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Nanoparticle-based Technologies for Retinal Gene Therapy

Jeffrey Adijanto and Muna I Naash*

University of Oklahoma Health Sciences Center, Department of Cell Biology, Oklahoma City, OK 73126-0901

Abstract

For patients with hereditary retinal diseases, retinal gene therapy offers significant promise for the prevention of retinal degeneration. While adeno-associated virus (AAV)-based systems remain the most popular gene delivery method due to their high efficiency and successful clinical results, other delivery systems, such as non-viral nanoparticles (NPs) are being developed as additional therapeutic options. NP technologies come in several categories (e.g., polymer, liposomes, peptide compacted DNA), several of which have been tested in mouse models of retinal disease. Here, we discuss the key biochemical features of the different NPs that influence how they are internalized into cells, escape from endosomes, and are delivered into the nucleus. We review the primary mechanism of NP uptake by retinal cells and highlight various NPs that have been successfully used for *in vivo* gene delivery to the retina and RPE. Finally, we consider the various strategies that can be implemented in the plasmid DNA to generate persistent, high levels of gene expression.

1. Introduction

Gene replacement therapy holds great promise for the treatment of many inherited retinal diseases. This approach directly targets the root of the disease, rather than treating symptoms, and is therefore theoretically the closest approach to a cure. In practice however, gene replacement therapy is far from perfect. Beside potential safety concerns, practical limitations exist. These include limited uptake and distribution of the gene expression vector, attenuated expression of the therapeutic gene over time, and the difficulty of treating patients after the onset of degeneration. When it comes to gene therapy, the two major gene delivery methods are viral (e.g., adeno-associated virus (AAV)) and non-viral (nanoparticles (NPs)). Each system comes with its own set of advantages and disadvantages. While AAV-based therapies typically have better transfection efficiencies than NP-based systems [1], NP technology offers a unique set of advantages. NPs are easy to synthesize and their molecular structures can be easily manipulated due to accessible functional groups. Furthermore, they generally have a low production cost compared to AAV systems, can accommodate large vector sizes, and possess a favorable safety profile (low immunogenicity and no risk of

*Corresponding author: muna-naash@ouhsc.edu.

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insertion mutagenesis) (reviewed in [2]). An additional layer of complication is conferred by the content of the plasmid DNA itself, and great effort has been placed on optimizing the DNA content of gene delivery plasmids to optimize persistence and levels of gene expression after delivery.

The overall effectiveness of a NP-based gene delivery system is dependent on three key factors: (1) cellular uptake of NPs, (2) escape of NPs from endosomal vesicles into the cytosol, (3) transfer of the plasmid DNA to the nucleus. NPs that have been formulated for gene therapy fall into one of several categories: (1) metal NPs; (2) lipid NPs; (3) polymer NPs. They differ in size, charge, shape, and structure, but all possess a mechanism to enter the cell, avoid or escape from endosomes, and deliver the plasmid cargo into the nucleus for gene expression. In this review, we discuss retinal diseases that are suitable for gene therapy. Next, we highlight the mechanisms (e.g., endocytosis, phagocytosis) through which most NPs are taken up by cells in the retina, followed by a discussion of the key features of the different NP technologies that have been evaluated as vehicles for gene transfer to the retina. Finally we assess the influence the plasmid content has on therapeutic efficacy.

2. Ocular gene therapy approach for retinal diseases

Retinal diseases can be entirely genetic or caused by a combination of genetic and environmental factors. Of the latter, the most prevalent include diabetic retinopathy and age-related macular degeneration, in which the genetic component is not necessarily causative, and mutations in associated genes only contribute to risk of developing the disease (reviewed in [3, 4]). On the other hand, most monogenic hereditary diseases can be traced in patients' genealogical pedigrees, and whole genome sequencing of samples from the patient and family members accelerates the identification of causal mutations and subsequent evaluation of disease mechanisms. Retinal degenerative diseases can be broadly categorized into two major groups depending on whether the disease initially targets rod (rod-cone dystrophy) or cone photoreceptor cells (cone-rod and cone dystrophies) (reviewed in [5]). Rod photoreceptors are responsible for night vision and are the dominant cell type in the peripheral (extramacular) region of the retina (>90%), whereas the macular (central) region of the retina is densely packed exclusively with cone photoreceptor cells. Patients with rod-cone dystrophies such as retinitis pigmentosa initially present with night-blindness followed by progressive loss of peripheral vision [6]. As the disease progresses to the advanced stages, patients are left with a small visual field that eventually disappears. Patients with cone-rod dystrophies such as Stargardt disease and Leber's congenital amaurosis (LCA) on the other hand present with rapid loss of central vision early in life followed by a progressive loss of peripheral vision [7–9].

To date, almost 300 unique genes have been associated with major retinal diseases including RP, LCA, and Stargardt disease (<http://sph.uth.tmc.edu/retnet/disease.htm>). These mutations are often in proteins directly responsible for a critical photoreceptor or retinal pigment epithelium (RPE) function, or in other proteins within the same functional network. Usher syndrome, for example, is associated with early onset retinitis pigmentosa and is caused by homozygous or compound heterozygous mutations within 10 different proteins (MYO7A, harmonin, CDH23, PCDH15, SANS, CIB2, usherin, VLGR1, whirlin, and clarin-1)

(reviewed in [10, 11]), all of which are thought to interact with one another within a protein complex that maintains photoreceptor structure and mediates material transport between photoreceptor inner and outer segments (OSs). While homozygous mutations in any one of the Usher-associated genes will cause retinitis pigmentosa, the onset of the disease, its progression, and the degree of severity can vary significantly from gene to gene and mutation to mutation. The heterogeneity of the disease is linked to the different functional roles of each protein within the complex. Similarly, mutations in genes involved in key developmental or physiological functions in the retina are also known to cause retinal degeneration, for example, mutations in retinal genes associated with photoreceptor development (e.g., CRX and CRB1) and phototransduction (e.g., GUCY2D) cause cone-rod dystrophy that is characteristic of LCA (reviewed in [12]). Mutations in genes expressed in the adjacent RPE can also cause retinal degeneration. Loss-of-function mutations in RPE genes such as RPE65 and LRAT (both involved in retinoid cycle) and in MERTK (regulates photoreceptor OS phagocytosis by the RPE) are known to cause LCA. The monogenic nature of many hereditary retinal diseases makes them a highly desirable target for gene therapy. But for each disease-associated gene, there can be up to hundreds of clinically-verified pathogenic single nucleotide polymorphisms (SNPs) scattered across the entire length of the gene. Therefore, for autosomal recessive retinal diseases, the most economical and straightforward strategy is the restoration of a fully functional version of the protein via DNA-based gene-therapy. In cases when the disease is caused by a dominant genetic mutation, gene-editing strategies (e.g., zinc-finger nucleases) can be implemented to correct the DNA mutation at the chromosomal level [13, 14]. Alternatively, shRNA knockdown of the mutant message with concurrent expression of a knockdown-resistant copy of the wild-type gene [15, 16] is a widely tested approach.

From a technical perspective, retinal gene therapy is feasible and is aided by the systemic isolation and immune privilege of the retina [17]. Gene-therapy vectors (and their carriers) delivered directly to the retina are less susceptible to elimination through the immune system and systemic excretion, and therefore they usually have a longer half-life, improved effectiveness, and higher bioavailability compared to vectors delivered systemically. In addition, potential side effects from non-specific delivery of the gene to other organs, such as the liver or kidney, can also be avoided. Although this local delivery improves safety profiles (since the systemic immune system is not activated), this is somewhat counteracted by the fact that intraocular delivery of therapeutics is highly invasive. Targeting cells in the outer retina (photoreceptors and RPE cells) requires subretinal injections [18, 19]. Although advancements have been made in the injection technique, repeated injections are prone to complications and should be avoided. Therefore, the success of a gene therapy strategy is contingent on its ability to achieve, from a single injection, persistent, high levels of gene expression and phenotypic correction in the target retinal cell type. Optimizing these parameters is the goal of preclinical gene therapy trials, and with the availability of non-invasive tools for accessing retinal morphology (e.g., optical coherence tomography) and function (electroretinography; ERG), gene-therapy plasmids and packaging methods can be easily evaluated for their effectiveness in treating animal models of the disease prior to human use.

3. NP vs. viral strategies for ocular gene therapy

Assessments of non-viral NP-based gene-therapies often begin with a discussion comparing NPs to viral systems. This is due largely to the success of AAV gene therapy studies: as an example the first successful clinical application of retinal gene-therapy was achieved using AAV-based delivery of the *RPE65* gene for the treatment of LCA in 2008 [20–22]. Patients from these studies retained a high level of visual function up to three years after treatment and did not develop any major health complications [23–25]. AAV-based therapies are currently being developed for various retinal diseases, including rod-cone dystrophy, Stargardt disease, and juvenile retinoschisis (reviewed in [26]). As a result of this success, AAV-based therapies have become the benchmark for other gene-therapy approaches. However, a major drawback to AAVs as a carrier is that they can only accommodate relatively small sized genetic cargo (< 5 kbp) [27]. The most recent advancements in AAV technology have extended the total transgene capacity to 10 kbp with the use of dual AAV vectors, wherein the gene is delivered in two fragments (< 5kbp each AAV) and subsequently recombine in the infected cells using homologous recombination or trans-splicing strategy, or both [28]. On the other hand, NPs can easily accommodate plasmids with sizes up to 20 kbp [29]. In addition to the size limitations of AAVs, there are also some issues with safety. Studies have shown that AAV vector DNA was detected in the serum of injected animals within 15 min after subretinal injection [30]. Viral genomes were also detected in nasal and lacrimal fluids. Other studies show that intravitreal injection of AAV carrying a GFP reporter gene into mice and dogs induced GFP expression in the optic nerve and brain [1, 31]. The threats are compounded by the potential for insertional mutagenesis [32]. On the other hand, NP-mediated gene transfer therapy has been effective in inducing long term protein expression without encountering safety issues commonly associated with viral-based delivery [2, 33]. Viral delivery of plasmid DNA however, is extremely efficient, as viruses have evolved specialized mechanisms for endosome escape and DNA delivery into the nucleus, whereas non-viral systems have a tendency to be entrapped in endosomes. Nonetheless, NP and plasmid DNA technologies have also advanced significantly in the past decade to improve efficacy, with some proof-of-concept studies demonstrating their effectiveness in gene delivery into mouse retina that parallels that of AAV systems [1]. Given the need for the development of clinically viable large capacity gene delivery vehicles to deliver the many large retinal disease genes (e.g., *usherin* and *abca4*), further optimization of the NP approach is desirable.

4. General mechanisms for NP uptake into the outer retina

In general, cells take up NPs from their surroundings either by phagocytosis or endocytosis (reviewed in [34]). The mechanism of choice depends on the cell type and the physical properties (size, charge, and shape) of the particles (reviewed in [35, 36]). Of cells in the eye, the RPE possesses both phagocytic and endocytic machinery [37, 38] whereas photoreceptors and Müller cells rely primarily on endocytosis [39–41]. A major role of the RPE is the phagocytosis of shed photoreceptor OS, which are digested within phagolysosomes to yield raw materials (nucleic acids, amino acids, lipids) that can either be utilized by the RPE or recycled back to the photoreceptors (reviewed in [42]). RPE phagocytosis of rod OSs is mediated by a mechanism that involves $\alpha\beta5$ integrin, a receptor

on the RPE apical surface that binds ligands (i.e., milk fat globule EGF-factor 8, MFG-E8) on the rod OS surface [38, 43]. Although OSs are the natural substrate for RPE phagocytosis, early *in vitro* studies of rat RPE primary cultures showed that RPE cells can also phagocytize bacteria (1 μm diameter), yeast (5 μm diameter), and even algae (8 μm) [44]. In general, particles less than 250 nm are phagocytized less efficiently and are taken up via endocytosis [36]. However *in vivo*, RPE cells are known to take up small particles, including compacted DNA NPs (~10 nm diameter) and naked supercoiled plasmid DNA (~150 nm diameter [45]) [19, 46]. While the dominant particle uptake mechanism in the RPE appears to be phagocytosis, it is not clear whether uptake of smaller NPs by the RPE is achieved via phagocytosis or endocytosis. Further studies are needed to correlate the NP size to the mechanism used in its uptake by the RPE.

In other non-phagocytic cell types such as photoreceptors, NPs less than 250 nm typically enter the cell via endocytosis [36], which can be subdivided into several distinct pathways that are defined by the proteins and lipids involved, the cargo they carry, and the morphological appearance of the endocytic structures [34]. The major forms of endocytosis are clathrin- and caveolae-mediated endocytosis (respectively, CME and CvME), but other CME and CvME-independent pathways also contribute to material uptake. In most cell types, CME accounts for a large fraction of all endocytic events, which can be triggered when particles bind a cell-surface receptor (receptor-mediated CME) or when particles establish ionic or dipole-dipole interactions with the plasma membrane (receptor-independent CME) (reviewed in [47]). Formation of vesicles in CME is mediated by clathrin proteins (assembled in a triskelion shape) that are recruited to the plasma membrane by the AP-2 protein complex upon NP-receptor binding. There, clathrin and its adaptor proteins (e.g., epsin, amphiphysin, SNX9) interact to form a polyhedral lattice basket-like structure around the NPs, eventually encapsulating them within vesicles that are internalized into cells. Many NPs for targeted drug delivery have been designed to take advantage of receptor-mediated CME, by chemically conjugating the NPs with receptor ligands such as lactoferrin and folate (reviewed in [48]). Alternatively, NPs can be coated with specific antibodies that bind surface receptors on the target cell (reviewed in [49]). Non-functionalized NPs rely on their surface charge to establish ionic interactions with the plasma membrane and trigger CME. In this regard, cationic particles can interact with negatively charged phospholipids on the plasma membrane surface and are therefore more effectively endocytosed compared to anionic or neutral particles [50]. However, as the endosomes mature and fuse with lysosomes to become endolysosomes, NPs that are processed through the endosome system are subject to degradation by acid hydrolases in a harsh acidic environment. NPs that enter via CvME on the other hand, are encapsulated within caveolae vesicles and are transferred to either early endosomes or to a large hydrolase-free organelle that targets to the endoplasmic reticulum (reviewed in [51, 52]), the latter of which allows the NPs to avoid degradation. CvME is regulated by caveolin (Cav) proteins, and in most cell types (other than muscle cells), caveolin isoform 1 (Cav-1) is primarily responsible for formation of caveolae vesicles. In the outer retina, Cav-1 is highly expressed in Müller glia cells, photoreceptors and the RPE [53]. Thus CvME may be a pathway for NP uptake in the outer retina.

In phagocytosis or endocytosis, NPs that eventually escape from endolysosomes will release the plasmid DNA cargo into the cytosol. However, the plasmid must enter the nucleus before any gene expression can occur. This is not an issue in proliferating cells since the nuclear envelope is disassembled during mitosis thus creating an opportunity for plasmid DNA to enter the nucleus [54]. However, both the RPE and photoreceptors are post-mitotic, so DNA entry into the nucleus is a rate-limiting step that diminishes the effectiveness of non-viral gene therapy [55]. Early studies showed that supercoiled plasmid DNA interacts with nuclear pore proteins and enters the nucleus via the same nuclear-entry pathway as karyophilic proteins and RNA [56, 57]. A more recent study implicates importin 7 in the nuclear transport of endogenous mitochondrial DNA or exogenous plasmid DNA [58]. In addition, plasmid DNA can also interact with cytosolic core histones and the histone chaperone NAPI for transportin-mediated delivery into the nucleus [59]. Another approach to enhance nuclear targeting is to exploit transcription factors to serve as chaperones for nuclear delivery of plasmids into the nucleus by incorporating nuclear localization signals in the plasmid DNA (reviewed in [60]). For example, the viral SV40 enhancer commonly used in gene expression plasmids is composed of a 72bp sequence that contains the nuclear localization signal recognized by several ubiquitously-expressed transcription factors (e.g., AP-1, AP-2, AP-3, Tef-1, Tef-2, Oct1, and NF κ B) [60]. Transcription factors are in turn recognized by a class of proteins known as karyopherins (importins), which bind the transcription factor-plasmid complex for targeted delivery to the nuclear pore complex. Plasmids without transcription factor binding sites or the SV40 enhancer do not translocate to the nucleus; they remain indefinitely in the cytoplasm until the nuclear envelope dissolves during cell mitosis [61, 62]. Therefore, the overall effectiveness of the NP-based gene therapy is dependent on both the NP formulation and plasmid DNA design. In the following section, we discuss and highlight the different NP technologies that have been evaluated for retinal gene transfer therapy.

5. NPs for retinal gene therapy

Nanoparticles of widely varying composition, charge, and effectiveness have been designed and tested for their ability to deliver drugs and genes to various cells. Many have been tested in the retina, and those we will discuss here are summarized in Table 1 and pictorially in Figure 1.

5.1. Metal NPs

5.1.1. Gold NPs—Over the past decade, metal NPs have been developed as a non-toxic drug or gene nanocarrier with various therapeutic applications. Among inert metals, gold is a popular choice due to its biocompatibility and easy synthesis process (reviewed in [63, 64]). In the context of ocular therapy, early studies have demonstrated that gold NPs, when injected intravitreally or subretinally to rabbit eyes, were non-toxic to the retina and RPE [87, 88]. Furthermore, intravenously-administered gold NPs (20 nm in diameter) that entered the retina by crossing the blood-retina-barrier also did not induce retina toxicity in mice [89]. An important advantage of gold (and metal) NPs is their optical properties that allow them to be detected using various imaging techniques including dark-field microscopy [90], Rayleigh light scattering microscopy [91], photothermal microscopy [92], plasmon

resonance coupling [93], and surface Raman scattering [94]. Furthermore, gold and metal NPs possess a high electron density and can therefore be visualized under a transmission electron microscope, revealing key information about the cell's NP uptake efficiency and the subcellular location of the NPs [88, 95]. In the retina, gold NPs can be detected using a non-invasive imaging technique called photothermal optical coherence tomography [96], thus allowing one to monitor the distribution and location of gold NPs in the retina after injection.

For gene therapy applications, gold NPs are commonly functionalized with cationic polyamines such as polyethylenimine (PEI) [97] and poly-L-lysine (PLL) [98] that can bind and condense negatively-charged plasmid DNA and confer protection from DNase activity. The surface of gold NPs can also be conjugated with various ligands (e.g., transferrin) [99], antibodies [100, 101], and cell penetrating peptides (e.g., TAT) [97] to facilitate cell entry via receptor-mediated endocytic pathways. Once inside endosomes, PEI, with its high amine density, can function as a “proton sponge” to buffer and trap protons [102]. The protons depolarize the endosome membrane and drive entry of chloride ions and osmotically-obliged fluid into the endosomal vesicle, causing membrane lysis and release of the NP-plasmid into the cytosol. While the high density of amine groups in PEI greatly improves the NP's ability to escape from endosomes, they are also known to be cytotoxic to cells. In light of this issue, Peng and colleagues demonstrated that the toxic effects of PEI on dermal stem cells can be mitigated when it is delivered as a conjugate on gold NPs [97]. In another study, the transfection efficiencies achieved using gold-PEI NPs in COS-7 cells (a monkey kidney cell line) have been found to be several fold higher than transfecting using PEI NPs alone [103], although the increase in transfection efficiency was associated with decreased cell viability. More recently, Sharma and colleagues showed that gold-PEI NPs were effective in transfecting human corneal cells *in vitro* and rabbit cornea *in vivo* [104]. Although gold-PEI NPs have not been tested for the retina and RPE, the technology remains a promising non-viral option for retinal gene therapy.

5.2. Lipid-based NPs

5.2.1. Liposomes—Liposomes are among the most popular and widely studied gene transfection system due to their ease of use, commercial availability, low cost, and efficient delivery mechanisms. Most liposome formulations are composed of two basic lipids, a cationic lipid (e.g., DOTAP, MVL5) and a neutral “helper” lipid (e.g., DOPE, cholesterol). The most well-known commercial liposome, lipofectamine, is a 3:1 mixture of a polycationic lipid (with 5 positive charges) and a neutral lipid [105]. When the lipid mixtures are added to negatively charged DNAs, it was found that most lipid-DNA complexes exist as single- or multi-lamellar nanostructures (depending on the formulation), in which DNA is sandwiched within multiple layers of cationic membrane bilayers to form a spherical NP ranging from 100–200 nm in diameter [106]. Generally, liposomes attach to the cell surface by electrostatic interactions and are subsequently endocytosed via CME [107, 108]. In the endosomes, the cationic lipids interact and fuse with the negatively charged endosome membrane to release the DNA cargo into the cytosol. In the hallmark studies by the Safinya group (reviewed in [65]), it was found that the key to a liposome's transfection efficiency is its charge density, a parameter defined by the ratio of cationic to

neutral lipids, and the number of charges present on the cationic lipid headgroup. Liposomes require a high charge density to effectively fuse with endosome membranes for release of DNA into the cytosol (the primary endosome escape mechanism), but having an excessively high charge density will hinder the dissociation of DNA from the liposome complex after endosome escape [109]. By testing different cationic lipids in liposome formulations, it was found that formulations incorporating multivalent cation lipids gave the highest transfection efficiencies [109, 110]. This finding gave rise to the development of highly charged multivalent cationic lipids with dendritic headgroups that carry up to 16 positive charges [111]; liposomes formulated with these dendritic polycation lipids were able to transfect cells that are notorious for being difficult to transfect, including mouse embryonic fibroblasts and many other mouse and human cells in culture [112].

Masuda and colleagues were among the first to compare the efficacy of three cationic liposome formulations (TMAG, DDAB, and DC-cholesterol) for gene transfer to rat ocular tissues via different administration routes: topical application, or injection into anterior chamber, subretinal, and intravitreal injections [113]. The authors show that subretinal and intravitreal injections of all three liposomes resulted in expression of the lacz reporter gene in the ganglion cells and RPE, with TMAG liposomes being the most efficient. However, none were successful in transfecting photoreceptor cells [113]. More recently, Kachi and colleagues performed an extensive study to evaluate the safety and effectiveness of ocular gene transfer using commercial cationic liposome reagents (lipofectamine 2000 from Invitrogen and NeuroPorter from Gene Therapy Systems) [18]. Intravitreal injection of the lipofectamine-packaged plasmid (CMV promoter -driven lacz) induced β -galactosidase expression only in the ganglion cell layer. On the other hand, subretinal injection of the complex induced high levels of gene expression in photoreceptors and RPE cells within 3 days post-injection. However, lipofectamine 2000 was found to be highly toxic to photoreceptor cells (even at low concentrations), as indicated by the thinning of the outer nuclear layer (ONL) and a concomitant decrease in ERG a- and b-wave amplitudes (which reflect photoreceptor and bipolar cell activities, respectively) at 14 days post injection [18]. NeuroPorter on the other hand, was non-toxic to the retina, but it was only able to transfect the RPE. Currently, there is no liposome formulation that has been shown to safely transfect photoreceptor cells. While liposome technology remains widely popular for *in vitro* applications, tissue toxicity and low *in vivo* efficiency remains the major hurdles that prevent its use in human patients.

5.2.2. Solid Lipid NPs—Since the advent of the first generation solid lipid NPs (SLNs) in the 1990s, the technology have been developed as an efficient, non-toxic drug and gene delivery system for use in a wide range of applications including cancer, lung, and neuronal diseases [114–118]. Unlike liposomes, SLNs are highly stable nanostructures that can withstand sterilization by autoclave and maintain their activity for several years when refrigerated. A SLN comprised a solid lipid core that is encapsulated by biocompatible surfactants (e.g., Tween® 80 and Pluronic® F-68) (reviewed in [66, 67]). For gene therapy applications, various cationic lipids such as octadecylamine, precirol ATO 5, DOTAP, DOPE, DC-cholesterol, stearylamine, et al., have been used in combination with neutral lipids to generate a solid lipid core that can effectively bind negatively charged plasmid

DNA and oligonucleotides [115, 119–121]. In some SLN formulations, the plasmid DNA was condensed with protamine for improved packaging into the solid lipid core [120, 122]. This process has been shown to enhance gene transfection for some cell types by promoting DNA translocation to the nucleus [120, 123]. Other biocompatible molecules such as chitosan and dextran (with protamine) have also been shown to improve transfection for some cell types [114, 124–126]. Chloroquine can also be added to the SLN to promote endosome escape and improve transfection efficiency [127]. Finally, the surfactant on the SLN surface can be functionalized with various molecules such as cell penetrating peptides or cell surface receptor ligands (e.g., transferrin, folate, hyaluronic acid) to facilitate cell uptake [128–131]. Despite these advances in SLN technology, few studies have explored the potential of SLNs in retinal gene therapy. Early studies found that SLNs were able to transfect the human RPE cell line, ARPE-19, albeit with low efficiency [132]. More recently, Delgado and colleagues developed a dextran and protamine-based SLN that can successfully induce eGFP expression in the RPE and photoreceptor cells after subretinal injection into the rat eye [114]. While these findings demonstrate that SLN technology can deliver genes to cells in the retina, more studies are needed to compare the *in vivo* transfection efficiency and safety profile of SLN to other lipid- or polymer-based nanoparticles.

5.3. Polymer-based NPs

Polymer-based NPs can be composed of different monomers ranging from proteins (gelatin (reviewed in [70]), albumin (reviewed in [69])), carbohydrates (e.g., dextran and chitosan, reviewed in [74]), or small chemical compounds (poly-lactic acid (PLA) and poly-lactic-co-glycolic acid (PLGA), reviewed in [77, 78]). For gene therapy applications, nucleic acids can either be adsorbed onto the NP surface or encapsulated within the NPs. While encapsulation is a popular option as the plasmid DNA is protected from enzymatic degradation, the encapsulation efficiency and capacity of these NPs for plasmid DNA can be low. All polymer-based NPs for therapeutic applications are designed to be biodegradable and biocompatible, and several (e.g., gelatin, albumin, PLGA) have been FDA approved for drug delivery in humans (reviewed in [133]). Polymer-based NPs can be modified in several ways to obtain the desired characteristics. For example, the rate of plasmid release from the particle can be controlled by modifying the molecular weight (MW) of the polymer (the higher the MW, the slower the release) and crosslinking density (the higher the density, the slower the release) [134, 135]. For co-polymer based NPs such as PLGA, the cargo release rate can also be altered by changing the ratio of lactic acid to glycolic acid monomer (increasing lactic acid content decreases the degradation rate of the NPs) [77]. PLGA NPs can also be modified by incorporating other cationic polymers such as PEI and chitosan to form a hybrid polymer NP with enhanced plasmid DNA encapsulation efficiency and endosome escape. Similarly, cationic molecules such as spermine can be incorporated into anionic dextran polysaccharide NPs to improve DNA binding [136, 137]. Alternatively, excess positive charges (which tend to cause cell toxicity) on highly cationic NPs (e.g., type A gelatin NPs) can be neutralized by incorporating anionic polymers such as chondroitin sulfate and dextran sulfate [138]. For protein-based NPs (albumin and gelatin), the choice of crosslinker can have a dramatic effect on the size and size distribution, and cytotoxicity of the NP [69, 70]. Furthermore, increasing the crosslinker used in the formulation generally

decreases the NP size. The surface of polymer-based NPs can be modified to alter their size and surface charge (e.g., by coating with polyethylene glycol (PEG)), or functionalized with antibodies and ligands for targeting cell-surface receptors [139].

5.3.1. Albumin NPs—Albumin-based NPs are generally thought to be a safe vehicle for ocular gene delivery since albumin makes up a large percentage (60–70%) of all proteins in the vitreous and albumin particles are completely biodegradable (reviewed in [69]). Albumin also contains many charged amino acids (including lysine) that can interact with plasmid DNA for gene therapy. As with other polymer NPs, cationic polymers (e.g., PEI) can be incorporated into albumin NPs for improved DNA capacity and endosome escape [140]. In addition, albumin NPs can be surface functionalized with PEG and receptor ligands such as folate and transferrin for improved NP uptake [141–143]. The first evaluation of albumin-based NPs in ocular gene delivery was performed by Mo and colleagues [68], who encapsulated an SOD1 expression plasmid into spherical NPs (~120 nm diameter; zeta-potential of –44 mV) generated from crosslinked-human serum albumin (HSA) protein. While the authors showed that intravitreal injection of their HSA-NPs could induce protein expression in the mouse retina, it is not known which retinal cell types are actually transfected because whole retinal extracts were used for western blot analysis. In a separate study, it was shown that freshly isolated retinas and photoreceptor cells have the ability to internalize albumin via endocytosis [144], suggesting that photoreceptor cells can take up albumin NPs via the same mechanism. Although Mo and colleagues demonstrated that ARPE-19 cells endocytose HSA-NPs using both CME and CvME pathways [68], it is not known which endocytic pathway (e.g., CME or CvME) is involved in retinal uptake of HSA-NPs. Further studies are needed to determine the mechanisms for cellular uptake and endosome escape utilized by HSA-NPs in the retina. Other hybrid albumin NPs have been prepared by coating the NP surface with positively charged polymers (e.g., PLL [145] and PEI [146]) or by incorporating cationic chemicals (e.g., protamine free base [147, 148]) to enhance nucleotide binding. These hybrid albumin NPs have been shown to be effective carriers for oligonucleotides and small interfering RNA, but plasmid DNA loading has not been tested.

5.3.2. Gelatin NPs—Gelatin NPs (GNPs) can be produced from two major forms of gelatin, type A (acidic) or type B (basic), that are derived from acidic or lime (alkaline) treatment of bovine or porcine skin tissue, respectively. Type A GNPs are enriched in cationic amine groups whereas type B GNPs are enriched in anionic carboxyl groups, and both have been used for gene therapy applications (reviewed in [70]). Kommareddy and Amiji developed a modified version of type B gelatin NPs that could encapsulate plasmid DNA for efficient delivery into the cytosol of mouse fibroblast cells (NIH3T3) [149]. By using thiolated gelatin monomers in the GNP formulation, the authors introduced sulfhydryl groups that form disulfide bonds within the NP, thus stabilizing the polymer structure until after the GNP is released from endosomes in to the cytosol. Once in the cytosol, the disulfide bonds are dissolved by cytosolic glutathione. The GNP surface can also be coated with targeting peptides for cell-type specific delivery [150]. Type B thiolated GNPs have been extensively characterized for use in *in vitro* and *in vivo*, and have been shown to be effective as an anti-cancer gene therapy [151, 152]. Type A GNPs are enriched in positively

charged amine groups that effectively bind and encapsulate plasmid DNA, and confer the proton-sponge effect that enables NPs to destabilize endosomes and escape into the cytosol. However, highly cationic NPs tend to be associated with increased toxicity [153, 154], thus anionic polymers (e.g., chondroitin sulfate and dextran sulfate) can be incorporated into type A GNPs to neutralize the amine groups and improve the toxicity profile of the GNP [138]. This hybrid form of type A GNPs has been successfully used for the delivery of a plasmid DNA expressing MUC5AC (a type of gel-forming mucin secreted by conjunctiva goblet cells) to the cornea and conjunctiva *in vivo* as a treatment for dry-eye disease [155, 156]. However, the potential application of GNPs for retinal gene therapy has not been evaluated.

5.3.3. Polysaccharide NPs—Dextran and chitosan are the two most popular polysaccharides for the formulation of NPs for gene therapy applications. Dextran is a high molecular weight polymer composed of α -1,6- and α -1,3- linked glucopyranose units that are naturally derived from lactic acid bacteria (reviewed in [74]). Both dextran and chitosan (and other polysaccharides) are known to be biodegradable, biocompatible, and non-immunogenic, properties that result in a favorable safety profile for polysaccharide-based NPs for therapeutic applications. Dextran is neutral at physiological pH and therefore does not effectively bind DNA, thus cationic molecules or polymers are commonly incorporated into dextran NPs to improve their effectiveness in gene therapy applications. Dextran NPs incorporating cationic methacrylate monomers and spermine (a tetravalent amine) have been used for the delivery of plasmid DNA *in vitro* (COS7 and HEK293) and *in vivo* (trachea and lung, and colorectal cancer) [157–159]. Other dextran NPs conjugated to cationic polymers such as PEI or poly- β -amino ester also show high binding efficiency to plasmid DNA, as well as the ability to escape endosomes [160, 161]. Although hybrid cationic dextran NPs are effective gene carriers, chitosan NPs are significantly more popular due to their cationic nature. Chitosan is a water-soluble product derived from partial deacetylation (to generate amine groups) of chitin, the main structural component of crustacean exoskeleton and fungi cell wall. It is a linear polymer formed by randomly distributed D-glucosamine and N-acetyl-D-glucosamine monomers. Although chitosan NPs are cationic, they do not exhibit strong endosomal buffering capacity compared to other cationic polymers (e.g., PEI) and therefore do not efficiently escape from endosomes [162, 163]. Introducing amine groups into chitosan by modification of functional groups on glucosamine with imidazole-4-acetic acid (to generate secondary and tertiary amine groups) has been shown to improve plasmid DNA and siRNA delivery to lung and liver tissues, as indicated by increased gene expression or knockdown compared to unmodified chitosan NPs [164, 165].

For retinal gene therapy, Mitra and colleagues have recently evaluated an ethylene-glycol-modified chitosan NP (with enhanced water solubility compared to unmodified chitosan NPs) as a vehicle for gene delivery to the retina [71]. A chicken β -actin-driven GFP reporter plasmid was compacted into glycol-chitosan NPs and subretinally injected into mice. The NPs protected the plasmid DNA from DNase and induced GFP expression specifically in RPE cells at 14 days post-injection without causing any overt toxicity to the retina, as evaluated by biochemical analyses of RPE health, and histological and functional (using ERG) analyses of the retina. In this study, the authors highlighted a major issue with chitosan NPs for retinal gene therapy, in that polymerized chitosan forms a highly viscous

hydrogel that prevents the NPs from achieving a wide distribution across the retinal surface; protein expression was confined to a small area surrounding the injection site. Thus for applications in the retina, hybrid chitosan NPs containing other cationic polymers that disrupt polysaccharide gel formation may improve NP distribution in the retina. In a study by Jin and colleagues, a PLGA-chitosan hybrid NP was formulated for intravitreal delivery of a plasmid expressing an anti-VEGF peptide (plasminogen kringle 5; K5) into rat retina as a treatment strategy for retinal neovascular disease [72]. These NPs induced K5 peptide protein expression in retinal cells at the retinal ganglion cell (RGC) and inner nuclear layers (INL) (representing ganglion, horizontal, amacrine, and bipolar cells), and attenuated key pathological effects (e.g., area of vascularized lesion, VEGF expression, inflammatory cytokine release) caused by laser-induced choroidal neovascularization. The same group has also previously used these PLGA-chitosan NPs carrying a K5 expression plasmid to inhibit neovascularization in a rat model of oxygen-induced angiogenesis at the inner retina, the major cause of retinal damage in diabetic retinopathy [73]. Taken together, these studies demonstrate that chitosan NPs are safe and effective in the retina, and that hybrid chitosan NPs that do not form hydrogels are more suitable for retinal gene therapy.

5.3.4. PLA and PLGA NPs—PLGA is a solid polymer composed of two monomers, lactic acid and glycolic acid, both of which are naturally metabolized in cells. PLGA NPs can be formulated with different lactic acid to glycolic acid ratios to alter the NP degradation rate, with PLA NPs being the slowest to degrade. PLGA/PLA NPs have been extensively characterized in various systems and have exhibited excellent safety profiles in *in vivo* applications [166, 167]. With the approval of PLGA for use in drug delivery systems by the US Food and Drug Administration and the European Medicine Agency, PLGA NPs have become among the most popular NPs for drug delivery. In the PLGA polymer, lactic acid and glycolic acid are linked by ester bonds. Since ester bonds have a very high pKa (~25), PLGA NPs are neutral at physiological pH, but as they are internalized by cells and enter acidic endolysosomes, acid-catalyzed hydrolysis of the esters in the PLGA backbone produces positively-charged intermediates on the NP surface that destabilize the endosomes and allow the NPs to escape [168]. However, since PLGA NPs lack cationic charge, they do not effectively bind and encapsulate DNA. Thus cationic polymers (i.e., PEI or chitosan) are commonly incorporated to improve DNA encapsulation capacity and efficiency [169, 170]. Like other polymer based NPs, PLGA can be surface-modified with PEG for functionalization with protein ligands or antibodies to target cell-specific receptors [139, 171].

Bourges and colleagues were the first to evaluate the potential of PLA NPs for drug delivery to the retina [172]. They showed that PLA NPs (with two discrete sizes: 150 and 300 nm diameter), when injected intravitreally, were able to deliver their cargo (fluorescence molecules or a membrane dye) to the retina and RPE within 24 hours. This finding was unexpected because the outer limiting membrane (OLM) of the retina, consisting of adherens junction and desmosomes between Müller cells and photoreceptors, forms a continuous semi-permeable barrier that spans across the entire retina, effectively blocking movement of particles larger than 10 nm between the inner retina and outer retina [173]. Evaluation of injected retinas revealed that intravitreal injection of saline or PLGA NPs both

induced Müller cell activation, as indicated by the upregulation of glial fibrillary acidic protein (GFAP; a marker of retinal stress) in the Müller cells within one hour post-injection [172]. Furthermore, infiltrating macrophages were found in the vitreous and retina, indicating that the inflammatory response originated from the injection process, rather than the NPs. Nevertheless, this study demonstrates that disruption of OLM permeability, such as in disease conditions, could provide intravitreally-injected NPs access to photoreceptor cells and the RPE. Support for this idea comes from the previously discussed study by Jin and colleagues in which the laser-induced damage to the retina and RPE provided an access-point for trans-retinal diffusion of PLGA-chitosan NPs (carrying a plasmid that expresses the anti-VEGF K5 peptide) from the vitreous to the RPE [72]. In a similar study, Zhang and colleagues used PLGA polymer to encapsulate two plasmids, one that expressed an shRNA against hypoxia-inducible factor 1- α (HIF-1 α ; a major component of the angiogenesis pathway upstream of VEGF) and the other to express a GFP reporter [75]. When the NPs were injected intravitreally into rats with laser-induced choroidal neovascularization (CNV), GFP was detected in both photoreceptors and RPE at three days post-injection. At 14 days after laser-damage, the NP-treated retinas exhibited significantly reduced CNV lesion sizes compared to control. While the intravitreal route is commonly used for delivery of NPs to the retina, Singh and colleagues explored whether PLGA NPs (containing a plasmid that expresses an anti-VEGF peptide) could be specifically delivered to the laser-treated retinas when administered via the intravenous route [76]. The authors made two key observations regarding intravenous NP delivery, the first being that NP uptake into the RPE only occurred in laser-treated eyes with leaky blood vessels near the site of the lesion, and secondly, surface functionalization of the PLGA NP with an RGD peptide or transferrin significantly enhanced NP uptake by the RPE cells at the CNV site. As with any NPs that are administered intravenously, PLGA NPs were detected in the liver and kidney. This is not normally an issue however, since PLGA is known to have very low toxicity in all organ tissues tested, including liver and kidney [167]. Another concern is ectopic expression of the transgene in other organs, but this potential issue can be avoided by using a retina-specific promoter (as discussed in section 6.2).

5.3.5. Dendrimer NPs—Dendrimers are a unique class of NPs that are synthesized by sequential polymerization of monomers to a core (initiator) molecule (reviewed in [81, 82]). Monomers are first covalently attached to the surface layer, and with each layer/generation, addition of monomers creates branched polymers that expand in the outward direction, resulting in a highly branched spherical structure with internal cavities that can hold a variety of therapeutic agents, including small molecules, proteins, and plasmid DNA. Dendrimers that have a high number of primary amine surface groups can form electrostatic interactions with negatively charged plasmid DNA. Polyamidoamine (PAMAM) dendrimers, consisting of alkyl-diamine core and tertiary amine branches, are among the most popular dendrimers for gene delivery *in vitro* [174]. However other dendrimers composed of different structural units (e.g., saccharides [175], PLL [176], PEI [177], and polypropylimine (PPI) dendrimers [178]) have also been used for gene therapy applications. Dendrimers generally have sizes ranging from 1–100 nm (depending on the number of layers called generations), and have a positive surface charge that interacts with the negatively charged cell membrane to initiate uptake via CME. In addition, dendrimers can

also be surface-functionalized with peptide ligands to take advantage of receptor-mediated endocytic pathway for cell-specific NP delivery [175, 179]. Once inside endosomes, PAMAM and PEI dendrimers can escape from endosomes using the “proton sponge” effect [102, 180]. Incorporating chloroquine (an inhibitor of endosomal acidification) or fusogenic peptides into the dendrimer have been shown to facilitate endosome escape and increase transgene expression for some cell lines [181, 182].

In vitro studies show that PAMAM dendrimers exhibit high transfection efficiency for plasmid delivery into a variety of cell lines [183, 184] and even stem cells [185]. PAMAM dendrimers were also effective *in vivo* [183, 186], although higher generation dendrimers were found to be cytotoxic due to their highly cationic structure [187]. PEI dendrimers on the other hand have been successfully used *in vivo* to deliver nucleic acids (plasmid DNA, siRNA, and long dsRNA) into various mouse tissues (e.g., lung, brain, liver, kidney, heart) [188–190]. PEI dendrimers have also been tested as a vehicle for delivery of an shRNA expression plasmid to retinal ganglion cells via intravitreal injection [80]. The knockdown effect was observed in as early as 16 hours post-injection and was sustained through 2 months. Another dendrimer that was successfully used in *in vivo* for ocular gene therapy was a modified PLL dendrimer that incorporates lipoamino acid (LAA) in its structure [79]. In this study, Marano and colleagues showed that PLL-LAA dendrimers carrying an anti-VEGF oligonucleotide were able to reach the RPE at laser-damaged sites after intravitreal delivery and suppress CNV.

5.3.6. CK30-PEG compacted-DNA NPs—While many NP technologies are designed to encapsulate DNA for gene delivery, CK30-PEG (a 30-mer cationic poly-lysine conjugated with 10kDa PEG (CK30-PEG)) produces compacted-DNA NPs by neutralizing most (>90%) of the DNA’s negative charges (reviewed in [33]). Without the repulsive negative charges, the charge-neutral DNA folds upon itself and condenses into a compact nanostructure that is DNase-resistant and easily endocytosed by cells. CK30-PEG NPs can be formulated as ellipsoidal or rod-like NPs, by using trifluoroacetate or acetate, respectively, as the lysine counterions [29]. Rod-shaped CK30-PEG NPs are preferred for retinal gene therapy because they can effectively transfect both the RPE and photoreceptor cells, while ellipsoidal NPs were found to transfect only the RPE [19]. When different size plasmids are compacted with CK30-PEG, it was found that the length of the NP product increases with the plasmid size, whereas the width of the NP remains relatively constant (5.3 kb plasmid yields 8.4×184 nm rods; 20.2 kb plasmid yields 11.3×537 nm rods) [29]. While the size, charge, and shape of NPs are important determinants of their cell-entry pathway and their ability to escape endosomes, the effectiveness of CK30-PEG NPs are less dependent on these parameters than other nanoparticles are as the CK30-PEG NPs rely on a unique nucleolin-dependent endocytic process in which the NPs are directly transported into the nucleus [191]. In airway epithelial cells, CK30-PEG DNA NPs have been shown to directly bind nucleolin, a surface protein enriched in lipid rafts, and the NP-nucleolin complex is endocytosed and subsequently trafficked along microtubules directly into the nucleolus, where the plasmid DNA can be expressed [192]. There are two advantage of this mechanism over other uptake pathways: (1) it bypasses the conventional endosome pathway, thus avoiding damage to the plasmid DNA cargo in the cytosol and acidic

endosome environment, and (2) CK30-PEG DNA NPs are directly transported to the nucleus, where gene expression occurs. *In vitro* studies of CK30-PEG NP uptake demonstrated the high efficiency of the nucleolin pathway: most NPs were delivered to the nucleus by 1 hour post-administration [191]. This same NP formulation has also been shown to effectively deliver a luciferase- or lacZ- reporter plasmid to mouse alveolar and airway epithelial cells *in vivo* [193].

The uptake of CK30-PEG NPs by retinal cells and the underlying mechanisms have also been extensively studied. In *Rds*^{+/-} mice, an animal model of retinitis pigmentosa caused by haploinsufficiency in the retinal degeneration slow (*Rds*) gene, subretinal injection of CK30-PEG NPs carrying an *Rds* expression plasmid was able to induce *Rds* gene expression in photoreceptor cells in as early as 8 hour post-injection (Naash lab unpublished data and [33]). Robust protein expression can be detected in two days post-transfection. In the mouse retina, nucleolin is expressed in all cell layers suggesting that this uptake pathway could be used to mediate CK30-PEG DNA NP uptake in virtually all cell types of the retina [33]. Indeed, an initial study showed that subretinal injection of CK30-PEG NPs containing CMV-driven GFP reporter plasmid induced robust GFP expression in photoreceptor and RPE cells two days post-injection [19]. Intravitreal injection of the same NPs induced GFP expression primarily in ganglion cells of the retina (and other ocular tissues including cornea, trabecular meshwork, and lens). Studies are ongoing to determine whether this retinal uptake is mediated by the nucleolin pathway.

CK30-PEG compacted DNA NPs have also been extensively tested as a gene therapy for retinal degenerative diseases. We have successfully used CK30-PEG NPs to achieve persistent (tested up to 2 years) high level expression of the *RPE65* gene in the RPE (using the RPE-specific VMD2 promoter) and promote phenotypic rescue in the *rpe65*^{-/-} mouse model of LCA [46, 83]. This same NP technology was also used for the delivery of a gene-therapy plasmid carrying the human *ABCA4* gene (driven by the photoreceptor-specific IRBP promoter) into the retina of *abca4*^{-/-} mice [84]. In NP-treated mice, *ABCA4* was stably expressed (at both the mRNA and protein level) in the retina up to 8 months post injection. Importantly, the NPs rescued many features of the Stargardt-associated retinal degeneration in these mice [84]. Using the same strategy, subretinal injection of CK30-PEG NPs carrying the *RDS* gene induced sustained gene expression and partial rescue of photoreceptor degeneration in *Rds*^{+/-} mice, as indicated by elevated ERG a- and b-wave amplitudes and improved retinal morphology compared to control *Rds*^{+/-} retinas (injected with naked DNA) [85, 86]. Importantly, CK30-PEG NPs were found to be non-toxic to the retina [194], even when a second subretinal injection of the NPs was delivered 30 days after the first injection [195]. Considering the effectiveness of nucleolin-dependent endocytosis in gene transfer to photoreceptor cells, other forms of DNA-NPs can be formulated to potentially exploit this mechanism for cell internalization. This could be achieved by conjugating the NPs with an anti-nucleolin antibody, nucleolin-binding DNA aptamer (AS1411) [196], or a peptide ("tumor-homing" F3) that binds cell surface nucleolin and induces trafficking into the nucleus [197].

6. Plasmid design for non-viral gene therapy

6.1. S/MAR sequence for long term transgene expression

Although there are many different ways to virally and non-virally compact nucleotides for delivery to the eye, the content of the DNA is also critical and has a significant effect on the efficacy of the treatment. The ideal theoretical plasmid for non-viral ocular gene therapy has several characteristics: (1) it is maintained as an episome (without genomic integration), (2) it promotes cell-specific expression, (3) it induces high levels of protein expression, and (4) it establishes stable long term protein expression without gene silencing. Maintenance of plasmid DNA as episomes provides several advantages over genomic integration, including averting potential safety issues associated with insertional mutagenesis. In addition, multiple copies of episomal vectors can be maintained in the nucleus thereby allowing high transgene expression (reviewed in [198]). Finally, they are also less susceptible to epigenetic silencing by genomic DNA elements. Studies show that a plasmid DNA can be maintained as an episome in the nucleus of cells *in vitro* and *in vivo* when it contains a scaffold/matrix attachment region (S/MAR) sequence [199–201]. S/MAR sequences are found throughout the genome, where they serve as attachment points for nuclear scaffold proteins (scaffold attachment factors A and B (SAF-A and B)) on the nuclear matrix [202, 203]. When incorporated into an expression vector, S/MARs can enhance gene expression in several ways: (1) vectors with S/MAR sequence attaches to the nuclear matrix, where they can replicate episomally (depending on cell type) and do not integrate into the genome, (2) S/MAR sequences contain AT-rich regions that relieve the superhelical strain of the plasmid, allowing the plasmid DNA to unwind easily for gene transcription [204], (3) plasmids containing S/MARs are more likely to associate with a subset of modified histones (e.g., H3K4m1, H3K4m3, H3K36m3) that are found in transcriptionally-active sites of the chromosome (euchromatin), thus the plasmid is less susceptible to epigenetic silencing [205]. For applications in ocular gene therapy, it has been shown that plasmid DNA containing an S/MAR sequence was retained episomally in RPE cells for up to a year after transfection [206]. Further, the authors demonstrate that incorporating the S/MAR sequence into the plasmid DNA was able to significantly improve the longevity of the transgene expression in the RPE.

One of the most popular episomal plasmid vector for gene therapy is the pEPI vector [203], which was generated by replacing the SV40 enhancer in the commercially available CMV-GFP expression vector with a 2 kbp long S/MAR sequence derived from the human β -interferon gene. Variations of the pEPI vector have found success in *in vitro* and *in vivo* applications. It has been demonstrated that the pEPI vector can achieve long term transgene expression in a wide variety of cell lines including Chinese hamster ovary (CHO-K1) cells [200], HeLa cells [207], mouse fibroblasts (iMEF), and even in human primary fibroblast cells. In most cells tested, the pEPI vector was episomally maintained at 5–10 copies per cell and is mitotically stable over hundreds of generations. Long term gene knockdown can also be achieved by modifying the pEPI vector to express an shRNA hairpin [208]. Successful *in vivo* expression of luciferase gene using pEPI vector was achieved in adult mouse liver after delivery of the naked pEPI plasmid via hydrodynamic tail vein injection [201]. For applications in ocular gene therapy, the pEPI vector has demonstrated high effectiveness in

establishing long term gene expression in photoreceptor and RPE cells as a potential treatment strategy for various retinal degenerative diseases, including LCA [46], Stargardt's disease [84], and retinitis pigmentosa [86].

6.2. Cell type-specific vs. ubiquitous promoter

Based on results from various studies on retinal gene delivery, it was found that subretinally injected NPs, small or large, ellipsoid or rod shaped, are easily taken up and expressed in the RPE, likely due to the phagocytic activity of the RPE. Even uncompact plasmids (when injected subretinally) can induce GFP protein expression in the RPE within two days after injection [19, 46]. This becomes an issue if gene delivery to photoreceptor cells is desired, since ectopic expression of a retinal gene in the RPE, depending on the gene's function, could cause undesirable side effects. To avoid potential problems, one can induce photoreceptor-specific expression by incorporating photoreceptor specific promoters into the expression plasmid. Commonly used promoters for this purpose include rhodopsin (RHO) [209], rhodopsin kinase (GRK1) [210], CRX [211], and the human interphotoreceptor retinoid-binding protein (IRBP) [212]. For NPs (such as PLGA and liposomes) that deliver the plasmid via endocytosis pathways to the cytoplasm (rather than directly into the nucleus), photoreceptor-specific transfection can also be achieved by incorporating the nuclear localization signal for a photoreceptor-specific transcription factor, such as CRX, into the plasmid DNA [213, 214]. It is important to note that while these cell-specific promoters and transcription factors are highly active in healthy photoreceptor cells, their activities can be significantly diminished in diseased conditions, during which cells are programmed to shut down specialized functions (and downregulate cell-specific genes) in favor of survival genes [215, 216]. In this regard, constitutive promoters (such as CMV and the human elongation factor 1 alpha (EF1A) promoter) have an advantage in that their ability to drive gene expression is less affected by the condition of the cells [217–220], but at the expense of non-specific expression in the RPE and potentially other retinal cells. The CMV promoter is widely used due to its ability to induce robust protein expression in a wide variety of cell types. However CMV is rapidly inactivated by CpG methylation *in vivo*, and protein expression is dramatically reduced within the first week of transfection [221]. This problem with *in vivo* silencing can be partially circumvented by removal of CpG motifs in the CMV sequence or by using other constitutive promoters that are less susceptible to methylation, such as the murine phosphoglycerate kinase-1, human EF1A, human ubiquitin C, and chicken β -actin promoters [217].

6.3. Avoiding epigenetic silencing of the gene expression vector

While methylation of CpG motifs within the promoter and the gene coding regions of the plasmid contribute to *in vivo* silencing, studies by the Kay lab demonstrated that the bacterial sequences in the plasmid DNA backbone exert an inhibitory effect on the transcription of the mammalian transgene [222]. They show that the bacterial portion of the plasmid forms a “heterochromatin-like” DNA structure that interacts with modified-histones and proteins commonly found in heterochromatin (inactive) domains of the genomic DNA, resulting in condensation and epigenetic modification of the plasmid DNA, ultimately resulting in transcriptional repression of the mammalian transgene. To circumvent this issue, DNA elements called insulators have been incorporated into plasmid DNA [222, 223].

Insulators are non-unique DNA sequences found throughout the genome that function as a barrier that prevents the promoter from being influenced by the regulatory elements of adjacent genes (reviewed in [224]). Some insulators also have the ability to block advancement of nearby condensed heterochromatin, thus protecting the gene from epigenetic silencing. Chen and colleagues showed that transgene silencing by the plasmid bacterial backbone was partially attenuated when the gene expression cassette (promoter/enhancer and transgene) was flanked by two of the same 1.2 kbp insulator elements derived from the chicken β -globin locus control region (cHS4) [222]. Alternatively, one can avoid the issue with epigenetic silencing by eliminating the bacterial backbone altogether by using minicircle plasmids [225, 226], which is a class of episomal DNA vectors that contains only the mammalian gene expression cassette. Minicircles are also inherently more efficient in inducing gene expression due to their smaller size, and have been shown to drive high level *in vivo* gene expression in skin, muscle, heart, and liver [225, 227, 228]. Minicircles with the S/MAR sequence have also been shown to promote significantly better transgene expression compared to minicircles without S/MAR in mouse liver [229].

7. Summary and Conclusion

Over the past several decades, a myriad of DNA nanocarriers have been developed as additional methods for use alongside viral-based gene transfer technologies. However, few have been systematically characterized and optimized for *in vivo* transfection efficiency and cytotoxicity, and none have so far been able to match the level of transfection achieved with viral systems. This issue is partly attributed to the extensive number of parameters (e.g., crosslinker, monomer ratio, NP:DNA ratio, cationic molecule additives) that affects the biophysical properties of the final NP product – size, shape, and charge – all of which ultimately define the transfection efficiency and toxicity profile of the NP. Even changes to the NP formulation process, such as inclusion of an additional water/oil emulsion step in PLGA NP preparation, can dramatically change the NP properties. On the other hand, the high level of customization in the NP formulation can also be advantageous as it allows for optimization and improvements to the technology. While this can be achieved by high-throughput *in vitro* screening methods, it is well known that the ability of NPs to safely transfect cells *in vitro* often do not correlate with their performance in *in vivo* applications, and vice versa. Thus the only viable strategy is to first test the plasmids for transgene expression *in vitro* followed by systematic *in vivo* evaluation of the NPs in the tissue of interest for three key parameters: (1) expression level; (2) long-term expression; and (3) cytotoxicity.

In the context of retinal gene therapy, various NPs (from PLGA to liposomes) have been tested for gene delivery to the retina and the RPE via intravitreal or subretinal injections. In most cases, efficient transfection of cells in the GCL can be achieved by intravitreal injections, whereas transfecting cells in the outer retina (photoreceptor and the RPE) require the more invasive subretinal injection. The exception to this generalization is when the OLM of the retina is breached, such as in laser-induced CNV models, thereby granting intravitreally-injected NPs access to photoreceptor and RPE cells. However, despite significant advances in NP technologies, PLGA and CK30-PEG compacted DNA NPs remain the only NP technologies that can safely deliver genes to both photoreceptor cells

and the RPE; others were only successful in transfecting the RPE. A major reason is that RPE cells, with their high phagocytic activity, likely rapidly internalize a large fraction of injected NPs. This issue can be ameliorated by using a higher NP concentration, at the expense of increased cytotoxicity. The toxicity is primarily associated with the density of cationic charge that destabilizes the plasma membrane and causes cell damage, but a high cationic density is required for DNA-loading, NP uptake into cells, and endosome escape. Thus, NP efficiency is often sacrificed to reduce toxicity. An alternative strategy is to increase the rate of NP uptake by photoreceptor cells, which can be achieved by functionalizing the NPs with an antibody or a peptide ligand (e.g., transferrin) for a photoreceptor-specific receptor to trigger rapid receptor-mediated CME and subsequent NP-internalization. Even with these strategies, NP uptake by RPE cells cannot be avoided, thus using a photoreceptor-specific promoter in the expression plasmid can prevent unwanted transgene expression in the RPE.

While the NP formulation defines the efficiency of DNA transfer into cells, it is often the design of the plasmid DNA that determines the level and persistence of transgene expression. Plasmid vectors that contain an S/MAR sequence such as pEPI have been shown to be highly effective in inducing long term transgene expression in the retina. However, even with this vector, gene and protein expression progressively decline over time, likely due to epigenetic silencing (e.g., methylation or by association with inactive histones). More recently, additional DNA elements (e.g., insulator, chromatin-opening element (UCOE)) have been tested in the pEPI vector [230]. In addition, an improved version of pEPI with 60% less CpG motifs has also been developed for a more persistent transgene expression *in vitro* and *in vivo* [231]. However, there are several other DNA elements, such as the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) [232–234], intronic enhancers (e.g., intron of GDEP gene) [235], and curved sequences [236, 237], that have yet to be extensively evaluated in the retina. Future work will involve systematic evaluation of the modified pEPI vectors compacted in CK30-PEG NPs for retinal gene expression. Despite recent improvements in NP technology and plasmid design, AAV-based gene delivery is currently more effective than non-viral systems. Optimization of NP formulations for safety and efficiency, and improvements in the gene expression plasmid are the key aspects in the development of NP technology for retinal gene therapy.

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List of Abbreviations

AAV	Adeno-Associated Virus
NP	Nanoparticles
LCA	Leber's Congenital Amaurosis
RPE	Retinal Pigment Epithelium

OS	Outer Segment
SNP	Single Nucleotide Polymorphism
ERG	Electroretinogram
CME	Clathrin Mediated Endocytosis
CvME	Caveolae Mediated Endocytosis
ONL	Outer Nuclear Layer
SLN	Solid Lipid Nanoparticle
PLA	Poly-lactic Acid
PLGA	Poly-lactic-co-glycolic Acid
PEG	Polyethylene Glycol
HSA	Human Serum Albumin
GNP	Gelatin Nanoparticle
K5	Plasminogen Kringle 5
RGC	Retinal Ganglion Cell
INL	Inner Nuclear Layer
OLM	Outer Limiting Membrane
GFAP	Glial Fibrillary Acidic Protein
CNV	Choroidal Neovascularization
PAMAM	Polyamidoamine
PPI	Polypropylimine
LAA	Lipoamino acid
RDS	Retinal Degeneration Slow
S/MAR	Scaffold/Matrix Attachment Region
SAF	Scaffold Attachment Factor

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Highlights

- Monogenic retinal diseases are great targets for genetic therapies.
- Viral and non-viral (nanoparticle) approaches have been tested.
- Various types of nanoparticles have been tested successfully in the eye.
- The retinal pigment epithelium is the easiest retinal cell type to transfect.
- Some nanoparticles such as CK30-PEG also transfect photoreceptors.

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Figure 1. Diagram depicting cross-section of the retina with layers and cell types labeled. Listed NPs are those that have been shown to transfect the layer indicated.

Table 1

Summary of NPs for Gene Therapy and their Characteristics

Nanoparticles	Characteristics	Successfully Transfected Retinal Cells	Review References
Gold-PEI	<ul style="list-style-type: none"> • Easy synthesis • Optical properties for imaging • Low cytotoxicity 	<ul style="list-style-type: none"> • N/A 	Reviewed in [63, 64]
Liposome	<ul style="list-style-type: none"> • Easy preparation • Commercially available • high cytotoxicity 	<ul style="list-style-type: none"> • RPE (Neuroporter®) [18] 	Reviewed in [65]
Solid Lipid	<ul style="list-style-type: none"> • Stable under heat sterilization • Many options for customization • Simultaneously deliver drugs and plasmid DNA 	<ul style="list-style-type: none"> • N/A 	Reviewed in [66, 67]
Albumin	<ul style="list-style-type: none"> • High biocompatibility • FDA-approved • Easy to chemically modify • Options for hybrid nanoparticles 	<ul style="list-style-type: none"> • Retina (cell type unknown) [68] 	Reviewed in [69]
Gelatin	<ul style="list-style-type: none"> • High biocompatibility • FDA-approved • Low material cost • Easy to chemically modify • Options for hybrid nanoparticles 	<ul style="list-style-type: none"> • N/A 	Reviewed in [70]
Polysaccharide	<ul style="list-style-type: none"> • High biocompatibility • Tendency to form viscous hydrogels • Easy to chemically modify • Options for hybrid nanoparticles 	<ul style="list-style-type: none"> • RPE (glycol-chitosan NP) [71] • Cells in inner retina (PLGA-chitosan NP) [72, 73] 	Reviewed in [74]
PLA/PLGA	<ul style="list-style-type: none"> • High biocompatibility • Well-described formulations • FDA- and EMA-approved • tLow cytotoxicity • Options for hybrid nanoparticles • Simultaneously deliver drugs and plasmid DNA 	<ul style="list-style-type: none"> • RPE (PLGA NP) [72, 75] [76] • Photoreceptor (PLGA NP) [75]Soli 	Reviewed in [77, 78]
Dendrimer	<ul style="list-style-type: none"> • Difficult to synthesize • Commercially available (expensive) • High cytotoxicity 	<ul style="list-style-type: none"> • RPE (PLL-LAA dendrimers) [79] • Retinal ganglion cells (PEI dendrimers) [80] 	Reviewed in [81, 82]
CK30-PEG	<ul style="list-style-type: none"> • Easy synthesis 	<ul style="list-style-type: none"> • RPE (CK30-PEG) [19, 46, 83] 	Reviewed in [33]

Nanoparticles	Characteristics	Successfully Transfected Retinal Cells	Review References
	<ul style="list-style-type: none">• Low cytotoxicity• Directly trafficked to the nucleus	<ul style="list-style-type: none">• Photoreceptor (CK30-PEG) [19, 84–86]• Retinal ganglion cells (CK30-PEG) [19]	

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