment, and the retinal phenotype can be indicative of a specific disorder. It is classified into three clinical subtypes based on the severity and age of onset: profound deafness and vestibular dysfunction in Usher type 1, mild-to-severe to severe hearing loss in type 2, and milder, progressive deafness in type 3.[74] Yet, this classification system only partially captures the complexity of Usher syndrome.[75] The syndrome follows an autosomal recessive inheritance pattern and affects approximately 1 in 12 000 to 1 in 30 000 individuals, with mutations in genes such as *MYO7A* and *USH2A* identified as key players.[76] However, over 17 genes are under discussion to be associated with the different types of Usher syndrome.[75] These mutations affect proteins crucial for cellular transport and maintenance, affecting both photoreceptors and hair cells.

Stargardt's disease is a type of macular dystrophy that causes loss of central vision. It is one of the most frequently observed macular dystrophies in young adults and develops gradually over time. Its occurrence is estimated to be approximately one in every 8 000-10 000 individuals.[77] Patients with Stargardt's disease experience significant difficulty with tasks requiring central vision compared with those with RP who have peripheral vision loss.[78] Mutations in *ABCA4*, located on chromosome 1p22.1, contribute to STGD, cone-rod dystrophy, and RP. *ABCA4* encodes an ATP-binding cassette (ABC) transporter protein that is crucial for removing toxic substances such as lipofuscin from photoreceptor cells. Variations in *ABCA4* activity led to diverse clinical presentations, with mild reductions causing STGD in 95% of cases, moderate loss resulting in cone-rod dystrophy, and complete loss leading to RP. Other mutations in genes such as *STGD4, ELOVL4*, and *PRPH2* also contribute to STGD, affecting the production of essential fatty acids and cell function, with *ELOVL4* mutations causing clumping and cell death. These mutations highlight the complex genetic landscape that underlies inherited retinal diseases.[77]

Leber congenital amaurosis is a severe congenital retinal condition and the primary cause of childhood blindness. Symptoms manifest early within the first six months of life, including nystagmus, strabismus, and photophobia. Diagnosed in 2–3 infants per 100 000 births, LCA affects both sexes equally and currently lacks cure.[79] Ongoing research has focused on genetic and molecular studies to identify genetic variations for effective treatment. Innovative technologies such as CRISPR offer promising avenues for genetic editing. Notably, the FDA-approved Voretigene neparvovec (Luxturna ®), the first vision-restoring therapy targeting the *REP65* gene, showed significant success in clinical trials for LCA caused by mutations in *RPE65*, maintaining vision for at least three years in participants aged 4–44.[79,80]

In summary, IRDs such as RP, Stargardt disease, Usher syndrome, and LCA represent a complex group of genetic disorders with significant clinical heterogeneity. Advances in genetic characterization and therapeutic strategies, including gene therapy, offer hope for the treatment and management of these conditions.

2. Delivery of Free Nucleic Acids or Using Viral Vectors

2.1. Transfection of Free Nucleic Acids

Gene therapy encompasses four primary strategies. The first, known as gene replacement or augmentation, involves introducing a functioning copy of a defective gene to enhance the production of normal protein. The second approach, gene editing, focuses on rectifying mutations within a gene or decreasing the expression of the mutated protein to alleviate a disease state. The third method, gene silencing, utilizes the RNAi mechanism to suppress the abnormal expression of targeted pathogenic proteins in acquired disorders. Lastly, modifier gene therapies employ a modifier gene that can influence pathways upstream or downstream of the impaired gene, enabling the alteration of multiple gene expressions through a single intervention.[81] Nucleic acids used to treat retinal diseases includes plasmid DNA (pDNA), antisense oligonucleotides (ASOs), small interfering RNA (siRNAs), microRNAs (miRNAs), and short hairpin RNA (shRNAs). pDNA is typically used for long-term gene expression in dividing cells (e.g., retinal pigment epithelial cells), while siRNA and antisense oligonucleotides are more suitable for silencing gene expression in the cytoplasm of non-dividing cells, which is important for diseases like macular degeneration.

The RNA molecules regulate gene expression through mechanisms such as RNA interference (RNAi) and post-transcriptional gene silencing, which can lead to the inhibition of pathological processes such as angiogenesis and inflammation, which are implicated in retinal diseases.[82]

Interestingly, although siRNA therapy has been noted for its high specificity in silencing genes, shRNA has been used to 'knock down' genes with autosomal dominant gain-of-function mutations. Additionally, ASOs are used to correct splicing defects and translational read-through inducing drugs (TRIDs) to increase the expression of full-length proteins from genes with premature stop codons.[82a] pDNA, which offers long-term expression, may be suitable for chronic conditions but requires a delivery system due to its larger size and risk of immune response. In contrast, mRNA provides transient expression, ideal for applications requiring short-term gene editing with reduced risk of integration into the genome, and can be delivered more easily, sometimes even as naked RNA.[81]

However, in gene therapy for retinal diseases, plasmids and viral vectors are commonly used as delivery vehicles to introduce therapeutic genes into the target cells. Plasmids, which are circular DNA molecules capable of self-replication, offer advantages, such as ease of manipulation and safety, owing to their non-viral nature. On the other hand, viral vectors derived from viruses, such as AAV or lentivirus, efficiently deliver genes into target cells but may raise concerns regarding immunogenicity and potential integration into the host genome and are limited in their loading capacity.[83]

Current progress in genome editing for retinal disorders emphasizes the utilization of nucleic acids, specifically pDNA and mRNA, which encode endonucleases like CRISPR-associated proteins, delivered alongside guide RNA. Various gene editing methodologies have been established, including CRISPR/Cas9, transcription activator-like effector nucleases (TALENs), zinc finger nucleases (ZFNs), and homing endonucleases or meganucleases.[84] Among these, the CRISPR/Cas9 system stands out as the most renowned gene editing technique, demonstrating promise in gene therapy. CRISPR/Cas9 operates as a dual-component system, comprising a guide RNA tailored to the target gene and an endonuclease that generates a site-specific double-stranded DNA break, enabling genetic modification. This approach facilitates precise and lasting alteration or elimination of mutations linked to specific diseases.[85]

The use of naked DNA/RNA in gene therapy is challenging because of several factors. First, nucleases can easily degrade these molecules, thereby reducing their effectiveness.[86] Additionally, the difficulty in penetrating cell membranes efficiently hampers their uptake by the target cells. Furthermore, immune responses triggered by foreign DNA/RNA can result in inflammation and potential adverse reactions despite the eye being an immune-privileged site.[87]Another significant obstacle is the effective targeting of specific tissues or cells, and avoiding off-target effects. Overcoming these challenges is essential for the successful application of naked DNA/RNAs in gene therapy.

Despite these challenges, the direct introduction of genetic material into the retina through naked DNA delivery offers a novel non-viral approach for treating inherited retinal diseases. This method avoids the use of viral vectors that are commonly employed in gene therapy.[88] The success of this approach includes the ability to avoid immunogenic responses typically associated with viral vectors as well as the potential for targeted and minimal damage delivery, as demonstrated by the use of pulsed femtosecond laser microbeams.[88] Femtosecond laser-based optoporation involves creating temporary openings in cellular membranes through dielectric breakdown using concentrated laser pulses. Previous research by the team demonstrated the application of near-infrared (NIR) ultrafast laser beams for localized transfection of opsin-encoding plasmids into mammalian cells and *ex vivo* retinal tissue.[89]

Additionally, non-viral vectors, such as minicircles, offer the advantage of reduced immune responses owing to the absence of bacterial DNA, which is a significant consideration in the development of gene therapies.[90] An example of this would be AGN211745, which targets VEGF receptor 1 (VEGFR1/FLT1), and another siRNA to combat AMD (bevasiranib), which was administered as a naked nucleic acid designed to inhibit VEGF itself. These drugs can also be used to target diabetic macular edema (DME). A phase III clinical study of bevasiranib, which was the first siRNA therapeutic to reach phase III, was terminated because of inefficiency, and clinical trials of AGN211745 were not continued. However, both drugs were well tolerated with no reported side effects.[91]

However, several challenges are associated with the delivery of naked DNA. One of the main issues is the efficiency of gene transfer, which is often lower than that of the viral methods. Overcoming the physical and physiological barriers within the eye to achieve effective delivery to the neural retina remains a key obstacle.[90] Furthermore, although non-viral methods such as ultrasound-targeted microbubble destruction (UTMD) are promising, they are still in the early stages of application for ocular diseases, and several problems must be addressed before they can be widely adopted.[92]

In summary, naked DNA delivery methods offer certain advantages over viral vectors, such as reduced immunogenicity and potential for targeted delivery when combined with specific carriers or targeting strategies. However, it should be noted that the immunogenicity of the delivery system will depend significantly on the identity of the nucleic acid and the carriers used. They also face significant challenges including low efficiency and the need to overcome ocular barriers. Advancements in non-viral delivery techniques and a better understanding of their mechanisms are essential for improving their translational capacity and realizing their potential in the treatment of inherited retinal diseases.

AGN211745 (Sirna-027) was one of the first siRNA drugs to enter clinical trials and target the eyes. However, chemically modified siRNA yields better efficiency. This RNAi-based treatment has been studied for the treatment of choroidal neovascularization (CNV) resulting from neovascular AMD by targeting the VEGF receptor 1 (VEGFR1/FLT1). In a clinical trial, intravitreal injection of a single dose of Sirna027 was well tolerated and no dose-limiting toxicity was found.[93] These findings were in accordance with preclinical toxicity studies of Sirna-027, with no evidence of ocular inflammation. Observations also included stabilization or improvement in visual acuity and foveal thickness.[93] Thus, carrier systems improve transfection efficiency, and further studies on the specific functioning of siRNA in the eye could lead to novel therapeutic approaches. Naked siRNA injected into the eye has shown some potential, though transfection efficiency remains a challenge. The use of carrier systems could significantly improve transfection efficiency and enhance therapeutic outcomes. Further studies on the specific functioning of both naked siRNA and siRNA delivered via carrier systems in the eye could lead to novel therapeutic approaches. Over the years, several siRNAs and antisense oligonucleotides (ASO) have been used in clinical trials for the treatment of eye diseases (AMD, glaucoma, diabetic macular edema, RP, LCA, etc.), and issues remain regarding the use of these drugs, necessitating further research into their mechanisms of action, delivery methods, and stability in ophthalmology.[94] Olsen et al. illustrated that the human sclera exhibits permeability to high-molecular-weight molecules, specifically those with a molecular weight of up to 70 kDa.[95] In a similar study, Asaharaet et al. used iontophoresis to deliver 6-carboxyfluorescein-labeled phosphorothioate oligonucleotides (S-ODNs) and a 4.7 kb plasmid expressing green fluorescent protein (GFP) to the eyes of rabbits. S-ODNs were detected in the anterior chamber, vitreous, and posterior retina at 5, 10, and 20 min post-iontophoresis, respectively.[96] Iontophoresis has emerged as a promising technology in drug delivery, particularly for ocular applications. This noninvasive technique enhances the penetration of pharmacological molecules through anatomical barriers using a small electric current. It allows for precise control over drug dosage, providing a flexible and tolerable method for delivering medications.[97] An additional delivery method used by Voigt *et al.* is transcorneoscleral coulomb-controlled iontophoresis (CCI), a variant of the basic iontophoretic technique, designed to maximize drug transfer across the cornea and sclera while preventing tissue burns.[98] Rat anti-nitric oxide synthase II oligonucleotides (anti-NOSII ODNs) were more efficiently delivered in a rat model of endotoxin-induced uveitis (EIU) owing to enhanced penetration. Nitrite levels in the aqueous humor of rats that received anti-NOSII ODN treatment were significantly lower than those in control rats due to the downregulation of NOSII mRNA expression.[98a] These collective findings suggest that nucleic acids, oligonucleotides, or genes may represent promising candidates for transscleral electrophoretic or iontophoretic delivery to the posterior segment without loss of physical integrity or biological function.

However, current methods such as electroporation can cause significant damage and high cell death rates.[99] Palanker et al. employed a novel approach for the delivery of plasmid DNA to retinal cells, including RPE and photoreceptors. This involved the use of an array of microelectrodes placed behind the eyeball which produces synchronized pulses of mechanical stress and high electric field in proximity to the targeted cells for transfection (electron avalanche technique) while avoiding sensitive anterior eye structures. This study used animal models, particularly rabbits, to test transfection protocols, including subretinal injection, electroporation, and the newly developed electron avalanche transfection method. The primary drawback of electroporation is the elevated risk of cell death. Additionally, the study involved the use of the chorioallantoic membrane (CAM) of fertilized chicken eggs as a model tissue to observe the efficiency of transfection under tensile stress. In CAM, electron avalanche transfection was 10,000-fold more efficient and produced less tissue damage than electroporation did. This study showed that this method enables efficient and safe DNA transfer, particularly to the rabbit retina, after subretinal injection and transscleral electron avalanche transfection. The safety of the method was confirmed through electroretinography (ERG) and histology, and no signs of damage were observed.[99] Electron avalanche transfection has the potential to be a promising non-viral method for gene therapy, especially for the treatment of retinal degenerative disease; however, more studies are necessary for further evaluation.

2.2. Viral Vector-Delivered Plasmids

Viral vectors have traditionally been the preferred method for transferring nucleic acids into targeted tissues, and have dominated the field for a long time.[100] Even in clinical trials, patients responded positively to viral vector-delivered therapy.[101] Vectors utilizing adeno-associated viruses efficiently transduce various retinal cell subtypes even after a single intraocular administration. Most previous studies have focused on AMD, DR, RP, and LCA. Recombinant AAV (rAAV) vectors serve as versatile gene transfer tools because capsid proteins from numerous AAV serotypes can be easily exchanged. This interchangeability has led to the development of recombinant vectors with individual transduction properties.[102] Due to variations in interactions with different cellular receptors, the behavior of different AAV serotypes in ophthalmological applications is different.[103] Recombinant adeno-associated virus 2 (rAAV2) vectors are highly effective carriers for retinal gene delivery. Among the hybrid rAAV 2 variations (cross-packing of AAV type 2 into other AAV serotypes), rAAV-2/4 and -2/5 showed the highest efficiency when administered via subretinal injection in rats.[104] Subretinal injections into different animals, including mice, rats, monkeys, and dogs, have been studied in many groups with different rAAV2. In summary, rAAV-2/2 and rAAV-2/5 resulted in transduction of both RPE and photoreceptor cells, whereas

rAAV-2/4 and rAAV-2/1 showed transgene expression solely in the RPE.[102,105] Rolling et al. investigated the biodistribution of rAAV (rAAV-2/4. CMV.gfp or rAAV-2/5.CMV.gfp) following subretinal or intravitreal injection in rats and large animals. After subretinal or intravitreal injections, vector sequences were absent in the liver and gonads but were detected in peripheral blood mononuclear cells (PBMC). The findings revealed unexpected detection of vector sequences in the optic nerve after subretinal injection and in the brain, along the visual pathway, following intravitreal injection in dogs; however, this raises safety concerns for future gene therapy trials for retinal diseases.[106] This study highlights the need for thorough evaluation of vector biodistribution and its potential impact on the development of gene therapy trials for retinal diseases. The same authors conducted a follow-up study, in which they delivered AAV2/8. gfp vector in rats and dogs subretinally.[107] New rAAV serotype vectors have emerged, among which rAAV8 stands out. Vectors derived from AAV8 are particularly noteworthy for their exceptionally efficient gene transfer capability in vivo.[108] AAV2/8 leads to efficient gene transfer in the RPE, photoreceptors, cells of the inner nuclear layer, and RGCs. In dogs, gene transfer has also been observed at locations distant from the injection site, specifically within neurons of the lateral geniculate nucleus of the brain. Because green fluorescence was visible along the visual pathway within the brain, DNA was extracted from brain slices. It could be shown that vector sequences were predominantly present in the left hemisphere (after injection into the right eye). Transduction of RGCs was not observed in previous studies with other rAAV vectors, and none of these vectors showed such high transgene expression in the brain, suggesting that rAAV8 vectors can transit along neurons of the visual pathway.[107]

2.3. AAV-Based siRNA Delivery

In AAV-delivered siRNA gene therapy, siRNA is not directly encapsulated due to its instability and size constraints. Instead, a plasmid containing a short hairpin RNA (shRNA) expression cassette is delivered by the AAV vector. Regarding the utilization of RNA interference, the downregulation of rhodopsin expression in vivo by AAV-delivered siRNA targeting an allele-independent treatment of Autosomal Dominant Retinitis Pigmentosa (ADRP) caused by mutations in the RHO gene was demonstrated. AAV serotype 5 expressing either an active siRNA for rhodopsin downregulation (siRNA 301) or an irrelevant siRNA was subretinally injected into the right eye of wild-type or RHO +/− heterozygote mice. This in vivo siRNA delivery to the retina resulted in a 60% decrease in rhodopsin content, as determined by western blotting, when active siRNA was used. However, only RHO +/− mice were used to validate the application of the siRNAs delivered by AAV. The use of an ADRP mouse model would require the delivery of therapeutic agents within the right time frame to prevent the substantial loss of photoreceptor cells in ADRP.[109]

Another study tested the adenovirus-mediated expression of shRNAs targeting VEGF in a mouse model using RNAi. AdVEGFRFP, AdBFPH1VEGFiNS, and AdBFPH1VEGFi were injected into the subretinal space of C57BL/6J mice and the effects of treatment on CNV in the retina were observed. AdVEGFRFP is an adenovirus construct designed to express VEGF165 and red fluorescent protein (RFP), whereas AdBFPH1VEGFiNS and AdBFPH1VEGFi are adenovirus constructs expressing nonspecific shRNA and specific shRNAs targeting VEGF, respectively. While adenovirus vectors expressing high levels of VEGF could induce CNV in mice within five days, co-injection of VEGF-expressing viruses into mice with shRNA targeting VEGF led to an 84% reduction in CNV. These findings indicate that the adenovirus-mediated shRNA constructs efficiently silenced high levels of VEGF, potentially allowing for the long-term suppression of overexpressed VEGF. Potential shRNA sequences that could silence VEGF in human RPE cells were also identified. However, the study also discusses potential challenges, such as immunogenicity and the need for further investigation into long-term effects and immune responses to shRNA expression.[110]

Despite some studies showing promising results, not all studies were effective in all aspects of disease treatment. While previous studies by Ali et al. showed improvement of photoreceptor function in young Prph2$^{Rd2/Rd2}$ animals, a model of recessive RP that lacks the gene *encoding peripherin 2 (Prph2)* after subretinal injection of rAAV encoding a *Prph2* transgene, unsatisfactory results in terms of transgene expression, transduction rate, and reduced loss of photoreceptor cells were observed in older animals in a follow-up study.[111] These studies further highlight the complexity of studying gene therapy in various ways. Although some promising reports have indicated the clinical potential of AAV-therapeutic approaches for ocular delivery, they still have several physical limitations. These include significant toxic side effects, such as immune response stimulation, inflammation, and the development of neutralizing antibodies, which can occur with repeated administration.[112] Such limitations of viral vectors are often observed in studies, and sometimes, even mortality is observed. A fatal systemic inflammatory response syndrome (SIRS) was observed in an 18-year-old male patient with partial ornithine transcarbamylase (OTC) deficiency following adenovirus-based gene therapy, The AAV contained human OTC cDNA and was injected into the right hepatic artery. Disseminated intravascular coagulation and multi-organ failure can lead to patient death. Vector DNA sequences were detectable in most tissues and death was directly attributed to AAV administration. This study highlights the unexpected and tragic consequences of the systemic administration of AAVs.[113]

3. Nanomaterials-Based Approaches

3.1. Cationic Polymers

When cationic polymers are used as non-viral carriers, nucleic acids (pDNA, mRNA, siRNA, etc.) condense into NPs by electrostatic interactions. These NPs or so-called polyplexes can be taken up by cells via endocytosis.[114] Clathrin-mediated endocytosis is often observed in positively charged NPs.[114c] Polyplexes that enter the cells via endocytosis often encounter problems associated with endosomal entrapment and lysosomal degradation.[115] Therefore, the development of suitable carriers that can induce the escape of material from the endosome before degradation by lysosomes is a major task in current research. As a specific pH value must be maintained in endosomes, these vesicles possess membrane-bound ion channels that can pump protons into the endosome.[114c] Due to the buffering capacity of

amine-containing polymers, endosomes can be destabilized due to a "proton-sponge effect" or by membrane disruption of positively charged polymers or polyplexes. According to proton sponge effect theory, it is assumed that the residual non-protonated amino groups of the polymers are protonated owing to a lower pH in the endosome, thus preventing the acidification of these vesicles. This leads to a constant influx of protons and passive entry of chloride ions. Osmotic swelling results in disruption of the membrane, release of genetic material into the cytoplasm, and successful transfection.[116]

3.1.1. Polyethylenimine (PEI)

PEIs are positively charged linear or branched polymers capable of forming nanoscale complexes with RNA and DNA.[117] In general, better transfection efficiencies as associated with liposomes are observed due to proton-sponge characteristics in the endosome leading to efficient endosomal escape. Although PEI is one of the most widely used polymers as a non-viral vector owing to its superior transfection efficiency, there is limited literature documenting its application in the ocular context. Similar to liposomes, intravitreal injection of PEI NPs and other cationic polymers often limits efficient nucleic acid delivery because the vitreous is a major barrier, most likely because of collagen fibers.[118]

PEIs can be synthesized differently, depending on the desired structure. Linear PEI was synthesized using precursor polymers (e.g., poly(2-oxazolines) and N-substituted poly(aziridines)). The post-polymerization modification of these precursor polymers can lead to linear PEI.[119] JetPEI ™ (molecular weight: ca. 22 kDa)[120] and in vivo-jetPEI™ are commercially available transfection agents that consist of linear, low-molecular-weight PEI. In one of the first studies on PEI in ophthalmic research, jet-PEI successfully delivered shRNA-expressing plasmid DNA to RGCs in vivo after intravitreal injection of PEI/DNA polyplexes in the eyes of mice.[121]

Branched PEI is produced via acid-catalyzed ring-opening polymerization of aziridine monomers, in which the reaction conditions determine the degree of branching.[122] Linear and branched PEI show different behaviors regarding their gene delivery performance in vitro and in vivo.[123] In the case of branched PEI, its high buffering capacity is caused by its specific polymer architecture, as it is a branched polymer consisting of primary, secondary, and tertiary amino groups exhibiting pKa values distributed over the entire physiological pH range.[124] However, as a consequence thereof, it also causes high cytotoxicity, which is associated with limitations and concerns in in vivo applications and with restrictions in clinical trials.[125]

Oligonucleotide-polyethyleneimine (ODN/PEI) complexes were prepared using branched PEI. FITC–ODN/PEI complexes were injected into the vitreous of rat eyes, and after 72 h, polyplexes were still found in the retina and glial cells; however, most fluorescence was observed in the nuclei of the superficial retina and in the inner nuclear layer. Additionally, *in vitro* delivery of TGF-b2 antisense oligodeoxynucleotide (As-ODN) to retinal Müller cells induced the specific downregulation of TGF-b2 production.[126]

Kim et al. developed a nanoscale polyRNAi-based therapy delivery system consisting of polymerized siRNA (polyRNA) complexed with branched PEI and coated with hyaluronic acid (HA) (**Figure 3**). Cy3-labeled polysiRNA-polyplexes were administered intravitreally to laser-induced CNV mice, and it was shown that these polyplexes overcome the barriers of the vitreous and retina and efficiently reach the subretinal space without being toxic to the retina. This was achieved by increasing the stability of the system first by polymerizing the siRNA by disulfide linkage and second by hyaluronic acid coating to enable penetration of retinal structures and to shield the positive charges to inhibit reactivity with the anionic collagen fibrils in the vitreous. Intravitreal injection of the anti-VEGF polysiRNA-polyplex significantly inhibited laser-induced CNV compared to the administration of monosiRNA and poly-siRNA (**Figure 4**).[127] These results again proved that the surface charge plays a significant role in the distribution of NPs after intravitreal injection. Previous studies have shown that positively charged PEI NPs strongly interact with negatively charged vitreous collagen fibrils.[128] However, cationic glycol chitosan and PEI/glycol chitosan NPs, with their surfaces modified by glycol groups, traversed the vitreous barrier and reached the inner-limiting membrane. This could be explained by the antifouling properties of these groups. However, they do not permeate the physical pores of the inner limiting membrane into the retinal structure. The two tested anionic nanoparticles, HA-NP (zeta potential: -26.2 ± 4.0 mV) and human serum albumin-NP (HSA; zeta potential: -20.9 ± 2.0 mV), showed superior penetration capabilities across the vitreous and retina up to the RPE due to limited interaction possibility between the anionic surface of the NPs and the anionic vitreal structures.[128] The selection and modification of nanomaterials, particularly considering parameters such as charge, size, and coating (e.g. with HA), seem to be crucial for ensuring a positive outcome in studies targeting the posterior eye segment.[128,129]

Based on these results, novel siRNA-based anti-VEGF nanoballs (siVEGF NB) were developed by the same authors using an siRNA hydrogel core coated with branched PEI and hyaluronic acid. Therefore, the constructs were large (660 nm). siVEGF NBs were used in a laser-induced CNV mouse model and were administered intravitreally. The therapeutic effects of siVEGF NBs lasted for up to two weeks and achieved high target accuracy for the subretinal space. siVEGF NBs enter cells through CD44 receptor-mediated endocytosis and bypass TLR3-dependent immune response, which is a crucial hurdle in siRNA therapy. in vitro and in vivo toxicity studies have revealed that siVEGF NBs are non-toxic to cell cultures and mice.[130]

3.1.2. Chitosan

Chitosan (a polysaccharide derived from the natural compound chitin) has been mostly studied for the treatment of anterior segment diseases due to mucoadhesion leading to prolonged time on the ocular surface after topical administration.[131] Only limited focus has been directed toward treatment strategies using chitosan for the back of the eye. Although some studies have concentrated on in vitro transfection of RPE cells with various chitosan NPs, the lack of complementary in vivo research may diminish the significance of these in vitro results.[132] In an in vivo study, Naash et al. synthesized glycol chitosan (GCS) NPs with pDNA expressing CBA-EGFP (EGFP = enhanced green

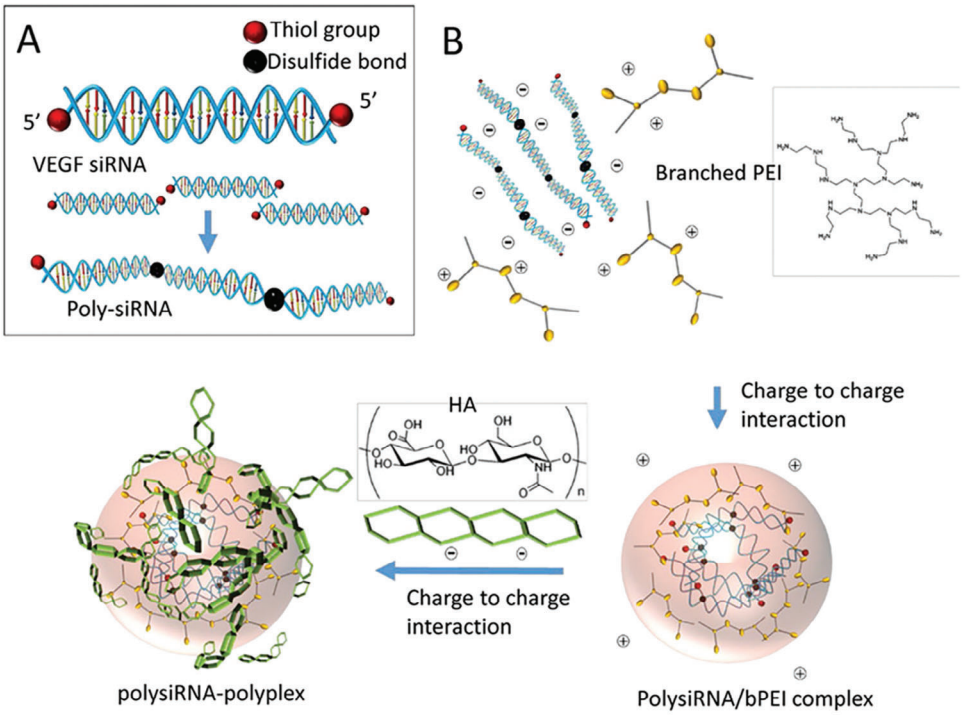

Figure 3. A) Synthesis of poly-siRNA using VEGF siRNA with thiol groups. B) Synthesis of the polysiRNA-polyplex, first reacting poly-siRNA and branched PEI to form a positively charged polyplex via electrostatic interactions. Subsequently, the positive charge was neutralized by coating it with negatively charged hyaluronic acid (HA). Reproduced with permission.[127] Copyright 2016, American Chemical Society.

fluorescent protein) and subretinally injected these NPs into adult wild-type mice. Fourteen days after injection, GFP expression was exclusively observed in the RPE.[133] This chitosan NP-mediated gene expression in RPE cells can last for at least six months post-injection.[134]

Oligochitosan (chitosan with a low molecular weight; NOVAFECT O25) was used to deliver the pCMS-EGFP plasmid into rat retinal cells. Subretinal and intravitreal administration was compared in terms of the type of transfected cells. Subretinal administration mainly led to the transfection of pigmented cells of the RPE and photoreceptor cells, whereas intravitreal injections transfected cells in the ganglion cell layer, blood vessels in the inner layers of the retina, and photoreceptors (**Figure 5**). This study showed that the method of administration influenced the distribution and efficiency of transfection. After 72 h, subretinal injections primarily targeted the outer layers of the retina, whereas intravitreal injections achieved higher transfection in the inner layers of the retina, as well as in the outer segments of the photoreceptors and RPE.[135] The effects of intravitreal injections of three different trimethyl chitosan (TMC)-hyaluronic acid (HA)

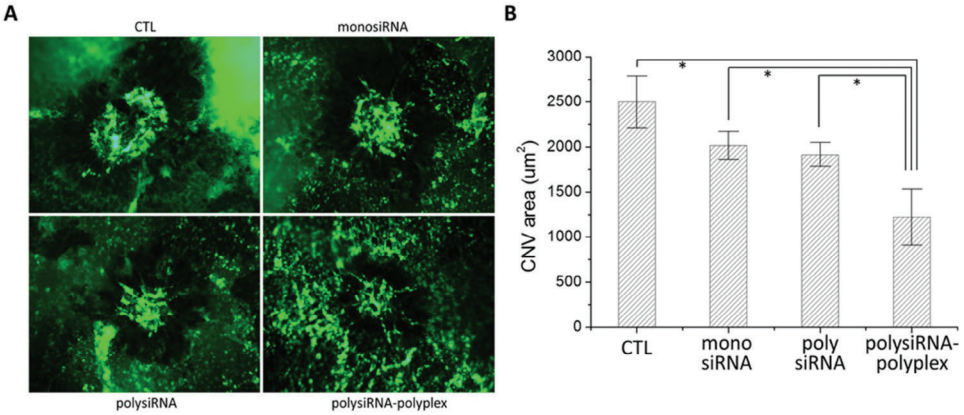

Figure 4. A) Representative image depicting CNV on day 12 after laser photocoagulation. Animals were assigned to groups that received intravitreal injections of control (CTL), free monosiRNA, free polysiRNA, or polysiRNA polyplexes. B) Quantitative assessment of CNV areas, indicating a significant inhibition of CNV by the polysiRNA polyplex compared to the control. Reproduced with permission.[127] Copyright 2016, American Chemical Society.

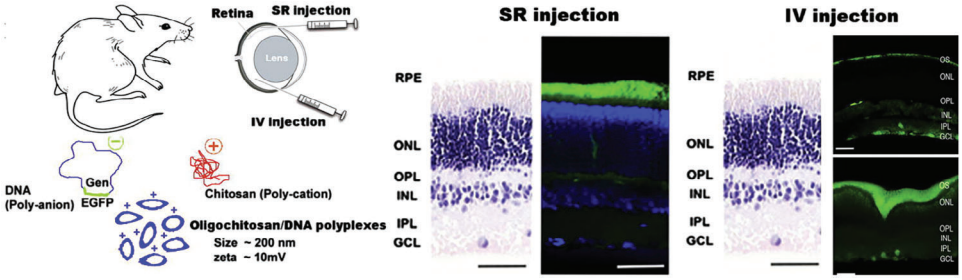

Figure 5. Left: Formation of polyplexes from chitosan (cation) and pDNA (anion) with sizes of approximately 200 nm and zeta potentials of +10 mV. Administration of oligochitosan/DNA polyplexes at a N/P ratio of 10 to rats by subretinal (SR) or intravitreal (IV) injection (schematic drawings). Right: in vivo gene expression of EGFP after SR or IV injection. Hematoxylin-eosin-stained cross-sections showing different layers of the retina. RPE (Retinal Pigment Epithelium layer), ONL (outer nuclear layer), OPL (outer plexiform layer), INL (inner nuclear layer), IPL (inner plexiform layer), GCL (ganglion cell layer), and fluorescence microscopy images of 5-µm treated retina cross-sections. EGFP expression in Hoechst 33342 staining of cell nuclei. Scale bar = 50 µm. Reproduced with permission.[135] Copyright 2012, Elsevier B.V.

polyplexes (weight ratios of 2:1, 1:2, and 1:1) were evaluated using a rat laser model of CNV. These VEGFR-2 siRNA polyplexes could cross both the vitreous and retinal barriers, probably because of the protection of the polyplex by hyaluronic acid. Additionally, TMC, a highly positively charged derivative of chitosan, was used to circumvent the problems associated with the low siRNA encapsulation ability. A reduction in the vascular area size of the CNV was observed for the polyplexes (36,501.67 ± 21,739.19 μm^2), as well as for free HA (71,346.97 ± 15,541.86 μm^2) and naked siRNA (46,287.39 ± 19,398.42 μm^2) with VEGFR-2 siRNA polyplex groups showing the significantly most reduced area of choroidal neovascularization lesions compared to control groups (113,728.15 ± 32,289.53 μm^2). Hyaluronic acid is crucial in numerous biological processes. Its interactions with cellular receptors and other extracellular partners are essential for organ and cell development, inflammatory responses, tissue injury reactions, cell movement, and resistance.[136] HA is recognized for its inflammation-reducing and tissue-regenerating qualities, which likely contribute to its capacity to diminish the vascular area in the CNV model. Inflammation significantly drives angiogenesis, the formation of new blood vessels. By inhibiting inflammatory cytokines such as VEGF (vascular endothelial growth factor), TNF-α, and interleukins, HA can reduce the size of the abnormal vascular region.[137] Additionally, HA is a vital component of the extracellular matrix, playing a role in tissue hydration and repair.[136] In a CNV model, HA may interact with the ECM to stabilize or repair damaged tissues, potentially preventing further neovascularization or promoting the regression of abnormal blood vessels.

Chitosan, poly(((cholesteryl oxocarbonylamido ethyl) methyl bis(ethylene) ammonium iodide) ethyl phosphate) (PCEP), a biodegradable polyphosphoester carrying a positive charge in its backbone and a hydrophobic cholesterol motif in the side chain, and magnetic iron oxide nanoparticles (MNPs) have been evaluated for the in vivo delivery of genes to the posterior eye segment.[138] Additionally, PCEP has already been successfully used for gene delivery, but not in the context of ocular delivery.[139] All three NP formulations have high potential for gene delivery to the posterior eye segment, while MNPs have the advantage that they are approved by the FDA because of their use as magnetic resonance imaging contrast agents.[140] While chitosan and PCEP are DNA-condensing agents due to their positive charge, MNPs have DNA tethered to a magnetic core. Rabbits were injected with NPs containing a plasmid DNA solution of EGFP, either intravitreally or subretinally. Eyes were analyzed for RPE abnormalities, retinal degeneration, and inflammation. In the case of intravitreal injections, formulations containing chitosan were the only ones that caused inflammation in most eyes. After subretinal injection, PCEP induced a lower transfection rate than MNPs did. Because the DNA-MNP was the only one in that study that showed significant transfection of RPE cells and photoreceptors, the author concluded that transfection with these NPs is the method of choice.[138]

3.1.3. Modified Peptides

Polycationic and lipophilic peptide dendrimers were developed by Toth et al. to combine the properties of lipids with those of peptides.[141] In these dendrimers, a branched poly(lysine) dendrimer head is attached to a linear peptide from lipoamino acids (Laa) (**Figure 6**). Dendrimers with different lipid residues, repeating units, and numbers of free amino acids on poly(lysine) can be synthesized via solid-phase synthesis. These modified peptides were used to deliver anti-VEGF ODNs (molar charge ratio of 5:1 (+/−)) into human RPE cells, as seen by reduced hVEGF-levels.[141] Rakoczy et al. used these peptide dendrimers for in vivo experiments to deliver an anti-VEGF oligonucleotide into the eyes of rats and inhibit laser-induced CNV.[142] Dendrimers successfully delivered ODN to retinal cells with zero to slight leakage for up to two months, indicating a reduction in the severity of CNV.[142a] Additionally, uptake, distribution, and retinal tolerance were examined in a long-term study.[142b] Compared to the two months of data, CNV inhibition was still present, but was reduced after four months. After six months, inhibition was no longer statistically significant.[142b] Extending the effective life of ODNs in vivo is a prerequisite for the development of efficient nucleic acid therapies using dendrimers. Another obstacle could be that divergent tendencies of toxicities/inflammation were reported with dendrimers.[142b,143]

To overcome these limitations, a new class of peptides has been developed. Naash et al. investigated the feasibility and effectiveness of compacted DNA NPs as neutrally-charged complexes containing a single molecule of plasmid DNA compacted with

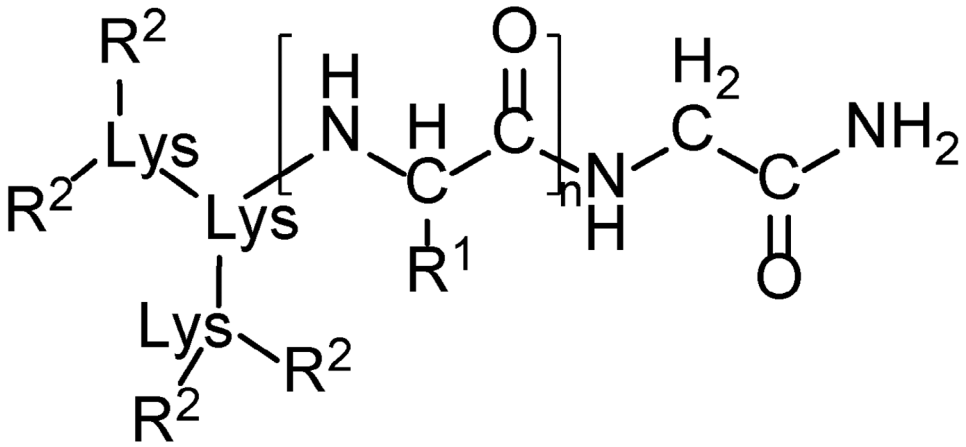

Figure 6. Polycationic Dendrimers on Lipophilic Peptide Core: (1) $R^1 = C_{10}H_{21}$, $R^2 = NH_2$, $n = 2$; (2) $R^1 = C_{10}H_{21}$, $R^2 = Lys\text{-}(NH_2)_2$, $n = 2$; (3) $R^1 = C_{12}H_{25}$, $R^2 = Lys\text{-}(NH_2)_2$, $n = 2$; (4) $R^1 = C_{10}H_{21}$, $R^2 = Lys\text{-}(NH_2)_2$, $n = 3$; (5) $R^1 = C_{12}H_{25}$, $R^2 = Lys\text{-}(NH_2)_2$, $n = 3$; (6) $R^1 = C_{12}H_{25}$, $R^2 = NH_2$, $n = 3$; (7) $R^1 = C_{16}H_{33}$, $R^2 = NH_2$, $n = 2$; (8) $R^1 = C_{16}H_{33}$, $R_2 = Lys\text{-}(NH_2)_2$, $n = 2$; (9) $R^1 = C_{16}H_{33}$, $R_2 = Lys\text{-}(NH_2)_2$, $n = 3$.[141]

polyethylene glycol-substituted 30-mer lysine peptides (PEG-CK_{30}). PEGylation was used to enhance the stability and make the particles more neutral, whereas cationic lysine was used to compact the DNA. PEGylation refers to the process of attaching polyethylene glycol (PEG) units to proteins or polymers. This modification can induce various effects on the target, such as enhancing hydrophilicity and modifying electrostatic binding characteristics. The NPs were stable in saline and serum, non-toxic following delivery, and capable of transfecting various ocular tissues, demonstrating their potential for non-viral gene transfer in the eye.[14] Prior to this study, their effectiveness and biocompatibility were shown in lung cells after in vivo delivery and in clinical trials in patients with cystic fibrosis (CF).[144] These NPs were generated in the presence of either acetate (AC) or trifluoroacetate (TFA) as counterions of the amino group of lysine during DNA compaction. The NPs exhibited different dimensional shapes based on the counterion used, with TFA particles being ellipsoids and short rods, while AC particles formed longer rod-like structures (**Figure 7**).[14,144b,145] The effect of the counterion on the morphology and shape of DNA NP is well known in the literature when using polycationic carriers.[145] The size and shape of compacted DNA NPs influence nuclear uptake and gene expression, with ellipsoidal NPs having a minimum diameter of 25 nm showing decreased efficiency, while rod-like NPs with a diameter smaller than the nuclear pore diameter facilitated gene transfer.[144b] Accompanied by the small size, it was shown that neutral PEG-CK_{30} nanoparticles enter cells via a different pathway (macropinocytosis) than that observed for positively charged nanoparticles, which could lead to higher transfection efficiency.[146]

Naash et al. utilized compacted DNA NPs in ocular gene therapy by conducting proof-of-principle experiments using a plasmid driven by the cytomegalovirus (CMV) promoter to express EGFP. This study also aimed to assess the potential of these NPs for targeted gene expression in various ocular tissues, including the retina, photoreceptors, and RPE, with the goal of providing a safe and efficient non-viral gene transfer approach for treating ocular diseases. Different tissues can be targeted for gene transfer by varying the injection sites in the eye. NPs (AC-GFP and TFA-GFP) were able to transfect nearly all cell types in the eye with dose-dependent gene expression levels. Subretinal injection resulted in the transfection of almost the entire photoreceptor population, with gene expression levels nearly matching those of the naturally highly expressed rod opsin gene in the retina (**Figure 8**).[14] However, despite the vast transfection efficiency, the expression was only temporary (two weeks).[147]

To increase the duration of gene expression and use a more disease-related context, the same group incorporated a therapeutic gene (wild-type retinal degeneration slow (*Rds*) gene) into these DNA NPs.[148] In a mouse model of RP, NPs were injected into the subretinal space of rds+/2 mice. Gene expression lasted for up to four months, with levels up to four times higher than those in the control groups. NPs are widely distributed in all photoreceptors and no toxic or side effects have been observed.[148a] Furthermore, the encapsulation of considerably larger vectors (13–14 kbp) containing the human ABCA4 cDNA resulted in prolonged transgene expression in RPE cells, lasting up to eight months.[149] Advanced work on the ocular safety profile was performed and no signs of local inflammatory responses and cellular toxic response associated with subretinal injection were observed, even after repeated dosing.[150]

Using a rhodopsin knockout (RKO) mouse model of RP, genomic sequences of the *rhodopsin* gene were encapsulated by PEG-CK_{30} and administered subretinally as murine rhodopsin cDNA or genomic DNA (gDNA) NPs. Functional and structural improvements in RKO eyes persisted for up to eight months following nanoparticle-mediated gDNA delivery, but not for the more commonly used cDNA delivery.[151] Such studies emphasize the importance of DNA and vector construction as well as carrier design in achieving efficient and long-lasting gene delivery.[151,152]

A comparative analysis of these NPs and AAVs revealed that gene expression was comparable and was observed for up to one year. However, while subretinally injected NPs remained in the eye, vector DNA and GFP expression in the visual pathways were found in the brains of some AAV-injected adult mice.[153]

In general, any type of nucleic acid (e.g., miRNA)[154] can be complexed by these polyethylene glycol-substituted 30-mer lysine

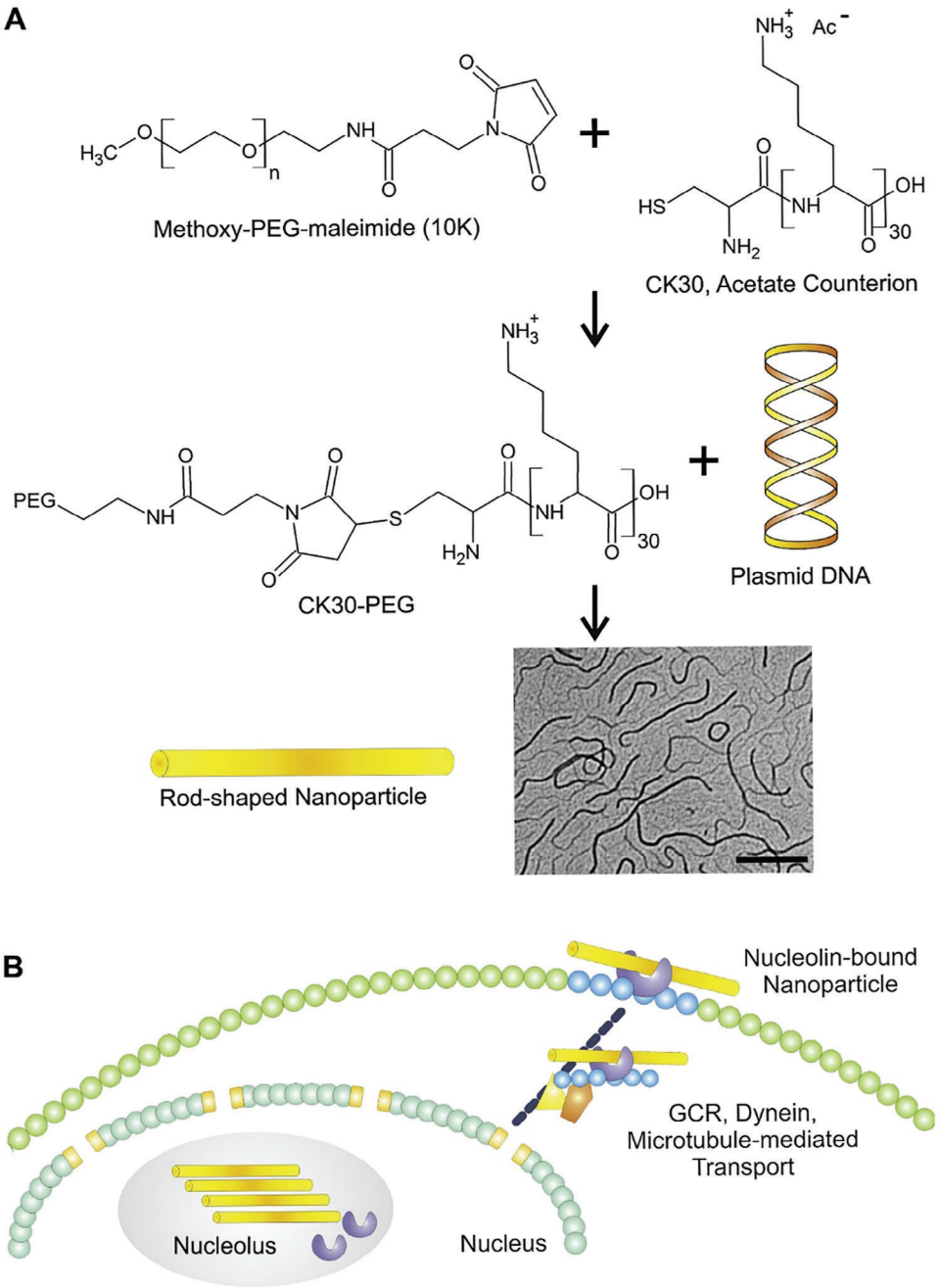

Figure 7. Synthesis and application of pDNA NPs using PEG-CK30. PEG-CK30 is synthesized by the reaction of maleimide-functionalized methoxy-PEG and CK30 with a thiol end group. In this figure, acetate is the lysine counterion, leading to rod-shaped NPs after complexation with pDNA (see the TEM image). NPs are internalized via a nucleolin-mediated pathway. These NPs bind to nucleolin within lipid rafts and are then internalized through raft-mediated endocytosis, although it remains unclear whether this pathway regulates RPE uptake. Subsequently, they are trafficked to the nucleus via the microtubules in conjunction with the glucocorticoid receptor (GCR) and dynein complexes. Within the nucleus, accumulation occurs primarily in the nucleolus. Reproduced with permission.[11] Copyright 2013, Elsevier.

peptides, and suprachoroidal injections are effective. High luciferase activity was detected in the retina, RPE, and choroid of eyes treated with suprachoroidally injected DNA NPs (rod and ellipsoid shapes) and subretinally injected DNA NPs (rod shape). The mean luciferase activity in the RPE choroid and retina was similar between suprachoroidal (sc) and subretinal (sr) administrations, and for both administration routes, transfection in the RPE choroid was ≈tenfold higher than that in the retina.[155] The significantly higher luciferase activity observed in the RPE-choroid compared to the retina suggests that the DNA NPs follow a concentration gradient after sc or sr injection, likely leading to a reduction in the number of DNA NPs reaching the inner

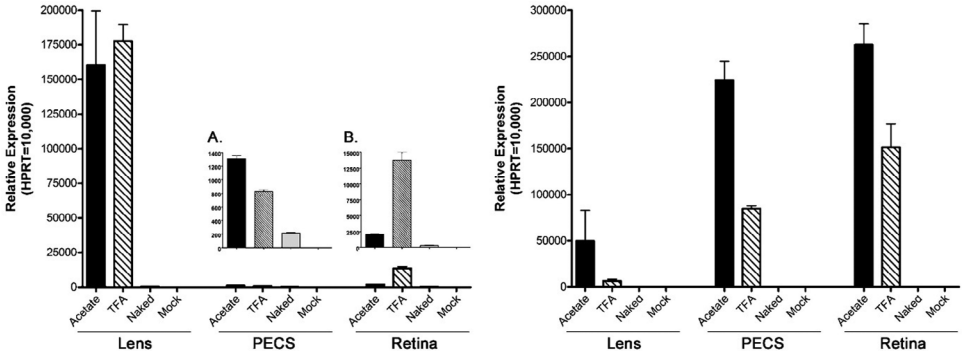

Figure 8. Left: Ocular expression levels of EGFP mRNA after intravitreal injection. AC-GFP and TFA-GFP nanoparticles promoted high levels of EGFP expression in the lens. Right: Ocular EGFP mRNA expression levels after subretinal injection. AC-GFP and TFA-GFP were very effective in enhancing EGFP expression in the retinal pigment epithelium, choroid, sclera (PECS), and retina. EGFP levels after AC-GFP delivery were twofold higher in the PECS and 1.5-fold higher in the retina than those in TFA-GFP. Reproduced with permission.[14]

retina. This could be due to the partial loss of DNA NPs from the suprachoroidal space, which warrants further investigation. However, the small size and neutral charge of the DNA NPs, combined with their potential for nucleolin-mediated nondegradative intracellular translocation, may facilitate their ability to cross ocular barriers such as the choroid, Bruch's membrane, and RPE. These properties likely enhance the particles' mobility through these layers, although further studies are needed to fully elucidate this transport mechanism.[155]

While the extensive results published between 2006 and 2016 by the Naash group indicate that PEG-CK30 DNA nanoparticles hold potential as a non-viral therapeutic approach for retinal diseases, to our knowledge, these findings have not spurred substantial follow-up research or commercial development. This may suggest challenges related to reproducibility or other obstacles that have hindered further progression toward clinical applications.

3.2. Cationic Lipid-Based Formulations

3.2.1. Liposomes and Lipid NPs

Liposomes are biocompatible and biodegradable lipid structures built by phospholipid bilayers. They can be internalized by cells through endocytosis, which makes them highly efficient in cellular uptake and transfection. Liposomes exhibit a wide range of sizes, from nanometers to micrometers, as they can exist as uni- or multilamellar structures. Liposomes are a highly adjustable delivery system capable of carrying both hydrophilic and lipophilic substances, ranging from small molecules to proteins and genetic material. They were the first nanotechnology-based drug delivery system to transition from theoretical concept to practical medical use. Several liposome-based drug treatments have been authorized for use and have been effectively integrated into healthcare.[156] An advancement of these liposomes are lipid NPs (LNPs). LNPs comprise a complex system of different lipids and substances: (1) An ionizable/cationic lipid; (2) a structural lipid/helper lipid; (3) cholesterol; (4) a PEGylated lipid; (5) the genetic material/nucleic acid payload.[157] LNPs can be customized to meet particular applications and therapeutic requirements. By altering the composition of the lipids, properties such as stability, drug-loading capacity, release kinetics, and targeting precision can be enhanced. One of the most prominent uses of LNPs is in delivering mRNA and siRNA. The most notable examples are the nucleoside-modified mRNA LNP vaccines against SARS-CoV-2, which were successfully developed by Pfizer-BioNTech and Moderna and authorized for use during the COVID-19 pandemic.[158] Within the broad field of lipid-based formulations (e.g., liposomes, LNP, etc), cationic lipids are used to form complexes with nucleic acids sometimes also referred to as lipoplexes. Lipoplexes typically include this cationic lipid/nucleic acid complex and a helper lipid. The key feature that distinguishes LNPs from lipoplexes is that the more advanced structure of LNPs cannot be easily predicted solely based on the chosen composition, in comparison with the simpler structures formed in lipoplexes.[159]

Examples of cationic lipids used for gene delivery are N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), [1,2-bis(oleoyloxy)-3-(trimethylammonio)propane] (DOTAP), 3β[N-(N′, N′-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol), and dioctadecylamidoglycylspermine (DOGS).[160] It is typical that quantities of neutrally charged lipids are included, especially dioleoylphosphatidylethanolamine (DOPE), owing to its pH-sensitive capability to destabilize lysosomal membranes following endocytosis.[161] Several studies exist on liposomal formulations with different lipids and ratios of lipids, and showed that the promising in vitro results depend on size, lipid composition, and bilayer fluidity, but they were not tested further in vivo.[162] Lipoplex performance was investigated after injection into the anterior chamber, subretinal space, and vitreous of the adult rats. Injection into the anterior chamber led to gene delivery to the basal layer of the corneal epithelium, ciliary epithelium, stroma of the ciliary body and iris, and RGCs. Injection into the vitreous or subretinal space results in gene expression in the ciliary epithelium, stroma of the ciliary body, iris, RGCs, and RPE cells.[163] DOTMA/DOPE (1:1 molar ratio) and DOTMA/cholesterol (Chol) (1:1 molar ratio) liposomes were prepared using an extrusion method to test these pDNA lipoplexes after intravitreal injection in rabbits. Transfection efficiency, measured by the luciferase activity of pDNA/DOTMA/Chol liposomes, was significantly higher than that of the negative

controls, wherein the highest gene expression was observed with complexes formed at a pDNA/cationic liposome ratio of 1:2. Luciferase activity with the highest dose of pDNA was observed in the aqueous humor, cornea, iris–ciliary body, lens, vitreous body, and retina.[164] In vitro studies have shown that DNA/liposome formulations can be taken up by RPE and corneal endothelial cells, leading to efficient gene transfection without toxic effects. However, efficiency of gene expression was highly dependent on the type of liposome used.[165] In a study comparing subretinal and intravitreal injections in rats, a formulation containing the cationic lipid 2,3-di(tetradecyloxy)propan-1-amine, polysorbate 80 with a plasmid (pCMS-EGFP) at different cationic lipid/DNA ratios was used. At 2/1 cationic lipid/DNA mass ratio, the resulting lipoplexes were 200 nm in size and efficiently transfected into ARPE-19 cells. After the subretinal injection, using the same lipoplexes, primarily photoreceptors and RPE cells, were transfected. Intravitreal administration resulted in a more uniform distribution, mainly in the ganglion cells and inner nuclear layer. The authors suggested that the PEG chains of polysorbate 80 prevent aggregation with fibrillar structures in the vitreous and therefore overcome this barrier. No toxicity was observed for either administration route.[166]

In general, intravitreal liposomal delivery methods have yielded lower transfection efficiencies in the retina and RPE. Topical application could transfer genes only to retinal ganglion cells.[163] In another study transgene expression was solely detected in in the iris, limbus, and conjunctiva.[167] An improvement was made using subretinal delivery.[11] These findings were underlined by Devoldere et al. The authors investigated the efficiency of chemically modified/stabilized mRNA encapsulated into lipoplexes using commercially available transfection agents Lipofectamine™2000 and Lipofectamine™MessengerMAX in vitro in human retinal cells, *ex vivo* in bovine retinal explants and in vivo in mouse retinas. Transgene expression was detectable for at least 20 days following a single administration of chemically modified mRNA in vitro. Subretinal administration into mice resulted in detectable mRNA expression in vivo, whereas mRNA expression was significantly lower following intravitreal administration. Based on *ex vivo* studies, the authors concluded that these findings can be attributed to the inner limiting membrane, which acts as a significant barrier for mRNA lipoplexes delivery by trapping mRNA complexes on the vitreous side.[168]

Lipid-based NPs for delivering messenger RNA or small interfering siRNA into the posterior eye were only rarely studied, but they showed more promising results than DNA liposomal formulations which was also observed for lipoplexes.[168,169] Sahay et al. injected seven different mRNA LNP-formulations subretinally into mouse eyes and luciferase expression was analyzed for up to five days. Formulations incorporating ionizable lipids characterized by low pKa values and unsaturated hydrocarbon chains demonstrated the highest levels of reporter gene transfection in the retina, with most of the expression found in the RPE.[169a] PEGylation is a method to shield the surface of the particles and alter protein absorption.[170] Different PEG-containing lipid NPs with varying sizes (ranging from 50 nm to 150 nm) were synthesized by modulating the polyethylene glycol (PEG) content from 5% to 0.5%. Surprisingly, subretinal administration of LNP-mRNA encoding luciferase demonstrated that LNPs containing 0.5% PEG exhibited the highest expression levels, most likely due to the most favorable combination of particle size and surface composition. Larger particle sizes facilitate increased mRNA encapsulation per particle, whereas the molar percentage of PEG on the particle surface influences the protein adsorption. The authors hypothesized that decreasing the mol% of PEG and increasing the surface concentration of 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) in lipid NPs enhances protein adsorption, thereby enabling more efficient internalization.[171] The same authors synthesized different PEGylated LNPs containing positively charged amine-modified PEG lipids (LNPa), negatively charged carboxyl (LNPz), and carboxy ester (LNPx) modified PEG lipids (**Figure 9**). Surface modifications with anionic PEG-lipids (LNPx-DSPE-PEG2k-Carboxy-NHS and LNPz-DSPE-PEG2k-Carboxylic acid) induced a considerable alteration in the surface potential of the LNPs. Additionally, the LNPx formulation bears an active ester (NHS) that is pH-sensitive and can hydrolyze to form carboxylic acids. These esters can also serve to conjugate substances such as proteins, antibodies, or peptides. These formulations were tested for their ability to deliver mCherry-mRNA by subretinal injection in the Nrl-locus GFP-tagged mouse model (NRL-GFP), which stably expresses GFP in photoreceptors. mCherry fluorescence was observed predominantly in the RPE when LNP (without PEGylation) and LNPa were administered, whereas it was localized to the photoreceptors for LNPx and LNPz, providing evidence that an anionic charge and/or different protein absorption leads to photoreceptor transfection.[172] Since publication of these findings by Sahay et al., this research group focused even more on optimizing LNP systems for retinal diseases including synthesizing novel ionizable lipids or manufacturing approaches using a preformed vesicle approach (PFV-LNPs), a solvent-free mixing process, which results in a superficial localization of mRNA.[173] PFV-LNPs achieved a similar level of gene editing compared to analogous LNPs while significantly improving the retinal tolerability.[173a] Looking ahead, these highly developed and advanced LNPs hold promise as non-viral vectors for intracellular nucleic acid delivery, particularly in their potential applications for mRNA-based gene therapies aimed at treating eye disorders.

PEGylated liposome-protamine-hyaluronic acid NPs (PEG-LPH-NPs) loaded with siRNA (132 nm in size) were tested in ARPE-19 cells in vitro and for the treatment of laser-induced CNV in rats after intravitreal injection. PEG-LPH-NP shielded the siRNA cargo and enabled the intracellular transport of siRNA, resulting in the suppression of VEGFR1 expression and reduction of CNV area while exhibiting minimal toxicity in the rat retina.[174]

To investigate a more disease-relevant model with liposomes, Human Antigen R (HuR), a member of the embryonic lethal abnormal vision (ELAV) protein family that regulates VEGF expression, was targeted. HuR downregulation via RNAi interference was employed as a therapeutic strategy for the management of diabetic retinopathy to prevent VEGF protein overexpression.[175] Liposomes with DOPE:Chol:DOTAP:DPPC = 4:2:2:2 and DOPE:Chol:DOTAP:DPPC = 4:2:4:2 (DPPC = 1,2-dipalmitoyl-*sn-glycero*-3-phosphatidylcholine) were formulated by combining a thin layer evaporation technique and extrusion through polycarbonate membranes and complexes with siRNA silencing HuR expression. Lipoplexes were intraocularly injected

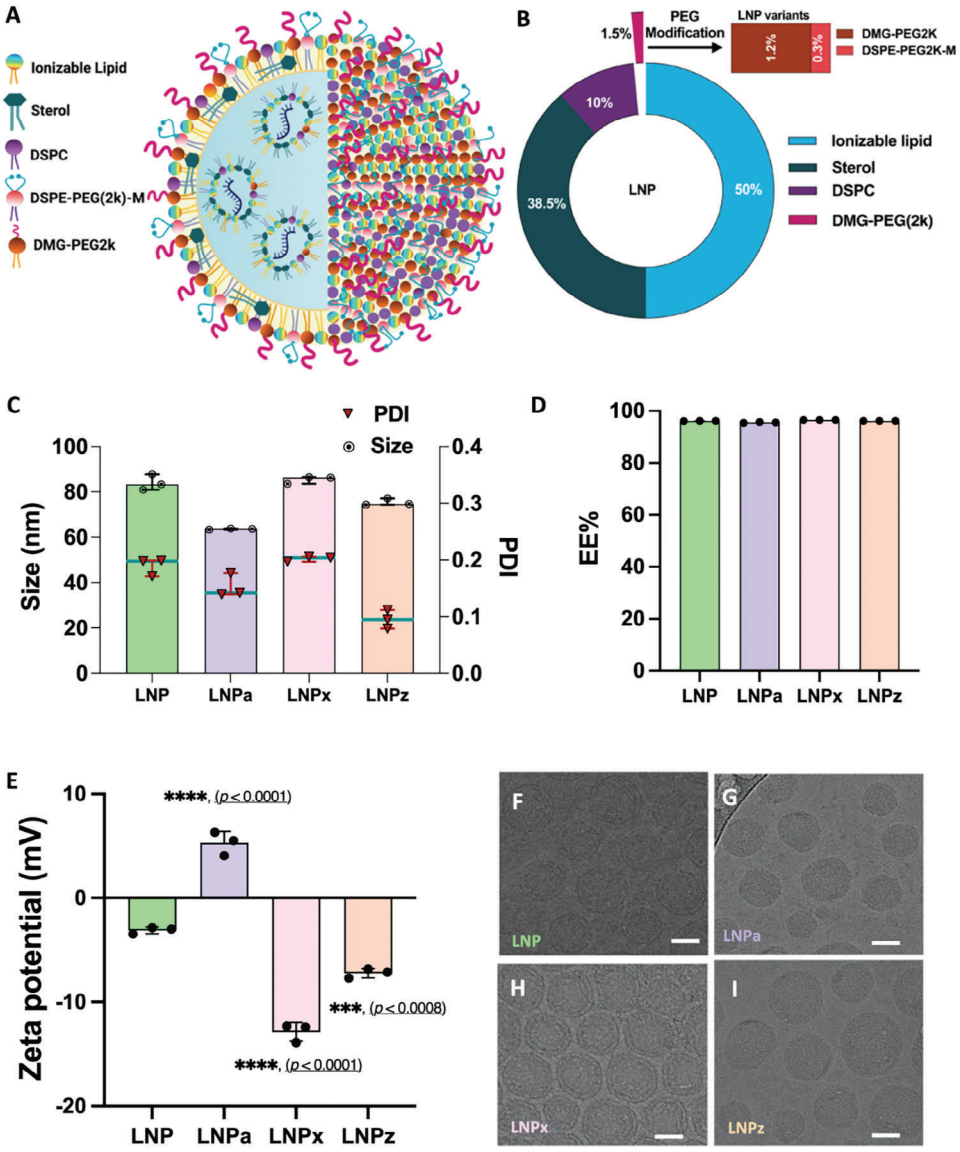

Figure 9. A) Structural organization of various LNP variants encapsulating the mRNA cargo. B) Lipid composition of LNP variants, emphasizing PEG modification. DSPE-PEG2k-M denotes PEG molecules with different functional groups. C) Hydrodynamic size (◉) and polydispersity index (PDI, ▼) of Cre mRNA-loaded LNP variants. D) CremRNA encapsulation efficiency of LNP variants. E) Zeta potential of Cre mRNA-loaded LNP variants. The statistical significance among the LNP variants was compared to that of the unmodified LNP. F-I) Cryo-transmission electron microscopy (cryo-TEM) images of LNP variants, with a scale bar indicating 20 nm. Reproduced with permission.[172]

into the eye of streptozotocin (STZ)-induced diabetic rats, and using an N/P ratio of 4/1, these lipoplexes significantly reduced HuR and VEGF protein content.[175b] Recently, the same HuR siRNA was used in a co-formulation of liposomes with cationic, branched polyethylenimine to form the so-called lipopolyplexes that form a polyplex-in-lipoplex structure. HuR siRNA encapsulated in PEI or liposome/PEI complexes were injected intravitreally into STZ-induced diabetic rats. Both siRNA-PEI and siRNA-liposome/PEI NPs showed better results than the untreated disease control group, but siRNA-PEI polyplexes achieved better downregulation of the target genes (HuR and VEGF) in vivo than lipopolyplexes. These findings are in accordance with the in vitro results using ARPE-19 cells, where the PEI-siRNA carrier also achieved better downregulation. The authors assumed that PE-Gylated lipids might be the reason for a more sustained release of HuR siRNA in lipopolyplexes, and the good endosomal escape ability of unshielded PEI in polyplexes might lead to a faster release of HuR siRNA.[175a] Further studies over longer periods are necessary to confirm this assumption.

3.2.2. Nioplexes

Niosomes (non-ionic surfactant vesicles) are formed through the organized assembly of surfactants with adequate insolubility in aqueous environments.[176] Niosomes are biodegradable,

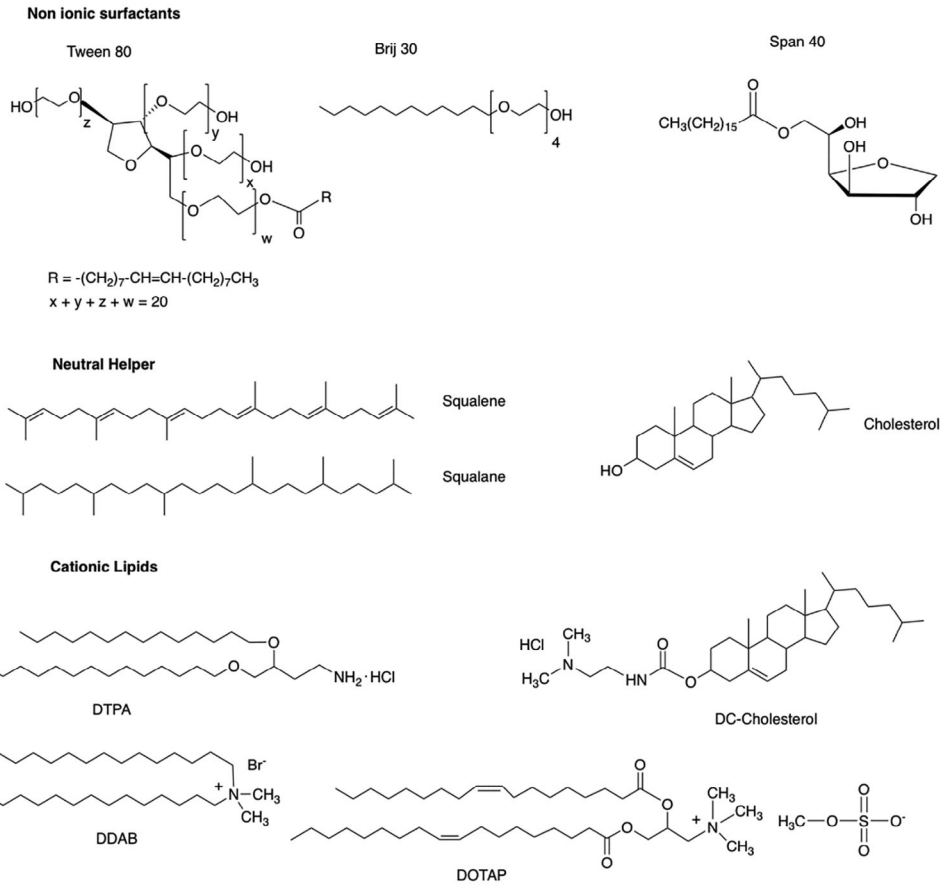

Figure 10. Different types of chemical structures (non-ionic surfactants, neutral helpers, cationic lipids) that can be utilized for building niosomes for gene/drug delivery purposes. Non-ionic surfactants are used for stability and reduced toxicity owing to the absence of charges. Helper lipids are uncharged and increase colloidal stability and cellular uptake. Cationic lipids are required if nucleic acids are encapsulated via electrostatic interactions. Reproduced with permission.[179]

biocompatible, non-immunogenic, and appear in various structures (unilamellar or multilamellar). They are built upon the hydration of synthetic non-ionic surfactants (Span, Tween, Brij),[177] with or without the incorporation of helper lipids, such as cholesterol (**Figure 10**). Cholesterol plays a crucial role in providing rigidity and stability to the bilayer structure of the niosomes. This vesicular system is similar to liposomes and can encapsulate different types of drugs. It is therefore often considered as a "non-ionic surfactant-based liposome" (phospholipids are replaced by non-ionic surfactants), but mostly showing higher stability, lower costs and increased membrane permeability than liposomes.[177b,178] Cationic niosomes have been investigated for their potential to transport ASOs, siRNAs, aptamers and pDNA.[179] Niosomes designed for gene delivery typically contain one or more cationic lipids (Figure 10). These lipids enable binding of nucleic acids via electrostatic interactions to form nioplexes.[180]

Niosome formulations for retinal nucleic acid delivery mainly contain various types of cationic lipids, non-ionic surfactant polysorbate (Tween), and helper lipids. Studies on different cationic lipids such as DOTMA, 1-(2-dimethylaminoethyl)-3-[2,3-di(tetradecyloxy) propyl]urea, 2-[[2-[(2-aminoacetyl)amino]acetyl]amino]-*N*-[2,3-di(tetradecoxy)propyl]-acetamide, 2, 3-bis (tetradecyloxy) propan-1-amine or *tert*-butyl-*N*-[2-[[2-[[2-[2,3-di(tetradecoxy)propylamino]-2-oxo-ethyl]amino]-2-oxo-ethyl]-amino]-2-oxo-ethyl]carbamate indicate that the structure of the cationic lipid (e.g. the polar head group) plays a fundamental role in tuning the transfection efficiencies in vitro.[181] Additionally, the helper lipid plays a crucial role and a variety of different structures were tested.[182] Squalene, cholesterol, and squalane were used to transfect the ARPE-19 cells with the pCMS-EGFP reporter plasmid. After cell transfection, the highest transfection efficiency was observed for nioplexes built with squalene, and high cell tolerability was observed for all tested helper lipids. Transfection without helper lipids was ineffective. The authors attributed the varying transfection efficiencies to the use of different helper lipids, as each exhibits distinct capabilities in facilitating endosomal escape and cell permeability. Another reason was that squalene nioplexes were internalized via micropinocytosis, resulting in less lysosomal accumulation.[182b] in vivo studies further supported the efficient transfection of rat retinas with squalene after intravitreal and subretinal administration. In these studies, EGFP expression was mainly observed in the ganglion cell layer and inner layers of the retina after intravitreal injection, indicating that only subretinal

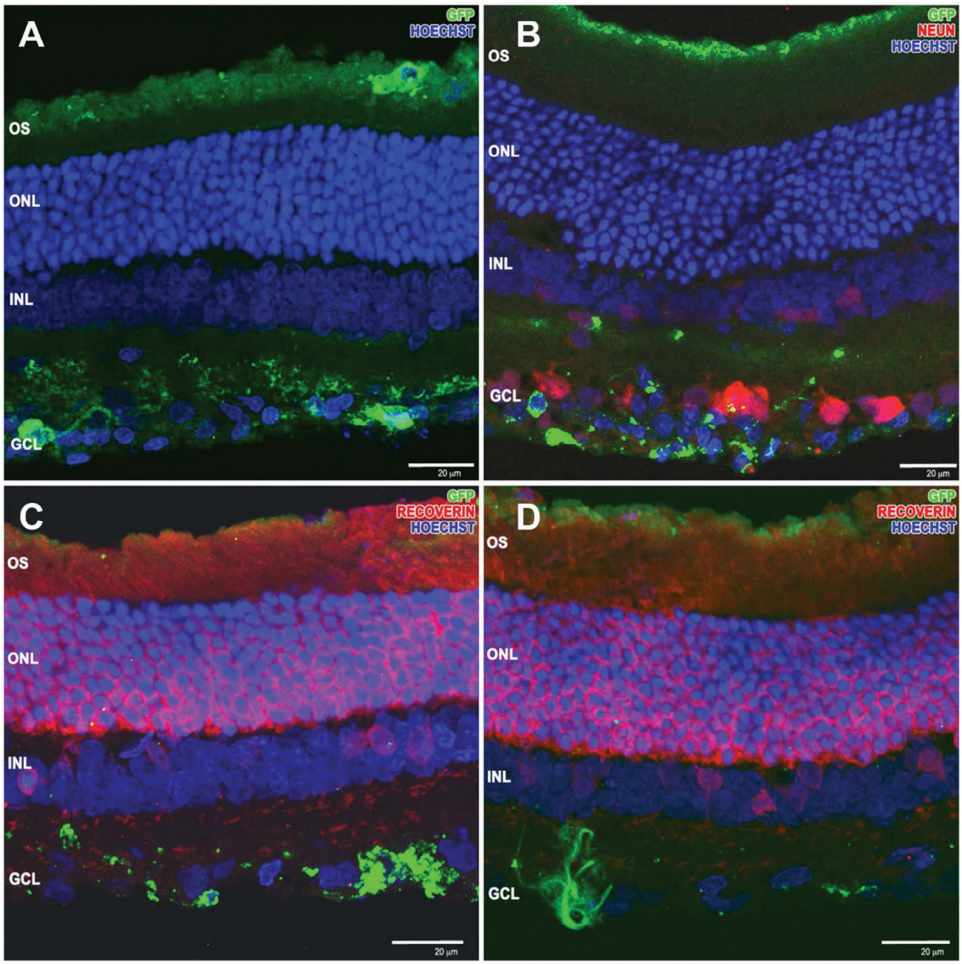

Figure 11. Confocal fluorescence images of retinal cross-sections three days after A,B) intravitreal and C,D) subretinal administration of DP60L nioplexes (niosome/DNA complexes). Intravitreal injections revealed fluorescence in glial cells within the ganglion cell layer (GCL) and outer segments (OS) of the photoreceptors. Subretinal injections also showed EGFP localization in photoreceptor outer segments (stained with recoverin, in red) and some microglial cells. The cell nuclei were counterstained with Hoechst 33342 (blue). Scale bars: 20 µm. Reproduced with permission.[182c] Copyright 2017, Elsevier B.V.

administration can reach the outer nuclear layer.[181a,183] Such niosomes were also successfully tested in delivering minicircle DNA.[184]

The incorporation of protamine into these squalene niosome/pDNA vectors was explored to assess the potential application of this innovative ternary formulation to deliver the pCMS-EGFP plasmid into the rat retina.[185] Protamine is a polycationic peptide consisting of two-thirds arginine residues and one-third other amino acids.[186] Compared with binary vectors based on niosome/DNA, the inclusion of protamine improved properties in terms of transfection efficiency, cell viability, and DNA condensation. Similar to previous studies, only subretinal administration mainly transfected photoreceptors and RPE at the injection site, whereas intravitreal administration resulted in protein expression in the ganglion cell layer and Müller cells or the inner retina. Examination of the retinas one month after subretinal administration indicated predominant GFP expression in ganglion cells. Furthermore, one month after intravitreal injection, GFP was partially localized in Müller glial cells.[185]

By incorporating lycopene as a helper lipid, nioplexes can condense, release, and protect pCMS-EGFP at a lipid/DNA mass ratio of 18/1 (size ≈100 nm). The authors discuss that the natural, non-polar lipid lycopene into the niosome bilaminar membrane can enhance membrane fluidity, disrupt membrane packing, and increase the vesicle's susceptibility to environmental stresses. The transfection efficiency in ARPE-19 cells was ≈35% without affecting cell viability; however, cell uptake was very low. Studies on internalization showed a preference for caveolae-mediated endocytosis and macropinocytosis, which could bypass lysosomal degradation.[182c] Both subretinal and intravitreal administration routes showed that the nioplexes were able to efficiently transfect the outer segments of the retina, offering hope for the treatment of hereditary retinal diseases by less invasive intravitreal injection (**Figure 11**).[182c] Other substances incorporated into cationic niosomes to further enhance gene transfection were

chloroquine, a known endosomal disrupting molecule and lysosomotropic agent.[187]

3.3. PLGA Nanoparticles

One of the first fundamental studies on NPs for ocular delivery was performed by Behar-Cohen et al. with poly(lactic) acid (PLA) and poly(lactide-co-glycolide) (PLGA) NPs.[188] PLGA is one of the most successfully used polymers to formulate polymeric NPs owing to its advantageous properties, including (1) biodegradability and biocompatibility, (2) approval by the FDA and European Medicine Agency for drug delivery systems via parenteral administration, (3) well-established formulations and production methods, (3) protection of drugs from degradation, and (4) potential for sustained release of drugs.[189] To reduce injection frequency, sustained drug delivery systems can be used to deliver drugs to the retina for prolonged periods.

pDNA-loaded NPs were prepared using a double emulsion system and solvent evaporation technique because PLA and PLGA do not contain any cationic groups. Thus, plasmid NPs were rather large, with sizes of ≈650 nm which could hinder efficient nucleic acid delivery. Gene expression was investigated using red nuclear fluorescence (RNFP) in rat RPE cells after intravitreal injection. Most of the expression was found within the RPE layer, whereas some fluorescence was detected in the inner retinal layers. Fluorescence was significantly increased compared to empty NPs or naked plasmid DNA encoding RNFP.[188] in vivo studies on CNV have utilized PLGA NPs with hypoxia-inducible factor 1α (HIF-1α) shRNA and GFP co-expressing pDNA (pshHIF-1α NPs sizes ≈300 nm). CNV was induced by laser photocoagulation in rats and pshHIF-1α NPs were injected intravitreally. Expression of GFP was localized in the RPE and lasted for four weeks, and the mean thickness of the CNV lesions in the pshHIF-1α NP-treated group was significantly smaller and showed decreased fluorescein leakage. Destruction of the retina was not detected.[190] in vivo studies with Rh-6G–loaded PLA NPs (140 nm, but also containing the PMMA-PMA copolymer; PMMA = poly(methyl methacrylate), PMA = poly(methacrylic acid)), prepared by nanoprecipitation, were performed to further study the kinetics of intraocular tissue and cellular localization after one intravitreal injection into healthy rat eyes. NPs migrated through the retinal layers and accumulated in the RPE cells. NPs were still present within the RPE cells for up to four months after a single intravitreal injection.[191] Disease models were not used in these studies. Related to these findings, in vitro studies have shown that PLGA NPs (250 nm) enhance the cellular delivery of an encapsulated VEGF antisense oligonucleotide that can inhibit VEGF secretion in ARPE-19 cells to target retinal neovascular diseases.[192] With PLGA, it is also possible to formulate siRNA/PLGA NP for nucleic acid delivery.[193]

In another approach, PLGA-chitosan NPs (260 nm) with plasminogen kringle 5 (K5) were produced using an emulsion-diffusion evaporation technique.[194] K5 demonstrated effective anti-angiogenic activity in neovascularization models.[195] Intravitreal injection of K5-NP resulted in high K5 expression in the inner retina of rats within a test period of four weeks. In rats with oxygen-induced retinopathy, K5-NP injection significantly reduced retinal vascular leakage and attenuated retinal neovascularization compared with contralateral eyes injected with control-NP. In STZ-induced diabetic rats, K5-NP administration led to decreased vascular endothelial growth factor and intracellular adhesion molecule-1 expression along with reduced leukostasis and vascular leakage, indicating that the expressed K5 is secreted into the extracellular space and has biological activity. These results were obtained after a single injection and no toxic effects on retinal structure and function were observed.[194] Similar results were observed in a follow-up study of PLGA-chitosan NPs, in which CNV was induced using a laser. K5 expression and down-regulation of VEGF expression were detected in the retina of K5-NP treated rats. Concurrently, CNV and vascular permeability were significantly decreased.[196]

4. Novel Approaches: DNA as Carrier

One biomolecule that has also been used for the creation of nano-objects over the last decade is DNA.[199] Owing to the sequence-specific base pairing and 3D structure of the double helix, nano-objects with defined functionalities can be designed and produced. Thus, DNA is widely used in multidisciplinary research, and its properties are extensively exploited not only for medical and diagnostic applications, but also in computer science and biophysics.[200] Until a few years ago, the therapeutic application of DNA nano-objects was almost exclusively focused on cancer.[201]

Based on lipid DNA nanotechnology, a drug delivery system, based on DNA as a carrier system, that addresses the problems of topical and retinal drug delivery to the eye has been developed.[202] These NPs are based on lipid-modified DNA strands, which, owing to microphase separation in an aqueous environment, assemble into micelles with a hydrophobic core and hydrophilic corona with a diameter of ≈10 nm (**Figure 12**). The DNA NPs used as carrier are based on self-assembly of DNA amphiphiles. The amphiphilic nature of the DNA strand is imparted by a hydrophilic, anionic phosphodiester backbone and hydrophobic units, which are attached to the nucleobases. The NPs consist of twelve nucleotides (12mer) of which four consisted of hydrophobic nucleotides at the 5′-end modified with a lipid chain.[202a] These biocompatible and easily degradable NPs have an intrinsic affinity for the ocular surface and can be simultaneously equipped with additional functionalities by a simple hybridization process of the single-stranded DNA corona. NPs can be loaded with drugs in several ways: by hydrophobic interactions, intercalation into the DNA double helix, or by the use of aptamers. Similar methods can be used to load functional nucleotides, such as aptamers, DNA motifs, miRNAs, or siRNAs, which are presently being studied.

First, lipid DNA NPs were investigated for their potential in the treatment of glaucoma. These NPs were loaded with the anti-glaucoma drug travoprost, which was bound to an aptamer that specifically targeted the drug. In another approach, for the anti-glaucoma drug, brimonidine, the first aptamers were tailored to bind to brimonidine. In the second approach, the drug was loaded into the NPs via hydrophobic interactions with double-stranded micelles. This binding to the NPs through non-covalent, specific interactions, without the need for any chemical modifications to the pharmaceutical ingredient, increased adhesion, uptake, and efficacy, and biosafety of the NPs was demonstrated

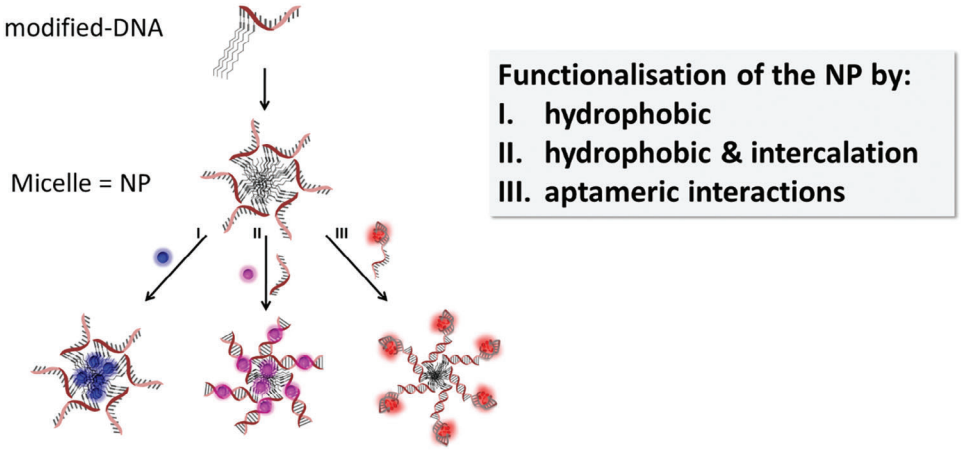

Figure 12. Loading of DNA nanoparticles with drugs: The lipid-modified DNA oligonucleotides self-assemble into micelle-like nanoparticles (NP). The drugs are then loaded via hydrophobic interactions by direct mixing of drug and NP in solution (I) or by intercalation into the DNA double strand (II), or the NP are functionalized with DNA aptamers and bound to the drug molecules (III).[203a]

both in vitro and in vivo, providing evidence for the feasibility of using a DNA-based drug delivery system in the field of ophthalmology to treat glaucoma.[203] Additionally, these NPs were evaluated for retinal applications. Following intravitreal injection, the NPs exhibited outstanding biodistribution, adherence, and persistence for up to five days in vivo. Lipid-DNA NP can rapidly reach and penetrate the retina, offering a potentially safe and long-lasting delivery system for small molecules or nucleotide-based therapies.[202b]

5. Future Perspectives

As retinal gene therapy advances, researchers and pharmaceutical companies are expected to concentrate on enhancing vector design to boost efficiency, expand vector targets, and increase overall product safety. Another crucial aspect for future gene therapy development involves discovering and implementing techniques to address genetic disorders without a specific diagnosis or to treat multiple mutations with a single product.[81]

The effectiveness of wide-ranging treatments is being demonstrated by current modifier gene therapies, with potential applications extending beyond eye disorders. So far, broad-spectrum modifier therapies tested in the retina work by adjusting multiple gene networks crucial for retinal functionality. This strategy is particularly well-suited for intricate conditions such as AMD, which involve several disrupted genetic pathways. Additionally, modifier gene therapy proves highly effective for single gene defects, where the primary mutation drives the disease progression, and other gene networks influence the outcome.[204] The success of existing modifier therapies in eye diseases will facilitate their application to other ocular and non-ocular conditions.[81]

Optogenetics, a potential gene therapy currently undergoing clinical trials, represents another promising technique. However, it still faces significant knowledge gaps that need to be addressed, due in part to the lack of efficient delivery options. Neuroscience has witnessed a revolutionary advancement with the introduction of optogenetics, a technique that allows researchers to manipulate neural activity. This is achieved by introducing light-sensitive optogenetic proteins into specific target cells through genetic modification. The ability to control neurons using light has opened up new possibilities, particularly in addressing retinal degenerative disorders, which stands out as one of the most promising potential applications of this technology.[205]

Various ocular delivery methods, including intravitreal, subretinal, and suprachoroidal approaches, are considered invasive to different extents. Future studies and therapeutic advancements should explore less intrusive administration routes, such as topical or oral applications, and investigate ways to enhance the prolonged efficacy of treatments to minimize the necessity for repeated injections. Innovative delivery systems are being developed by foldable sheets made of porous poly dimethyl silicon hexane, which can be inserted into the sub-tenon area using a syringe. This approach enabled prolonged delivery of ranibizumab to the retina over an extended period, demonstrating its potential as a substitute for repeated intravitreal injections.[206]

The exploration of nanomaterials for retinal targeting represents a promising avenue in ophthalmic research that has not reached their performance targets yet. Despite the fact that some of these materials were discovered decades ago and are yet to be commercialized, the potential for enhancing their efficacy through subtle modifications such as adjusting their topological structure, charge, size, or coatings is significant.[207] This iterative approach to material development demonstrates how scientific advancements can be built upon existing knowledge. This approach could be efficiently supported by machine learning algorithms in the future that analyze patterns and optimize outcomes based on historical data.[207] The refinement of these materials could unlock their potential as effective tools for retinal applications.

Moreover, the ongoing development of hybrid systems (polymer blends and polymeric lipid hybrid NPs)[208] and of new materials, such as cationic polymers derived from biogenic sources,[209] functionalization beyond cationic blocks,[210] stimuli-responsive systems,[207,211] and alternatives to PEG in polymeric and lipid NP formulations,[212] amongst many others which are currently being studied in general gene delivery research or for other targets, may also prove beneficial for ophthalmic research. The versatility and adaptability of

Table 1. Examples for successful intravitreal or subretinal administration of nanomaterials.

Type of nanoparticle	Composition	Zeta potential and size	Method of delivery	Therapeutic gene delivered	Outcome of the gene therapy
PEI polyplexes	branched PEI (bPEI)	Positive. Size: 150 to 350 nm	intravitreally	Anti TGF-β2 siRNA	in vitro delivery in retinal Müller cells→specific downregulation of TGF-β2 production[126]
	bPEI and hyaluronic acid coating	Negative Size: 260.7 ± 43.27 nm	intravitreally	Anti VEGF siRNA	polyplex at 1 and 7 days after laser photocoagulation inhibited laser-induced choroidal neovascularization[127]
PEI nanoballs	siRNA hydrogel coated with branched PEI and hyaluronic acid	Negative Size: 259.82 ± 33.35 nm	intravitreally	Anti VEGF siRNA	2 weeks after intravitreal injection analysis of the CNV area, demonstrating that the siVEGF NB inhibited CNV significantly[130]
HA nanoparticles	Amphiphilic hyaluronic acid conjugated with 5β-cholanic acid	Negative Size: 213.4 ± 10.3 nm	intravitreally	None: only fluorescently labeled	efficient penetration across the retina as well as the vitreous. Fast eliminatio after 72h[128]
HSA nanoparticles	Amphiphilic human serum albumin conjugated with 5β-cholanic acid	Negative Size: 326.3 ± 9.7 nm	intravitreally	None: only fluorescently labeled	superior penetration capabilities across the vitreous and retina up to the RPE[128]
Chitosan nanoparticles	glycol chitosan (GCS) and plasmid DNA	Positive Size: 253.3 ± 3.18 nm	subretinally	None: pscCBA–GFP (pDNA)	14 post-injection green fluorescent was observed exclusively in the RPE[133] Chitosan NP-mediated gene expression in RPE cells can last for at least six months.[134]
Oligochitosan polyplexes	Oligochitosan and plasmid DNA	Positive Size: 69–150 nm	intravitreally and subretinally	None: pCMS-EGFP	EGFP expression in different layers and cells, depending strongly on the administration route[135]
Lipoplexes	2,3-di(tetradecyloxy)propan-1-amine (cationic lipid), polysorbate 80 and plasmid DNA	Positive. Sizes up to 260 nm depending on the mass ratio of lipid/DNA	intravitreally and subretinally	None: pCMS-EGFP	Subretinal injection resulted in transfection in the RPE, photoreceptor outer segments. Intravitreal injections induced transfection in the ganglion cell layer after 72h[166]
LNP	N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA)/dioleoylphosphatidylethanolamine (DOPE) (1:1 molar ratio) liposomes and DOTMA/cholesterol (Chol) (1:1 molar ratio)	positive	intravitreally	None: pCMV-Luc	Transient expression of Luciferase, with a peak level at 3 days[164]
Nioplexes	three different synthetic cationic lipids, squalene, polysorbate 80, plasmid DNA	Slightly positive Sizes around 200 nm	intravitreally and subretinally	None: pCMS-EGFP	subretinal and intravitreal administration routes showed that the nioplexes transfected the outer segments of the retina:
	DOTMA, polysorbate 60, lycopene, plasmid DNA	Positive Size: 101.60 ± 2.48 nm			EGFP protein was observed mainly in GCL photoreceptors and RPE cells[181a,182c]

(*Continued*)

Table 1. (Continued)

Type of nanoparticle	Composition	Zeta potential and size	Method of delivery	Therapeutic gene delivered	Outcome of the gene therapy
	2,3-di(tetradecyloxy)propan-1-amine (cationic lipid), squalene, polysorbate 80, protamine bound to plasmid DNA	Depending on the ratio protamine/DNA Size:130 – 160 nm	intravitreally and subretinally	None: pCMS-EGFP	After 14 days: **subretinal**: Transfection of RPE cells and photoreceptor layer was seen. **intravitreal** GFP expression mainly in the ganglion cell layer and Müller cells. After 1 month: subretinal GFP expression predominantly in ganglion cells intravitreal injection, GFP partially colocalized with Müller glial cell[185]
PLA nanoparticles	Poly(D,L-lactide), random copolymer P(MMA-co-MA) containing 12 mol% methacrylic acid, Rh-6G	Negative Size: 140 ± 20 nm	Intravitreally	None: fluoro-chrome Rh-6G	NP were detected in RPE cells for up to four months[191]
PLGA/Chitosan nanoparticles	poly(lactide-co-glycolide), poly(vinyl acetate), plasmid DNA complexed to chitosan	Postive Size: 260 ± 30 nm	Intravitreally	K5 expression plasmid pK5	K5-NP significantly reduced retinal vascular leakage and attenuated retinal neovascularization up to 4 weeks[194]
Peptide-based NP	polyethylene glycol-substituted 30-mer lysine peptides (PEG-CK_{30}), plasmid DNA	Not determined Previous studies: Neutral Size:12-113 nm[197]	Subretinally	ABCA4	transgene expression in RPE cells of *Abca4*$^{-/-}$ mice, up to 8 months[198]
			subretinally	*Rhodopsin* gene	Transgene expression in *Rho* knock out mice, up to 8 month[151]

nanomaterials make them a continually evolving field, with the potential to offer innovative solutions for retinal diseases and conditions.

Additionally, nanoparticle formulations are already being evaluated with eye drops, contact lenses, and prostheses to produce a more sustained effect of therapies for anterior segment diseases and reduce infections associated with prostheses.[206,213] Combination therapeutics that combine molecular and cell therapies or molecular therapies with device delivery could be potentially powerful and more robust future treatment strategies for retinal diseases.

6. Conclusion

In the last three decades, significant advancements have been made in the development of viral vectors for gene therapy. Key advancements include the creation of new systems for producing genetically modified vectors with reduced immunogenicity and optimizing tissue-specific and inducible promoters to enhance in vivo transgene expression specificity and efficiency. Additionally, the development of alternative viral serotypes and identification of new virus types have aimed to circumvent immune responses and expand vector options. Despite these achievements, substantial challenges persist, including concerns about vector safety, precise target cell transduction, prediction of patient responses to inflammatory vectors, and the establishment of standardized investigations. Further research is needed to address these challenges by focusing on improving vector safety and enhancing the transduction efficiency. The question also arises of whether non-life-threatening diseases should be treated with potentially harmful viral vectors. Therefore, the development of non-viral vectors has increased in recent decades.

Research on NPs for nucleic acid delivery to the retina offers a promising approach to treating genetic eye diseases (**Table 1**). Polyplexes are NPs formed by condensing nucleic acids (e.g., pDNA, mRNA, and siRNA) into cationic polymers (e.g., PEI or chitosan) through electrostatic interactions. These NPs typically range in size from to 10–100 nm and are taken up by cells via endocytosis. Efficient cationic polymers can destabilize endosomes and promote the release of genetic material into the cytoplasm. LNPs and lipoplexes are formed by complexation of nucleic acids with cationic lipids. They can fuse with the plasma membrane, making them highly efficient in cellular uptake and transfection. Compacted DNA NPs are neutrally charged complexes containing a single molecule of plasmid DNA compacted with polyethylene glycol-substituted 30-mer lysine peptides. PEGylation is an effective technique to enhance stability and make particles more neutral, whereas cationic parts are used to compact nucleic acids.

Topical administration to the eye is the least invasive but also the least efficient method for retinal targeting due to the multiple barriers that limit drug permeation. These include the cornea and the vitreous, which the drug must penetrate and diffuse through. Additionally, a significant portion of the drug is absorbed into

the bloodstream through conjunctival and nasal blood vessels, further reducing the amount reaching the retina.[214]

Intravitreal and subretinal injections differ in terms of gene expression, based on the location and efficiency of transfection. Subretinal injections primarily target photoreceptor cells and RPE cells, which are crucial for disease-relevant applications, such as AMD and RP. In contrast, intravitreal injections result in a more uniform distribution, mainly in the ganglion cell and inner layers of the retina, and easier surgical intervention.

Both polyplexes, LNPs and lipoplexes have been tested for in vivo gene expression by using different administration methods and transfection efficiencies. Nanoparticles for intravitreal and subretinal injections require specific physicochemical characteristics to ensure effective drug delivery to the retina. The choice between polyplexes and lipoplexes depends on their specific applications and desired outcomes because each formulation has its own advantages and limitations. For intravitreal injections, nanoparticles with a size range of 150–180 nm have shown promising results.[167] These particles should be thermodynamically stable in the vitreous and able to traffic as single, non-aggregated units.[167] Surface properties play a crucial role in determining the distribution of nanoparticles in the vitreous and retina after intravitreal injection.[128] For subretinal injections, smaller nanoparticles may be more suitable. A study using 50-nm magnetic nanoparticles showed that they were cleared more quickly from the eye compared to 4-µm particles, suggesting that nanoparticle size affects clearance rates.[215]This faster clearance could be advantageous for avoiding long-term persistence and potential side effects. Depending on the material there is also a size-dependent retinal toxicity reported.[216] The surface charge and size of the NPs play a significant role in their distribution after injection, the therapeutic efficacy and also regarding cytotoxic effects. It has been demonstrated that cationic nanoparticles interact significantly with negatively charged vitreous collagen fibrils, limiting their distribution following intravitreal injection, and are typically more harmful than neutral or anionic nanoparticles.[217] Anionic or neutral NPs have shown superior penetration capabilities across the entire retina, up to the RPE. In contrast, glycol chitosan and PEI/glycol chitosan NPs, with their surfaces modified by glycol groups, traversed the vitreous barrier. Additionally, the shape of nanoparticles influences their toxicity, with fiber-shaped materials being more toxic to the lungs compared to spherical nanoparticles of the same chemical composition.[217] While this finding is specific to pulmonary inflammation, it suggests that particle shape may also be relevant for ocular applications.[218]

Due to the generally lower toxicity of non-viral vectors and lack of severe inflammatory effects, multiple injections could show promise in ocular gene therapy, particularly in the treatment of retinal diseases. Sustained gene expression in the retina and RPE for extended periods with no observed signs of local inflammatory responses or cellular toxic reactions could provide access for efficient disease treatment. However, it is important to note that further research is needed to fully understand the long-term effects and safety profile of multiple injections of non-viral vectors, especially in the context of specific ocular diseases and patient populations. Long-term effects, immune responses, and precise distribution of therapeutics are important factors to consider before their clinical application.

In conclusion, the future of nanomaterials in retinal targeting is bright, with the possibility of leveraging both established and emerging materials to create more effective and targeted therapies. A necessity in interplay between material science and ophthalmology will hold the promise of transformative advancements, and continued research and development in this area are crucial for unlocking the full potential of these materials for retinal applications.

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Conflict of Interest

S.S. is one of the inventors of the NPs presented in Chapter 5. The patent (US10285939B2, EP3057572B1) is owned by the Medical Faculty of the University of Tübingen, Germany. The author(s) declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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nanomaterials, nucleic acids, ocular gene therapy, transcription, retina

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[1] a) M. A. Mintzer, E. E. Simanek, *Chem. Rev.* **2009**, *109*, 259; b) Z. Liu, S. Wang, C. Tapeinos, G. Torrieri, V. Kankanen, N. El-Sayed, A. Python, J. T. Hirvonen, H. A. Santos, *Adv Drug Deliv Rev.* **2021**, *174*, 576; c) H. Zu, D. Gao, *AAPS J.* **2021**, *23*, 78; d) I. M. S. Degors, C. Wang, Z. U. Rehman, I. S. Zuhorn, *Acc. Chem. Res.* **2019**, *52*, 1750.
[2] S. M. Conley, M. I. Naash, *Progress in retinal and eye research* **2010**, *29*, 376.
[3] B. Sahu, I. Chug, H. Khanna, *Biomolecules* **2021**, *11*, 1135.
[4] D. Wang, P. W. L. Tai, G. Gao, *Nat Rev Drug Discov* **2019**, *18*, 358.
[5] G. A. Rodrigues, E. Shalaev, T. K. Karami, J. Cunningham, N. K. H. Slater, H. M. Rivers, *Pharm. Res.* **2018**, *36*, 29.
[6] N. A. Moore, N. Morral, T. A. Ciulla, P. Bracha, *Expert Opin. Biol. Ther.* **2018**, *18*, 37.
[7] J. W. Bainbridge, A. J. Smith, S. S. Barker, S. Robbie, R. Henderson, K. Balaggan, A. Viswanathan, G. E. Holder, A. Stockman, N. Tyler, S. Petersen-Jones, S. S. Bhattacharya, A. J. Thrasher, F. W. Fitzke, B. J. Carter, G. S. Rubin, A. T. Moore, R. R. Ali, *N. Engl. J. Med.* **2008**, *358*, 2231.
[8] K. Bucher, E. Rodriguez-Bocanegra, D. Dauletbekov, M. D. Fischer, *Prog Retin Eye Res.* **2021**, *83*, 100915.
[9] M. Pavlou, C. Schon, L. M. Occelli, A. Rossi, N. Meumann, R. F. Boyd, J. T. Bartoe, J. Siedlecki, M. J. Gerhardt, S. Babutzka, J.

Bogedein, J. E. Wagner, S. G. Priglinger, M. Biel, S. M. Petersen-Jones, H. Buning, S. Michalakis, *EMBO Mol. Med.* **2021**, *13*, 13392.
[10] K. Stieger, B. Lorenz, *Klin Monbl Augenheilkd* **2014**, *231*, 210.
[11] A. Koirala, S. M. Conley, M. I. Naash, *Biomaterials* **2013**, *34*, 7158.
[12] A. V. Oliveira, A. M. Rosa da Costa, G. A. Silva, *Mater Sci Eng C Mater Biol Appl.* **2017**, *77*, 1275.
[13] P. C. Issa, R. E. MacLaren, *Clinical & experimental ophthalmology* **2012**, *40*, 39.
[14] R. Farjo, J. Skaggs, A. B. Quiambao, M. J. Cooper, M. I. Naash, *PLoS One* **2006**, *1*, e38.
[15] D. M. Lipinski, M. Thake, R. E. MacLaren, *Prog Retin Eye Res.* **2013**, *32*, 22.
[16] K. Irsch, D. L. Guyton, in *Encyclopedia of Biometrics*, (Eds.: S. Z. Li, A. Jain), Springer US, Boston, MA **2009**, p. 11.
[17] J. P. Frangie, *Clin. Pharmacokinet.* **1995**, *29*, 130.
[18] C. Bucolo, F. Drago, S. Salomone, *Front. Pharmacol.* **2012**, *3*, 188.
[19] M. Löscher, C. Seiz, J. Hurst, S. Schnichels, *Pharmaceutics* **2022**, *14*, 134.
[20] a) N. K. Tram, C. J. Maxwell, K. E. Swindle-Reilly, *Curr. Eye Res.* **2021**, *46*, 429; b) M. El Sanharawi, L. Kowalczuk, E. Touchard, S. Omri, Y. De Kozak, F. Behar-Cohen, *Progress in retinal and eye research* **2010**, *29*, 443.
[21] Q. Xu, N. J. Boylan, J. S. Suk, Y.-Y. Wang, E. A. Nance, J.-C. Yang, P. J. McDonnell, R. A. Cone, E. J. Duh, J. Hanes, *J. Controlled Release* **2013**, *167*, 76.
[22] C. G. Wilson, L. E. Tan, J. Mains, in *Drug Product Development for the Back of the Eye*, (Eds.: U. B. Kompella, H. F. Edelhauser), Springer US, Boston, MA **2011**, p. 125.
[23] H. Koo, H. Moon, H. Han, J. H. Na, M. S. Huh, J. H. Park, S. J. Woo, K. H. Park, I. C. Kwon, K. Kim, *Biomaterials* **2012**, *33*, 3485.
[24] T. van Zyl, W. Yan, A. M. McAdams, A. Monavarfeshani, G. S. Hageman, J. R. Sanes, *Proc Natl Acad Sci U S A* **2022**, *119*, 2200914119.
[25] R. H. Masland, *Neuron* **2012**, *76*, 266.
[26] A. Bringmann, T. Pannicke, J. Grosche, M. Francke, P. Wiedemann, S. N. Skatchkov, N. N. Osborne, A. Reichenbach, *Prog Retin Eye Res* **2006**, *25*, 397.
[27] L. Liu, X. Liu, *Adv. Exp. Med. Biol.* **2019**, *1141*, 467.
[28] J. Cunha-Vaz, R. Bernardes, C. Lobo, *Eur. J. Ophthalmol.* **2011**, *21 Suppl 6*, S3.
[29] L. J. Rizzolo, S. Peng, Y. Luo, W. Xiao, *Prog Retin Eye Res* **2011**, *30*, 296.
[30] J. A. S. Rada, S. Shelton, T. T. Norton, *Exp. Eye Res.* **2006**, *82*, 185.
[31] S. Sun, J. Li, X. Li, B. Lan, S. Zhou, Y. Meng, L. Cheng, *Acta Biomater.* **2016**, *37*, 143.
[32] D. L. Nickla, J. Wallman, *Prog Retin Eye Res.* **2010**, *29*, 144.
[33] S. Duvvuri, S. Majumdar, A. K. Mitra, *Curr. Drug Metab.* **2004**, *5*, 507.
[34] J. Hillenkamp, A. A. Hussain, T. L. Jackson, J. R. Cunningham, J. Marshall, *Investigative ophthalmology & visual science* **2004**, *45*, 4529.
[35] J. J. Kang-Mieler, C. R. Osswald, W. F. Mieler, *Expert Opinion on Drug Delivery* **2014**, *11*, 1647.
[36] J. J. Kang-Mieler, K. M. Rudeen, W. Liu, W. F. Mieler, *Eye* **2020**, *34*, 1371.
[37] T. F. Patton, J. R. Robinson, *J. Pharm. Sci.* **1976**, *65*, 1295.
[38] a) T. Loftsson, H. H. Sigurdsson, F. Konrádsdóttir, S. Gísladóttir, P. Jansook, E. Stefánsson, *Pharmazie* **2008**, *63*, 171; b) M. E. Myles, D. M. Neumann, J. M. Hill, *Adv. Drug Delivery Rev.* **2005**, *57*, 2063.
[39] a) B. Begines, T. Ortiz, M. Pérez-Aranda, G. Martínez, M. Merinero, F. Argüelles-Arias, A. Alcudia, *Nanomaterials* **2020**, *10*, 1403; b) J. Cunha-Vaz, R. Bernardes, C. Lobo, *European journal of ophthalmology* **2011**, *21*, 3.
[40] W. Zhang, M. R. Prausnitz, A. Edwards, *J Control Release* **2004**, *99*, 241.
[41] R. Gaudana, H. K. Ananthula, A. Parenky, A. K. Mitra, *AAPS J.* **2010**, *12*, 348.
[42] E. Ramsay, E. M. Del Amo, E. Toropainen, U. Tengvall-Unadike, V. P. Ranta, A. Urtti, M. Ruponen, *Eur. J. Pharm. Sci.* **2018**, *119*, 83.
[43] a) G. A. Rodrigues, D. Lutz, J. Shen, X. Yuan, H. Shen, J. Cunningham, H. M. Rivers, *Pharm. Res.* **2018**, *35*, 245; b) M. Löscher, C. Seiz, J. Hurst, S. Schnichels, *Pharmaceutics* **2022**, *14*, 134.
[44] S. Friedrich, Y. L. Cheng, B. Saville, *Curr. Eye Res.* **1997**, *16*, 663.
[45] T. U. Krohne, F. G. Holz, C. H. Meyer, *Der Ophthalmologe* **2014**, *111*, 113.
[46] C. S. Amrutkar, S. B. Patil, *Indian J. Ophthalmol.* **2023**, *71*, 2355.
[47] K. G. Janoria, S. Gunda, S. H. Boddu, A. K. Mitra, *Expert opinion on drug delivery* **2007**, *4*, 371.
[48] D. Ghate, H. F. Edelhauser, *Expert opinion on drug delivery* **2006**, *3*, 275.
[49] K. De Clerck, G. Accou, F. Sauvage, K. Braeckmans, S. C. De Smedt, K. Remaut, K. Peynshaert, *Pharmaceutics* **2022**, *14*, 1716.
[50] a) S. S. Lee, P. M. Hughes, M. R. Robinson, *Curr. Opin. Ophthalmol.* **2009**, *20*, 511; b) H. Han, S. Li, M. Xu, Y. Zhong, W. Fan, J. Xu, T. Zhou, J. Ji, J. Ye, K. Yao, *Adv. Drug Delivery Rev.* **2023**, *196*, 114770.
[51] Y. Özsoy, S. Güngör, E. Kahraman, M. E. Durgun, in *Nanoarchitectonics in Biomedicine*, (Ed.: A. M. Grumezescu), William Andrew Publishing, Norwich, NY, **2019**, p. 85.
[52] R. Gaudana, J. Jwala, S. H. S. Boddu, A. K. Mitra, *Pharm. Res.* **2009**, *26*, 1197.
[53] W. Berger, B. Kloeckener-Gruissem, J. Neidhardt, *Progress in Retinal and Eye Research* **2010**, *29*, 335.
[54] D. Ghate, W. Brooks, B. E. McCarey, H. F. Edelhauser, *Investigative ophthalmology & visual science* **2007**, *48*, 2230.
[55] V. Gote, S. Sikder, J. Sicotte, D. Pal, *J. Pharmacol. Exp. Ther.* **2019**, *370*, 602.
[56] D. Waite, Y. Wang, D. Jones, A. Stitt, T. R. Raj Singh, *Ther Deliv* **2017**, *8*, 685.
[57] K. Y. Wu, A. Gao, M. Giunta, S. D. Tran, *Pharmaceuticals* **2024**, *17*, 1007.
[58] C. R. Wan, L. Muya, V. Kansara, T. A. Ciulla, *Pharmaceutics* **2021**, *13*, 288.
[59] Y. Ham, H. Mehta, J. Kang-Mieler, W. F. Mieler, A. Chang, *Asia Pac J Ophthalmol (Phila)* **2023**, *12*, 402.
[60] S. E. Hancock, C. R. Wan, N. E. Fisher, R. V. Andino, T. A. Ciulla, *Expert Opin Drug Deliv.* **2021**, *18*, 777.
[61] a) K. Stargardt, *Albrecht von Graefes Archiv für Ophthalmologie* **1909**, *71*, 534; b) A. Patel, K. Cholkar, V. Agrahari, A. K. Mitra, *World J Pharmacol* **2013**, *2*, 47; c) D. T. Hartong, E. L. Berson, T. P. Dryja, *Lancet* **2006**, *368*, 1795; d) R. Klein, K. J. Cruickshanks, S. D. Nash, E. M. Krantz, F. J. Nieto, G. H. Huang, J. S. Pankow, B. E. Klein, *Archives of ophthalmology* **2010**, *128*, 750.
[62] J. W. Yau, S. L. Rogers, R. Kawasaki, E. L. Lamoureux, J. W. Kowalski, T. Bek, S.-J. Chen, J. M. Dekker, A. Fletcher, J. Grauslund, *Diabetes care* **2012**, *35*, 556.
[63] a) S. Patel, H. Chen, N. H. Tinkham, K. Zhang, *Current diabetes reports* **2008**, *8*, 257; b) C. P. Wilkinson, F. L. Ferris III, R. E. Klein, P. P. Lee, C. D. Agardh, M. Davis, D. Dills, A. Kampik, R. Pararajasegaram, J. T. Verdaguer, *Ophthalmology* **2003**, *110*, 1677.
[64] a) W. L. Wong, X. Su, X. Li, C. M. G. Cheung, R. Klein, C.-Y. Cheng, T. Y. Wong, *The Lancet Global Health* **2014**, *2*, e106; b) K. L. Pennington, M. M. DeAngelis, *Eye and Vision* **2016**, *3*, 34; c) K. M. Gehrs, D. H. Anderson, L. V. Johnson, G. S. Hageman, *Ann. Med.* **2006**, *38*, 450.
[65] J. Ambati, B. J. Fowler, *Neuron* **2012**, *75*, 26.
[66] a) M. Fleckenstein, S. Schmitz-Valckenberg, U. Chakravarthy, *JAMA, J. Am. Med. Assoc.* **2024**, *331*, 147; b) V. Chaikitmongkol, S. B. Bressler, N. M. Bressler, in *Albert and Jakobiec's Principles and Practice of Ophthalmology*, (Eds.: D. M. Albert, J. W. Miller, D. T. Azar,

L. H. Young), Springer International Publishing, Cham **2022**, p. 3565.
[67] K. G. Falavarjani, M. Hashemi, M. Modarres, M. M. Parvaresh, M. Naseripour, H. Nazari, A. J. Fazel, *Middle East Afr J Ophthalmol* **2013**, *20*, 244.
[68] M. Tawfik, F. Chen, J. L. Goldberg, B. A. Sabel, *Naunyn Schmiedebergs Arch Pharmacol* **2022**, *395*, 1477.
[69] a) Y. Nuzbrokh, S. D. Ragi, S. H. Tsang, *Ann. Transl. Med.* **2021**, *9*, 1278; b) A. S. Brar, D. C. Parameswarappa, B. Takkar, R. Narayanan, S. Jalali, S. Mandal, K. Fujinami, S. K. Padhy, *Ophthalmology and Therapy* **2024**, *13*, 21.
[70] a) N. Cross, C. van Steen, Y. Zegaoui, A. Satherley, L. Angelillo, *Clin Ophthalmol* **2022**, *16*, 2909; b) W. Liu, S. Liu, P. Li, K. Yao, *Int. J. Mol. Sci.* **2022**, *23*, 4883.
[71] H. F. Mendes, J. Van Der Spuy, J. P. Chapple, M. E. Cheetham, *Trends Mol. Med.* **2005**, *11*, 177.
[72] C. Sanjurjo-Soriano, C. Jimenez-Medina, N. Erkilic, L. Cappellino, A. Lefevre, K. Nagel-Wolfrum, U. Wolfrum, E. Van Wijk, A. F. Roux, I. Meunier, V. Kalatzis, *HGG Adv.* **2023**, *4*, 100229.
[73] T. Ong, M. E. Pennesi, D. G. Birch, B. L. Lam, S. H. Tsang, *Pharm. Res.* **2019**, *36*, 34.
[74] T. A. C. Guimaraes, E. Arram, A. F. Shakarchi, M. Georgiou, M. Michaelides, *Br. J. Ophthalmol.* **2023**, *107*, 1403.
[75] E. Nisenbaum, T. P. Thielhelm, A. Nourbakhsh, D. Yan, S. H. Blanton, Y. Shu, K. R. Koehler, A. El-Amraoui, Z. Chen, B. L. Lam, X. Liu, *Ear Hear* **2022**, *43*, 1.
[76] a) Z. Saihan, A. R. Webster, L. Luxon, M. Bitner-Glindzicz, *Curr. Opin. Neurol.* **2009**, *22*, 19; b) D. Weil, S. Blanchard, J. Kaplan, P. Guilford, F. Gibson, J. Walsh, P. Mburu, A. Varela, J. Levilliers, M. Weston, *Nature 374*, 60; c) H. Bolz, *Der Ophthalmologe* **2009**, *106*, 496.
[77] S. H. Tsang, T. Sharma, in *Atlas of Inherited Retinal Diseases*, (Eds.: S. H. Tsang, T. Sharma), Springer International Publishing, Cham **2018**, p. 139.
[78] J. P. Szlyk, G. A. Fishman, S. Grover, B. I. Revelins, D. J. Derlacki, *Br. J. Ophthalmol.* **1998**, *82*, 1372.
[79] T. M. Schmitt, M. J. Ohns, J. J. DeVries, *The Journal for Nurse Practitioners* **2023**, *19*, 104560.
[80] J. G. Park, C. L. Xu, A. Boyd, L. Aghajanova, V. B. Mahajan, E. H. Wood, *Retina* **2022**, *42*, 2025.
[81] S. Drag, F. Dotiwala, A. K. Upadhyay, *Invest Ophthalmol Vis Sci* **2023**, *64*, 39.
[82] a) M. C. Gemayel, A. D. Bhatwadekar, T. Ciulla, *Expert Opin. Biol. Ther.* **2021**, *21*, 603; b) S. Guo, C. Li, C. Wang, X. Cao, X. Liu, X.-J. Liang, Y. Huang, Y. Weng, *Acta Pharm. Sin. B.* **2024**, *14*, 781.
[83] a) J. A. Sahel, T. Leveillard, S. Picaud, D. Dalkara, K. Marazova, A. Safran, M. Paques, J. Duebel, B. Roska, S. Mohand-Said, *Graefes Arch Clin Exp Ophthalmol* **2013**, *251*, 1669; b) E. Chaum, M. P. Hatton, *Survey of Ophthalmology* **2002**, *47*, 449; c) J. Adijanto, M. I. Naash, *Eur. J. Pharm. Biopharm.* **2015**, *95*, 353.
[84] T. Gaj, S. J. Sirk, S. L. Shui, J. Liu, *Cold Spring Harb Perspect Biol.* **2016**, *8*, a023754.
[85] B. L. da Costa, S. R. Levi, E. Eulau, Y. T. Tsai, P. M. J. Quinn, *Front Genome Ed.* **2021**, *3*, 775330.
[86] H. Y. Zhang, J. Vandesompele, K. Braeckmans, S. C. De Smedt, K. Remaut, *Chem. Soc. Rev.* **2024**, *53*, 317.
[87] a) M. A. Stepp, A. S. Menko, *Transl. Res.* **2021**, *236*, 52; b) D. Ren, S. Fisson, D. Dalkara, D. Ail, *Pharmaceutics* **2022**, *14*, 1973.
[88] S. Batabyal, S. Kim, W. Wright, S. Mohanty, *Journal of Biophotonics* **2021**, *14*, 202000234.
[89] K. Dhakal, S. Batabyal, W. Wright, Y.-t. Kim, S. Mohanty, *Light: Sci. Appl.* **2015**, *4*, e352.
[90] F. Staurenghi, M. E. McClements, A. Salman, R. E. MacLaren, *Int. J. Mol. Sci.* **2022**, *23*, 11673.
[91] J. C. Burnett, J. J. Rossi, K. Tiemann, *Biotechnol. J.* **2011**, *6*, 1130.
[92] C. Wan, F. Li, H. Li, *Mol. Med. Rep.* **2015**, *12*, 4803.
[93] P. K. Kaiser, R. C. Symons, S. M. Shah, E. J. Quinlan, H. Tabandeh, D. V. Do, G. Reisen, J. A. Lockridge, B. Short, R. Guerciolini, Q. D. Nguyen, *Am. J. Ophthalmol.* **2010**, *150*, 33.
[94] A. Gupta, K. N. Kafetzis, A. D. Tagalakis, C. Yu-Wai-Man, *Exp. Eye Res.* **2021**, *205*, 108482.
[95] T. W. Olsen, H. F. Edelhauser, J. I. Lim, D. H. Geroski, *Investigative ophthalmology & visual science* **1995**, *36*, 1893.
[96] T. Asahara, K. Shinomiya, T. Naito, H. Shiota, *Japanese journal of ophthalmology* **2001**, *45*, 31.
[97] D. Wei, N. Pu, S.-Y. Li, Y.-G. Wang, Y. Tao, *Drug Delivery* **2023**, *30*, 2165736.
[98] a) M. Voigt, Y. De Kozak, M. Halhal, Y. Courtois, F. Behar-Cohen, *Biochem. Biophys. Res. Commun.* **2002**, *295*, 336; b) F. F. Behar-Cohen, P. Milne, J.-M. Parel, I. Persaud, in *Drug Product Development for the Back of the Eye*, (Eds: U. B. Kompella, H. F. Edelhauser), Springer US, Boston, MA, **2011**, p. 361.
[99] T. W. Chalberg, A. Vankov, F. E. Molnar, A. F. Butterwick, P. Huie, M. P. Calos, D. V. Palanker, *Invest Ophthalmol Vis Sci.* **2006**, *47*, 4083.
[100] M. A. Kay, J. C. Glorioso, L. Naldini, *Nat. Med.* **2001**, *7*, 33.
[101] T. Shirakawa, A. Gotoh, S. Terao, N. Hinata, K. Goda, K. Tanaka, A. Takenaka, I. Hara, C. Kao, T. Gardner, *Cancer Res.* **2007**, *67*, LB.
[102] A. Auricchio, F. Rolling, *Curr. Gene Ther.* **2005**, *5*, 339.
[103] a) C. Summerford, R. J. Samulski, *J. Virol.* **1998**, *72*, 1438; b) R. W. Walters, S. M. P. Yi, S. Keshavjee, K. E. Brown, M. J. Welsh, J. A. Chiorini, J. Zabner, *J. Biol. Chem.* **2001**, *276*, 20610; c) G. D. Pasquale, B. L. Davidson, C. S. Stein, I. Martins, D. Scudiero, A. Monks, J. A. Chiorini, *Nat. Med.* **2003**, *9*, 1306; d) N. Kaludov, K. E. Brown, R. W. Walters, J. Zabner, J. A. Chiorini, *J. Virol.* **2001**, *75*, 6884; e) V. W. Choi, D. M. McCarty, R. J. Samulski, *Curr. Gene Ther.* **2005**, *5*, 299; f) G. Gao, L. H. Vandenberghe, J. M. Wilson, *Curr. Gene Ther.* **2005**, *5*, 285.
[104] J. E. Rabinowitz, F. Rolling, C. Li, H. Conrath, W. Xiao, X. Xiao, R. J. Samulski, *J. Virol.* **2002**, *76*, 791.
[105] a) R. R. Ali, M. B. Reichel, A. J. Thrasher, R. J. Levinsky, C. Kinnon, N. Kanuga, D. M. Hunt, S. S. Bhattacharya, *Hum. Mol. Genet.* **1996**, *5*, 591; b) R. R. Ali, M. B. Reichel, M. De Alwis, N. Kanuga, C. Kinnon, R. J. Levinsky, D. M. Hunt, S. S. Bhattacharya, A. J. Thrasher, *Hum. Gene Ther.* **1998**, *9*, 81; c) J. Bennett, A. M. Maguire, A. V. Cideciyan, M. Schnell, E. Glover, V. Anand, T. S. Aleman, N. Chirmule, A. R. Gupta, Y. Huang, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 9920; d) A. Auricchio, G. Kobinger, V. Anand, M. Hildinger, E. O'Connor, A. M. Maguire, J. M. Wilson, J. Bennett, *Hum. Mol. Genet.* **2001**, *10*, 3075; e) G. S. Yang, M. Schmidt, Z. Yan, J. D. Lindbloom, T. C. Harding, B. A. Donahue, J. F. Engelhardt, R. Kotin, B. L. Davidson, *J. Virol.* **2002**, *76*, 7651; f) M. Weber, J. Rabinowitz, N. Provost, H. Conrath, S. Folliot, D. Briot, Y. Chérel, P. Chenuaud, J. Samulski, P. Moullier, *Mol. Ther.* **2003**, *7*, 774.
[106] N. Provost, G. Le Meur, M. Weber, A. Mendes-Madeira, G. Podevin, Y. Cherel, M. A. Colle, J. Y. Deschamps, P. Moullier, F. Rolling, *Mol. Ther.* **2005**, *11*, 275.
[107] K. Stieger, M. A. Colle, L. Dubreil, A. Mendes-Madeira, M. Weber, G. Le Meur, J. Y. Deschamps, N. Provost, D. Nivard, Y. Cherel, P. Moullier, F. Rolling, *Mol. Ther.* **2008**, *16*, 916.
[108] a) H.-J. Nam, M. D. Lane, E. Padron, B. Gurda, R. McKenna, E. Kohlbrenner, G. Aslanidi, B. Byrne, N. Muzyczka, S. Zolotukhin, *J. Virol.* **2007**, *81*, 12260; b) Z. Wang, T. Zhu, C. Qiao, L. Zhou, B. Wang, J. Zhang, C. Chen, J. Li, X. Xiao, *Nat. Biotechnol.* **2005**, *23*, 321; c) T. C. Harding, A. S. Lalani, B. N. Roberts, S. Yendluri, B. Luan, K. E. Koprivnikar, M. Gonzalez-Edick, G. Huan-Tu, R. Musterer, M. J. VanRoey, *Mol. Ther.* **2006**, *13*, 956; d) M. Allocca, C. Mussolino, M. Garcia-Hoyos, D. Sanges, C. Iodice, M. Petrillo, L. H. Vandenberghe, J. M. Wilson, V. Marigo, E. M. Surace, *J. Virol.* **2007**, *81*, 11372; e)

M. Natkunarajah, P. Trittibach, J. McIntosh, Y. Duran, S. Barker, A. Smith, A. Nathwani, R. Ali, *Gene Ther.* **2008**, *15*, 463.
[109] M. Gorbatyuk, V. Justilien, J. Liu, W. W. Hauswirth, A. S. Lewin, *Vision Res.* **2007**, *47*, 1202.
[110] S. M. Cashman, L. Bowman, J. Christofferson, R. Kumar-Singh, *Invest Ophthalmol Vis Sci.* **2006**, *47*, 3496.
[111] a) G.-M. Sarra, C. Stephens, M. De Alwis, J. W. Bainbridge, A. J. Smith, A. J. Thrasher, R. R. Ali, *Hum. Mol. Genet.* **2001**, *10*, 2353; b) R. R. Ali, G.-M. Sarra, C. Stephens, M. d. Alwis, J. W. Bainbridge, P. M. Munro, S. Fauser, M. B. Reichel, C. Kinnon, D. M. Hunt, *Nat. Genet.* **2000**, *25*, 306.
[112] C. E. Thomas, A. Ehrhardt, M. A. Kay, *Nat. Rev. Genet.* **2003**, *4*, 346.
[113] S. E. Raper, N. Chirmule, F. S. Lee, N. A. Wivel, A. Bagg, G.-p. Gao, J. M. Wilson, M. L. Batshaw, *Mol. Genet. Metab.* **2003**, *80*, 148.
[114] a) O. Harush-Frenkel, N. Debotton, S. Benita, Y. Altschuler, *Biochem. Biophys. Res. Commun.* **2007**, *353*, 26; b) R. Rempe, S. Cramer, R. Qiao, H.-J. Galla, *Cell Tissue Res.* **2014**, *355*, 717; c) M. Sousa de Almeida, E. Susnik, B. Drasler, P. Taladriz-Blanco, A. Petri-Fink, B. Rothen-Rutishauser, *Chem. Soc. Rev.* **2021**, *50*, 5397.
[115] N. Oh, J.-H. Park, *Int. J. Nanomed.* **2014**, *9*, 51.
[116] E. C. Freeman, L. M. Weiland, W. S. Meng, *J Biomater Sci Polym Ed.* **2013**, *24*, 398.
[117] S. Höbel, A. Aigner, *Wiley Interdiscip. Rev.: Nanomed. Nanobiotechnol.* **2013**, *5*, 484.
[118] a) L. Pitkänen, M. Ruponen, J. Nieminen, A. Urtti, *Pharm. Res.* **2003**, *20*, 576; b) L. Peeters, N. N. Sanders, K. Braeckmans, K. Boussery, J. Van de Voorde, S. C. De Smedt, J. Demeester, *Investigative ophthalmology & visual science* **2005**, *46*, 3553.
[119] a) L. Tauhardt, K. Kempe, K. Knop, E. Altuntaş, M. Jäger, S. Schubert, D. Fischer, U. S. Schubert, *Macromol. Chem. Phys.* **2011**, *212*, 1918; b) B. Demeneix, J. P. Behr, *Advances in genetics* **2005**, *53*, 215.
[120] M. Chiper, N. Tounsi, R. Kole, A. Kichler, G. Zuber, *J. Controlled Release* **2017**, *246*, 60.
[121] H.-W. Liao, K.-W. Yau, *BioTechniques* **2007**, *42*, 285.
[122] a) D. S. Zhuk, P. A. Gembitskii, V. A. Kargin, *Russ. Chem. Rev.* **1965**, *34*, 515; b) R. Kircheis, L. Wightman, E. Wagner, *Adv. Drug Delivery Rev.* **2001**, *53*, 341; c) M. Jäger, S. Schubert, S. Ochrimenko, D. Fischer, U. S. Schubert, *Chem. Soc. Rev.* **2012**, *41*, 4755; d) B. Brissault, A. Kichler, C. Guis, C. Leborgne, O. Danos, H. Cheradame, *Bioconjugate Chem.* **2003**, *14*, 581.
[123] L. Wightman, R. Kircheis, V. Rössler, S. Carotta, R. Ruzicka, M. Kursa, E. Wagner, *The Journal of Gene Medicine: A cross-disciplinary journal for research on the science of gene transfer and its clinical applications* **2001**, *3*, 362.
[124] A. Akinc, M. Thomas, A. M. Klibanov, R. Langer, *The Journal of Gene Medicine* **2005**, *7*, 657.
[125] S. M. Moghimi, P. Symonds, J. C. Murray, A. C. Hunter, G. Debska, A. Szewczyk, *Mol. Ther.* **2005**, *11*, 990.
[126] A. L. Gomes dos Santos, A. Bochot, N. Tsapis, F. Artzner, R. A. Bejjani, B. Thillaye-Goldenberg, Y. de Kozak, E. Fattal, F. Behar-Cohen, *Pharm. Res.* **2006**, *23*, 770.
[127] J. Lee, N.-k. Ryoo, H. Han, H. K. Hong, J. Y. Park, S. J. Park, Y.-K. Kim, C. Sim, K. Kim, S. J. Woo, K. H. Park, H. Kim, *Mol. Pharmaceutics* **2016**, *13*, 1988.
[128] H. Koo, H. Moon, H. Han, J. H. Na, M. S. Huh, J. H. Park, S. J. Woo, K. H. Park, I. Chan Kwon, K. Kim, H. Kim, *Biomaterials* **2012**, *33*, 3485.
[129] a) L. Pitkänen, J. Pelkonen, M. Ruponen, S. Rönkkö, A. Urtti, *AAPS J.* **2004**, *6*, 72; b) G. Tan, D. Liu, R. Zhu, H. Pan, J. Li, W. Pan, *Acta Biomater.* **2021**, *134*, 605.
[130] N. K. Ryoo, J. Lee, H. Lee, H. K. Hong, H. Kim, J. B. Lee, S. J. Woo, K. H. Park, H. Kim, *Nanoscale* **2017**, *9*, 15461.
[131] a) A. E. q. de Salamanca, Y. Diebold, M. Calonge, C. García-Vazquez, S. Callejo, A. Vila, M. J. Alonso, *Investigative ophthalmology & visual science* **2006**, *47*, 1416; b) A. M. De Campos, A. Sánchez, M. a. J. Alonso, *Int. J. Pharm.* **2001**, *224*, 159.
[132] a) M. S. Jayaraman, D. J. Bharali, T. Sudha, S. A. Mousa, *Molecular Vision* **2012**, *18*, 2300; b) J.-Y. Lai, Y.-T. Li, T.-P. Wang, *Int. J. Mol. Sci.* **2010**, *11*, 5256.
[133] R. N. Mitra, Z. Han, M. Merwin, M. Al Taai, S. M. Conley, M. I. Naash, *ChemMedChem* **2014**, *9*, 189.
[134] A. V. Oliveira, G. A. Silva, *J. Bionanosci.* **2015**, *9*, 373.
[135] G. Puras, J. Zarate, A. Diaz-Tahoces, M. Avilés-Trigueros, E. Fernandez, J. Pedraz, *Eur. J. Pharm. Sci.* **2013**, *48*, 323.
[136] S. Garantziotis, R. C. Savani, *Matrix Biol.* **2019**, *78–79*, 1.
[137] a) G. Huerta Ángeles, K. Nešporová, *Carbohydr. Polym.* **2021**, *259*, 117697; b) M. Litwiniuk, A. Krejner, M. S. Speyrer, A. R. Gauto, T. Grzela, *Wounds* **2016**, *28*, 78.
[138] T. W. Prow, I. Bhutto, S. Y. Kim, R. Grebe, C. Merges, D. S. McLeod, K. Uno, M. Mennon, L. Rodriguez, K. Leong, G. A. Lutty, *Nanomedicine* **2008**, *4*, 340.
[139] J. Wen, H.-Q. Mao, W. Li, K. Y. Lin, K. W. Leong, *J. Pharm. Sci.* **2004**, *93*, 2142.
[140] D. L. Thorek, A. K. Chen, J. Czupryna, A. Tsourkas, *Ann. Biomed. Eng.* **2006**, *34*, 23.
[141] N. Wimmer, R. J. Marano, P. S. Kearns, E. P. Rakoczy, I. Toth, *Bioorg. Med. Chem. Lett.* **2002**, *12*, 2635.
[142] a) R. J. Marano, N. Wimmer, P. S. Kearns, B. G. Thomas, I. Toth, M. Brankov, P. E. Rakoczy, *Exp. Eye Res.* **2004**, *79*, 525; b) R. J. Marano, I. Toth, N. Wimmer, M. Brankov, P. E. Rakoczy, *Gene Ther.* **2005**, *12*, 1544.
[143] D. S. Shah, T. Sakthivel, I. Toth, A. T. Florence, A. F. Wilderspin, *Int. J. Pharm.* **2000**, *208*, 41.
[144] a) M. W. Konstan, P. B. Davis, J. S. Wagener, K. A. Hilliard, R. C. Stern, L. J. Milgram, T. H. Kowalczyk, S. L. Hyatt, T. L. Fink, C. R. Gedeon, *Hum. Gene Ther.* **2004**, *15*, 1255; b) T. L. Fink, P. J. Klepcyk, S. M. Oette, C. R. Gedeon, S. L. Hyatt, T. H. Kowalczyk, R. C. Moen, M. J. Cooper, *Gene Ther.* **2006**, *13*, 1048; c) A.-G. Ziady, C. R. Gedeon, T. Miller, W. Quan, J. M. Payne, S. L. Hyatt, T. L. Fink, O. Muhammad, S. Oette, T. Kowalczyk, M. K. Pasumarthy, R. C. Moen, M. J. Cooper, P. B. Davis, *Mol. Ther.* **2003**, *8*, 936; d) A.-G. Ziady, C. R. Gedeon, O. Muhammad, V. Stillwell, S. M. Oette, T. L. Fink, W. Quan, T. H. Kowalczyk, S. L. Hyatt, J. Payne, *Mol. Ther.* **2003**, *8*, 948.
[145] T. Kowalczyk, M. Pasumarthy, C. Gedeon, S. Hyatt, J. Payne, O. Muhammad, R. Moen, M. Cooper, *Mol. Ther.* **2001**, *3*, S359.
[146] M. Walsh, M. Tangney, M. O'neill, J. Larkin, D. Soden, S. L. McKenna, R. Darcy, G. O'sullivan, C. O'driscoll, *Mol. Pharmaceutics* **2006**, *3*, 644.
[147] X. Cai, S. Conley, M. Naash, *Vision research* **2008**, *48*, 319.
[148] a) X. Cai, Z. Nash, S. M. Conley, S. J. Fliesler, M. J. Cooper, M. I. Naash, *PLoS One* **2009**, *4*, e5290; b) X. Cai, S. M. Conley, Z. Nash, S. J. Fliesler, M. J. Cooper, M. I. Naash, *FASEB J.* **2010**, *24*, 1178.
[149] Z. Han, S. M. Conley, R. S. Makkia, M. J. Cooper, M. I. Naash, *J. Clin. Invest.* **2012**, *122*, 3221.
[150] a) X. Q. Ding, A. B. Quiambao, J. B. Fitzgerald, M. J. Cooper, S. M. Conley, M. I. Naash, *PLoS One* **2009**, *4*, e7410; b) Z. Han, A. Koirala, R. Makkia, M. J. Cooper, M. I. Naash, *Nanomedicine* **2012**, *7*, 521.
[151] Z. Han, M. J. Banworth, R. Makkia, S. M. Conley, M. R. Al-Ubaidi, M. J. Cooper, M. I. Naash, *FASEB J.* **2015**, *29*, 2535.
[152] a) M. Zheng, R. N. Mitra, N. A. Filonov, Z. Han, *FASEB J.* **2016**, *30*, 1076; b) R. N. Mitra, M. Zheng, E. R. Weiss, Z. Han, *Biomaterials* **2018**, *157*, 26; c) M. Zheng, R. N. Mitra, E. R. Weiss, Z. Han, *Mol. Ther.* **2020**, *28*, 523.
[153] Z. Han, S. M. Conley, R. Makkia, J. Guo, M. J. Cooper, M. I. Naash, *PLoS One* **2012**, *7*, e52189.
[154] R. N. Mitra, C. A. Nichols, J. Guo, R. Makkia, M. J. Cooper, M. I. Naash, Z. Han, *J Control Release* **2016**, *236*, 31.

[155] V. S. Kansara, M. Cooper, O. Sesenoglu-Laird, L. Muya, R. Moen, T. A. Ciulla, *Transl Vis Sci Technol* **2020**, *9*, 21.
[156] R. Tenchov, R. Bird, A. E. Curtze, Q. Zhou, *ACS Nano* **2021**, *15*, 16982.
[157] C. Hald Albertsen, J. A. Kulkarni, D. Witzigmann, M. Lind, K. Petersson, J. B. Simonsen, *Adv. Drug Delivery Rev.* **2022**, *188*, 114416.
[158] L. Schoenmaker, D. Witzigmann, J. A. Kulkarni, R. Verbeke, G. Kersten, W. Jiskoot, D. J. A. Crommelin, *Int. J. Pharm.* **2021**, *601*, 120586.
[159] M. Cárdenas, R. A. Campbell, M. Yanez Arteta, M. J. Lawrence, F. Sebastiani, *Curr. Opin. Colloid Interface Sci.* **2023**, *66*, 101705.
[160] D. A. Balazs, W. T. Godbey, *Journal of Drug Delivery* **2011**, *2011*, 1.
[161] U. B. Kompella, A. C. Amrite, R. Pacha Ravi, S. A. Durazo, *Prog Retin Eye Res.* **2013**, *36*, 172.
[162] M. Ramezani, M. Khoshhamdam, A. Dehshahri, B. Malaekeh-Nikouei, *Colloids Surf., B.* **2009**, *72*, 1.
[163] I. Masuda, T. Matsuo, T. Yasuda, N. Matsuo, *Investigative ophthalmology & visual science* **1996**, *37*, 1914.
[164] S. Kawakami, A. Harada, K. Sakanaka, K. Nishida, J. Nakamura, T. Sakaeda, N. Ichikawa, M. Nakashima, H. Sasaki, *Int. J. Pharm.* **2004**, *278*, 255.
[165] a) U. Pleyer, D. Groth, B. Hinz, O. Keil, E. Bertelmann, P. Rieck, R. Reszka, *Exp. Eye Res.* **2001**, *73*, 1; b) A. Urtti, J. Polansky, G. M. Lui, F. C. Szoka, *J. Drug Targeting* **2000**, *7*, 413; c) K. Abul-Hassan, R. Walmsley, M. Boulton, *Curr. Eye Res.* **2000**, *20*, 361.
[166] G. P. Ochoa, J. Z. Sesma, M. A. Díez, A. Díaz-Tahoces, M. Avilés-Trigeros, S. Grijalvo, R. Eritja, E. Fernández, J. L. Pedraz, *Pharm. Res.* **2014**, *31*, 1665.
[167] S. Alqawlaq, J. M. Sivak, J. T. Huzil, M. V. Ivanova, J. G. Flanagan, M. A. Beazely, M. Foldvari, *Nanomedicine: Nanotechnology, Biology and Medicine* **2014**, *10*, 1637.
[168] J. Devoldere, K. Peynshaert, H. Dewitte, C. Vanhove, L. De Groef, L. Moons, S. Y. Özcan, D. Dalkara, S. C. De Smedt, K. Remaut, *J. Controlled Release* **2019**, *307*, 315.
[169] a) S. Patel, R. C. Ryals, K. K. Weller, M. E. Pennesi, G. Sahay, *J Control Release* **2019**, *303*, 91; b) X. Huang, Y. Chau, *Mol Pharm* **2021**, *18*, 377.
[170] a) N. Hartl, F. Adams, O. M. Merkel, *Adv Ther (Weinh)* **2021**, *4*, 2000092; b) M. D. Howard, M. Jay, T. D. Dziubla, X. Lu, *J. Biomed. Nanotechnol.* **2008**, *4*, 133.
[171] R. C. Ryals, S. Patel, C. Acosta, M. McKinney, M. E. Pennesi, G. Sahay, *PLoS One* **2020**, *15*, 0241006.
[172] M. Gautam, A. Jozic, G. L. Su, M. Herrera-Barrera, A. Curtis, S. Arrizabalaga, W. Tschetter, R. C. Ryals, G. Sahay, *Nat. Commun.* **2023**, *14*, 6468.
[173] a) Y. Eygeris, M. I. Henderson, A. G. Curtis, A. Jozić, J. Stoddard, R. Reynaga, K. R. Chirco, G. L.-N. Su, M. Neuringer, A. K. Lauer, R. C. Ryals, G. Sahay, *Small* **2024**, *20*, 2400815; b) Y. Eygeris, M. Gupta, J. Kim, A. Jozic, M. Gautam, J. Renner, D. Nelson, E. Bloom, A. Tuttle, J. Stoddard, R. Reynaga, M. Neuringer, A. K. Lauer, R. C. Ryals, G. Sahay, *Proc. Natl. Acad. Sci. USA* **2024**, *121*, 2307813120.
[174] H.-a. Liu, Y.-l. Liu, Z.-z. Ma, J.-c. Wang, Q. Zhang, *Investigative ophthalmology & visual science* **2011**, *52*, 4789.
[175] a) S. Supe, A. Upadhya, S. Tripathi, V. Dighe, K. Singh, *Drug Deliv Transl Res.* **2023**, *13*, 1675; b) M. Amadio, A. Pascale, S. Cupri, R. Pignatello, C. Osera, D. A. V, D. A. AG, G. M. Leggio, B. Ruozi, S. Govoni, F. Drago, C. Bucolo, *Pharmacol Res.* **2016**, *111*, 713.
[176] N. O. Sahin, *Nanomaterials and nanosystems for biomedical applications*, Springer, Berlin, **2007**, p. 67.
[177] a) Z. S. Bayindir, N. Yuksel, *J. Pharm. Sci.* **2010**, *99*, 2049; b) G. H. Sagar, M. Arunagirinathan, J. R. Bellare, *Indian J Exp Biol* **2007**, *45*, 133.
[178] a) V. P. Chandu, A. Arunachalam, S. Jeganath, K. Yamini, K. Tharangini, G. Chaitanya, *International journal of novel trends in pharmaceutical sciences* **2012**, *2*, 25; b) A. Baillie, A. Florence, L. Hume, G. Muirhead, A. Rogerson, *J. Pharm. Pharmacol.* **1985**, *37*, 863.
[179] S. Grijalvo, G. Puras, J. Zárate, M. Sainz-Ramos, N. A. L. Qtaish, T. López, M. Mashal, N. Attia, D. Díaz Díaz, R. Pons, E. Fernández, J. L. Pedraz, R. Eritja, *Pharmaceutics* **2019**, *11*, 50.
[180] E. Ojeda, M. Agirre, I. Villate-Beitia, M. Mashal, G. Puras, J. Zarate, J. L. Pedraz, *Non-Viral Gene Delivery Vectors: Methods and Protocols*, Springer, Berlin, **2016**, p. 63.
[181] a) E. Ojeda, G. Puras, M. Agirre, J. Zarate, S. Grijalvo, R. Eritja, G. Martinez-Navarrete, C. Soto-Sánchez, A. Díaz-Tahoces, M. Aviles-Trigueros, *Biomaterials* **2016**, *77*, 267; b) E. Ojeda, G. Puras, M. Agirre, J. Zárate, S. Grijalvo, R. Pons, R. Eritja, G. Martinez-Navarrete, C. Soto-Sánchez, E. Fernández, *Org. Biomol. Chem.* **2015**, *13*, 1068.
[182] a) N. Al Qtaish, I. Gallego, I. Villate-Beitia, M. Sainz-Ramos, G. Martinez-Navarrete, C. Soto-Sanchez, E. Fernandez, P. Galvez-Martin, T. B. Lopez-Mendez, G. Puras, J. L Pedraz, *Eur. J. Pharm. Biopharm.* **2021**, *169*, 103; b) E. Ojeda, G. Puras, M. Agirre, J. Zarate, S. Grijalvo, R. Eritja, L. DiGiacomo, G. Caracciolo, J.-L. Pedraz, *Int. J. Pharm.* **2016**, *503*, 115; c) M. Mashal, N. Attia, G. Puras, G. Martínez-Navarrete, E. Fernández, J. L. Pedraz, *J. Controlled Release* **2017**, *254*, 55.
[183] G. Puras, M. Mashal, J. Zárate, M. Agirre, E. Ojeda, S. Grijalvo, R. Eritja, A. Diaz-Tahoces, G. M Navarrete, M. Avilés-Trigueros, E. Fernández, J. L. Pedraz, *J. Controlled Release* **2014**, *174*, 27.
[184] I. Gallego, I. Villate-Beitia, G. Martinez-Navarrete, M. Menendez, T. Lopez-Mendez, C. Soto-Sanchez, J. Zarate, G. Puras, E. Fernandez, J. L. Pedraz, *Nanomedicine* **2019**, *17*, 308.
[185] G. Puras, G. Martinez-Navarrete, M. Mashal, J. Zarate, M. Agirre, E. Ojeda, S. Grijalvo, R. Eritja, A. Diaz-Tahoces, M. Aviles-Trigueros, E. Fernandez, J. L. Pedraz, *Mol Pharm* **2015**, *12*, 3658.
[186] K. W. Park, *International Anesthesiology Clinics* **2004**, 42.
[187] M. Mashal, N. Attia, G. Martinez-Navarrete, C. Soto-Sanchez, E. Fernandez, S. Grijalvo, R. Eritja, G. Puras, J. L. Pedraz, *J Control Release* **2019**, *304*, 181.
[188] R. A. Bejjani, D. BenEzra, H. Cohen, J. Rieger, C. Andrieu, J.-C. Jeanny, G. Gollomb, F. F. Behar-Cohen, *Mol Vis* **2005**, *11*, 124.
[189] F. Danhier, E. Ansorena, J. M. Silva, R. Coco, A. Le Breton, V. Préat, *J. Controlled Release* **2012**, *161*, 505.
[190] C. Zhang, Y. Wang, H. Wu, Z. Zhang, Y. Cai, H. Hou, W. Zhao, X. Yang, J. Ma, *Gene Ther.* **2010**, *17*, 338.
[191] J.-L. Bourges, S. E. Gautier, F. Delie, R. A. Bejjani, J.-C. Jeanny, R. Gurny, D. BenEzra, F. F. Behar-Cohen, *Investigative ophthalmology & visual science* **2003**, *44*, 3562.
[192] J. V. Aukunuru, S. P. Ayalasomayajula, U. B. Kompella, *J. Pharm. Pharmacol.* **2003**, *55*, 1199.
[193] D. Cun, C. Foged, M. Yang, S. Frøkjær, H. M. Nielsen, *Int. J. Pharm.* **2010**, *390*, 70.
[194] K. Park, Y. Chen, Y. Hu, A. S. Mayo, U. B. Kompella, R. Longeras, J.-x. Ma, *Diabetes* **2009**, *58*, 1902.
[195] Y. Cao, A. Chen, S. S. A. An, R.-W. Ji, D. Davidson, Y. Cao, M. Llinás, *J. Biol. Chem.* **1997**, *272*, 22924.
[196] J. Jin, K. K. Zhou, K. Park, Y. Hu, X. Xu, Z. Zheng, P. Tyagi, U. B. Kompella, J.-x. Ma, *Investigative ophthalmology & visual science* **2011**, *52*, 6230.
[197] G. Liu, D. Li, M. K. Pasumarthy, T. H. Kowalczyk, C. R. Gedeon, S. L. Hyatt, J. M. Payne, T. J. Miller, P. Brunovskis, T. L. Fink, O. Muhammad, R. C. Moen, R. W. Hanson, M. J. Cooper, *J. Biol. Chem.* **2003**, *278*, 32578.
[198] Z. Han, S. M. Conley, R. S. Makkia, M. J. Cooper, M. I. Naash, *J. Clin. Invest.* **2012**, *122*, 3221.

[199] J. Fu, M. Liu, Y. Liu, H. Yan, *Acc. Chem. Res.* **2012**, *45*, 1215.
[200] a) T. Schnitzler, A. Herrmann, *Acc. Chem. Res.* **2012**, *45*, 1419; b) A. V. Pinheiro, D. Han, W. M. Shih, H. Yan, *Nat. Nanotechnol.* **2011**, *6*, 763.
[201] a) G. Zhu, J. Zheng, E. Song, M. Donovan, K. Zhang, C. Liu, W. Tan, *Proc Natl Acad Sci U S A* **2013**, *110*, 7998; b) Y. X. Zhao, A. Shaw, X. Zeng, E. Benson, A. M. Nystrom, B. Hogberg, *ACS Nano* **2012**, *6*, 8684.
[202] a) J. W de Vries, S. Schnichels, J. Hurst, L. Strudel, A. Gruszka, M. Kwak, K. U. Bartz-Schmidt, M. S. Spitzer, A. Herrmann, *Biomaterials* **2018**, *157*, 98; b) S. Schnichels, D. Simmang, M. Loscher, A. Herrmann, J. W. de Vries, M. S. Spitzer, J. Hurst, *Pharmaceutics* **2023**, *15*, 532.
[203] a) S. Schnichels, J. Hurst, J. W. de Vries, S. Ullah, K. Frossl, A. Gruszka, M. Loscher, K. U. Bartz-Schmidt, M. S. Spitzer, A. Herrmann, *ACS Appl. Mater. Interfaces* **2021**, *13*, 9445; b) S. Schnichels, J. Hurst, J. W. de Vries, S. Ullah, A. Gruszka, M. Kwak, M. Loscher, S. Dammeier, K. U. Bartz-Schmidt, M. S. Spitzer, A. Herrmann, *Nanomedicine* **2020**, *29*, 102260.
[204] S. Li, S. Datta, E. Brabbit, Z. Love, V. Woytowicz, K. Flattery, J. Capri, K. Yao, S. Wu, M. Imboden, A. Upadhyay, R. Arumugham, W. B. Thoreson, M. M. DeAngelis, N. B. Haider, *Gene Ther.* **2021**, *28*, 223.
[205] D. Sakai, H. Tomita, A. Maeda, *Int. J. Mol. Sci.* **2022**, *23*, 15041.
[206] N. Nagai, R. Daigaku, R. Motoyama, H. Kaji, T. Abe, *J. Mater. Sci.: Mater. Med.* **2022**, *34*, 5.
[207] Y. Li, R. Tian, J. Xu, Y. Zou, T. Wang, J. Liu, *Polym. Chem.* **2024**, *15*, 1908.
[208] a) M. C. Leyden, F. Oviedo, S. Saxena, R. Kumar, N. Le, T. M. Reineke, *Bioconjugate Chem.* **2024**, *35*, 897; b) Y. Jin, F. Adams, J. Moller, L. Isert, C. M. Zimmermann, D. Keul, O. M. Merkel, *Macromol. Biosci.* **2023**, *23*, 2200409; c) D. Sivadasan, M. H. Sultan, O. Madkhali, Y. Almoshari, N. Thangavel, *Pharmaceutics* **2021**, *13*, 1291.
[209] a) F. Adams, C. M. Zimmermann, D. Baldassi, T. M. Pehl, P. Weingarten, I. Kachel, M. Kranzlein, D. C. Jurgens, P. Braubach, I. Alexopoulos, M. Wygrecka, O. M. Merkel, *Small* **2024**, *20*, 2308775; b) T. Thomas, H.-A. Tajmir-Riahi, C. Pillai, *Molecules* **2019**, *24*, 3744; c) Y. Wang, E. Wagner, *Pharmaceutics* **2020**, *12*, 888.
[210] I. Porello, N. Bono, G. Candiani, F. Cellesi, *Polym. Chem.* **2024**, *15*, 2800.
[211] M. J. Mehta, H. J. Kim, S. B. Lim, M. Naito, K. Miyata, *Macromol. Biosci.* **2024**, *24*, 2300366.
[212] a) Z. Zhang, H. Sun, J. Giannino, Y. Wu, C. Cheng, *Journal of Polymer Science* **2024**, *62*, 2231; b) M. Bauer, C. Lautenschlaeger, K. Kempe, L. Tauhardt, U. S. Schubert, D. Fischer, *Macromol. Biosci.* **2012**, *12*, 986; c) S. Schottler, G. Becker, S. Winzen, T. Steinbach, K. Mohr, K. Landfester, V. Mailander, F. R. Wurm, *Nat. Nanotechnol.* **2016**, *11*, 372; d) M. Li, S. Jiang, J. Simon, D. Passlick, M. L. Frey, M. Wagner, V. Mailander, D. Crespy, K. Landfester, *Nano Lett.* **2021**, *21*, 1591.
[213] M. Whalen, M. Akula, S. M. McNamee, M. M. DeAngelis, N. B. Haider, *Bioengineering (Basel)* **2024**, *11*, 179.
[214] E. M. del Amo, A. Urtti, *Drug Discov. Today* **2008**, *13*, 135.
[215] H. B. Raju, Y. Hu, K. R. Padgett, J. E. Rodriguez, J. L. Goldberg, *Clin. Exp. Ophthalmol.* **2012**, *40*, 100.
[216] a) Z. Zhang, L. Zhao, Y. Ma, J. Liu, Y. Huang, X. Fu, S. Peng, X. Wang, Y. Yang, X. Zhang, W. Ding, J. Yu, Y. Zhu, H. Yan, S. Yang, *J. Nanobiotechnol.* **2022**, *20*, 146; b) S. W. Shin, I. H. Song, S. H. Um, *Nanomaterials* **2015**, *5*, 1351.
[217] H. M. Braakhuis, M. V. D. Z. Park, I. Gosens, W. H. De Jong, F. R. Cassee, *Part. Fibre Toxicol.* **2014**, *11*, 18.
[218] S. Zhu, L. Gong, Y. Li, H. Xu, Z. Gu, Y. Zhao, *Adv. Sci. (Weinh)* **2019**, *6*, 1802289.

José Hurst completed her studies of Human Biology at Ernst-Moritz Arndt University Greifswald, with a focus on microbiology, immunology, and biochemistry. She finished it 2008 with a diploma thesis at the Friedrich Löffler Institute, where she investigated intergenotype specific distinctions of the matrix protein from different genotypes of lyssavirus. José Hurst earned a Ph.D. from the Institute of Medical Virology and Epidemiology of Viral Diseases at the University Hospital of Tübingen in 2013, where her research focused on the regulation of cervical cancer cell growth via viral and cellular proteins. Currently, she serves as the Deputy Head of the Neuroprotection & Drug Delivery Lab at the University Eye Hospital Tübingen. Prior to this role, she worked as a Postdoctoral Researcher at the same institution, making significant contributions to the field of ocular therapeutics.

Friederike Adams completed her M.Sc. in Chemistry, specializing in organic and macromolecular chemistry, in 2015, and obtained her Ph.D. in 2019, both from the Technical University of Munich, Germany. After earning her Ph.D., she joined the Ludwig Maximilian University of Munich and worked as a postdoctoral researcher in the Department of Pharmacy. Following a research stay at Warwick University in England as a Postdoctoral Research Fellow, she became part of the University of Stuttgart and University Eye Hospital Tübingen in 2021. Here, she leads the independent junior research group "Precision Polymers for Pharmaceutics".