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Non-viral therapeutic approaches to ocular diseases: an overview and future directions

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Abstract

Currently there are no viable treatment options for patients with debilitating inherited retinal degeneration. The vast variability in disease-inducing mutations and resulting phenotypes has hampered the development of therapeutic interventions. Gene therapy is a logical approach, and recent work has focused on ways to optimize vector design and packaging to promote optimized expression and phenotypic rescue after intraocular delivery. In this review, we discuss ongoing ocular clinical trials, which currently use viral gene delivery, but focus primarily on new advancements in optimizing the efficacy of non-viral gene delivery for ocular diseases. Non-viral delivery systems are highly customizable, allowing functionalization to improve cellular and nuclear uptake, bypassing cellular degradative machinery, and improving gene expression in the nucleus. Non-viral vectors often yield transgene expression levels lower than viral counterparts, however their favorable safety/immune profiles and large DNA capacity (critical for the delivery of large ocular disease genes) make their further development a research priority. Recent work on particle coating and vector engineering present exciting ways to overcome limitations of transient/low gene expression levels, but also highlight the fact that further refinements are needed before use in the clinic.

Graphical Abstract

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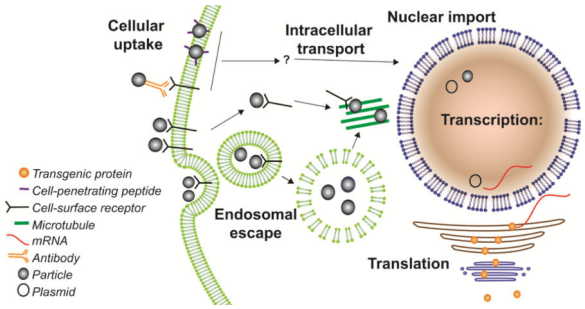

Keywords

non-viral gene therapy; eye; retina; subretinal delivery; nanoparticles

Introduction

Inherited retinal degeneration is a primary cause for debilitating impairments in vision among working age people in the developed world(1), and development of effective therapeutics for inherited retinal disease is a primary research goal. Retinal dystrophies are categorized roughly into two categories: 1) retinitis pigmentosa (RP), a broad term encompassing phenotypes that initially affect rod photoreceptors and peripheral vision, and may later be associated with cone cell death, and 2) macular dystrophy (MD), a term encompassing widely varying phenotypes in which macular or central vision is initially affected(2, 3). Though MD can be caused by defects in cone photoreceptors, it can also be caused by defects in the retinal pigment epithelium (RPE). For example, Stargardt's MD is associated with retinal pigment epithelium (RPE) atrophy (4) and subsequent degeneration of the cones in the macular region of the retina and leads to central vision loss. There are also various syndromic forms of inherited retinal diseases (particularly leading to RP) which exhibit degenerative processes not only in the eye but also in other organs (5). The most common form of syndromic RP is Usher syndrome which comprises hearing loss in addition to visual impairment (for review see(6)).

Inherited retinal diseases are complex. Causal mutations in more than a hundred genes have been identified for inherited retinal degeneration and there are still many unidentified (7). In addition, most disease genes contain multiple pathogenic mutations leading to a wide variety in the severity and age of onset of the disease (8, 9). Mutations in certain genes like the structural protein peripherin-2 can induce RP as well as MD, depending on the site of mutation involved (10, 11) and RP can be inherited in a dominant or recessive manner (5). There is also a large degree of intra- and inter-familial phenotypic heterogeneity and often incomplete penetrance even within groups of patients carrying the same mutation. This wide variability in phenotypes has led geneticists to look for modifiers or digenic/polygenic disease, topics that are still being explored (12-14). Many patients with RP develop symptoms in the third or fourth decade of life, however, there are more severe forms of retinal degeneration, such as Leber's congenital amaurosis (LCA), which can lead to complete loss of vision before patients reach puberty (for review see (15)). Gene therapy is a desirable treatment method, but the genetic and phenotypic heterogeneity in inherited retinal

degeneration makes the development of a single therapeutic approach a very challenging process.

Gene augmentation therapy is a theoretically straightforward option for the treatment of recessive disease (usually caused by loss-of-function mutations)or a dominant disease associated with haploinsufficiency phenotype (16, 17). In these cases, supplementation with a healthy copy of the gene should be effective, provided that the degeneration is not yet severe and that a sufficient amount of gene expression can be obtained from the therapeutic vector. More challenging is designing a treatment for dominantly inherited degenerations caused by gain-of-function mutations. Patients have one healthy and one mutated allele, and the mutant protein interrupts normal function leading to retinal degeneration. Multiple mechanisms (many as yet poorly understood) result in this degeneration, so many different treatment approaches must be considered. A common gain-of-function disease mechanism occurs when mutant proteins are misfolded/aggregated (18, 19)or are mislocalized (20) leading to endoplasmic reticulum (ER) stress, and ultimately cell death (21). Different pharmaceuticals can be used to alleviate ER stress and prolong the life of photoreceptors. Proof-of-principle for this therapeutic approach has been shown in multiple cases, for example, increasing the cellular unfolded protein response via pharmacological induction of the heat-shock protein rescued retinal function and morphology in a P23H rat model of rhodopsin-associated RP (22). Other dominant mutations cause cell death by other mechanisms, for example some mutations in guanylate cyclase lead to constitutive activity and overproduction of cGMP, causing LCA(23, 24). A key defining feature of these dominant diseases is that reintroduction of the WT allele is not sufficient to completely prevent retinal degeneration.

However, several gene therapy approaches have been tested for dominant diseases. For example, the administration of neurotrophic factors (either as purified proteins or as gene therapy vectors) has been beneficial in some cases (25), however, without the correction of the underlying disease mutations, these treatments merely delay the degenerative process. Thus other research has focused on ways to eliminate the undesirable transcript or mutation. Silencing RNA (siRNA) which is complementary to unwanted transcripts prevents their efficient translation,and knockdown approaches have been tested for dominant phenotypes (26). Geneknockdown in the retina has been tested using multiple modalities and multiple disease genes, including siRNA (rhodopsin, peripherin-2 and GCAP1) (27-30), and zinc-finger nucleases (for rhodopsin and USH1C)(31, 32). The recent development of additional genome editing systems such as CRISPR/Cas9 that can be used to correct the disease-causing mutation in the native allele also provide great hope for the development of effective treatments for dominant diseases (33). Preliminary results from the Bakondi group utilizing CRISPR/Cas9 genome editing to correct the RP phenotype in a rhodopsin mutant rat model (S334ter-3) are encouraging (Bakondi B, et al., Gene Editing Corrects The Retinal Dystrophy Phenotype in S334ter-3 Rats, ARVO2015, Abstract number 3183), and results from a complete study are highly anticipated. However, there are several limitations to knockdown technologies that remain to be overcome including the difficulty in designing a knockdown construct which can bind the mutated version but not the healthy sister allele, potential off-target effects, and concerns about delivery and expression levels which affect

all types of gene delivery strategies. The new technology is intensely studied right now and will hopefully lead to a major breakthrough in future therapy of retinal degeneration.

However, even with the new treatment possibilities, there are still obstacles for any type of gene therapy method including, 1) successful delivery of the therapeutic DNA to the affected tissue, 2) its uptake by the target cells and 3) efficient expression of the transfected vector. All three parts will be discussed in this review for non-viral vectors with a special emphasis on their applications in the eye.

1. Therapeutic approaches for ocular diseases with gene therapy: pre-clinical testing and clinical trials

The retina is an excellent system to test newly emerging gene therapy vectors due to its relative immune-privilege, the plethora of non-invasive procedures available to assess functional and structural rescue, and the ability to deliver therapeutic agents directly to specific tissues of the eye. Thus gene supplementation therapy in the eye is a mature field and several clinical trials are either completed or underway. Thus far, the ocular gene therapy trials have used viral delivery systems, either adeno-associated virus (AAV) or lentivirus. However, concerns regarding the immune response to viral vectors, limitations in payload capacity, and production costs have spurred investigation of non-viral alternatives. Though these non-viral approaches have not yet been tested in ocular clinical trials,they have been under investigation in animal models for several years and may be poised to enter the clinical trial phase in the near future.

1.1. Current gene therapy clinical trials for retinal degeneration

So far, most clinical trials for gene therapy in inherited retinal degeneration are in phase I or II to test possible toxicity and adverse effects of the applied vectors and gather preliminary data on the efficacy(34). However, one of the earliest successful therapies which uses AAV to target LCAhas successfully completed phase I and II in several different clinical trials (NCT00481546/NCT01496040/NCT00749957/NCT01208389/NCT00643747/ NCT00516477) ((see(35, 36) for the most recent information) and is now in phase III (NCT00999609). In 1997, a mutation in the RPE-specific gene RPE65 was identified as causative agent for LCA in several patients with a very early onset vision loss (37). The identification of similar phenotypes in Swedish briard dogs (38), a naturally occurring mouse model of LCA (39) with RPE65 mutations, and an RPE65 knockout mouse line (40)have facilitated the development of a feasible gene therapy vector for RPE65-associated LCA by providing the means to test it. Delivery of the RPE65 gene to RPE cells in the retina of dogs (41, 42) and mice (43, 44) showed long-term improvement of retinal function and few adverse effects leading to the initiation ofthe different clinical trials. Patients were treated with AAV containing the RPE65 cDNA and either a ubiquitously expressed promoter or the native RPE65 promoter(45-48). None of the trials revealed any severe adverse effects, and reported moderate improvement of visual function in some of the patients. Long-term follow-up studies showed that the increase in visual acuity is stable for up to 3 years after treatment(49) despite a progressive retinal degeneration (36). However, recently released follow-up for up to six years has showed continued diminishment in the

areas of improvement (50, 51). Given the already modest nature of the improvement, these results highlight the need for further understanding of the mechanism underlying the disease defect in order to improve on the design of therapy.

Three other retinal disease genes are currently being delivered by AAV in clinical trials. Choroideremia is an X-linked retinal degenerative disease with a prevalence of 1 in 50,000 and is associated with early onset photoreceptor and RPE degeneration and a marked choroidal atrophy (for review see (52)). Mutations in the Rab escort protein-1 (REP-1) are responsible for this disease (53) and cause a defect in membrane association of Rab GTPases which are crucial for proper protein trafficking inside the cell (54). Two different approaches were tested in a mouse model for choroideremia: a lentiviral vector carrying the elongation factor 1 promoter known to express in ocular cells and the REP-1 cDNA(55) and an AAV2 vector with a ubiquitously expressed promoter and the REP-1 cDNA(56). Both vectors showed successful transfection of RPE cells in mouse models, but AAV2 was chosen for the phase I/II clinical trial (NCT01461213). Though the trial is ongoing, the 6 patients with advanced retinal degeneration who were initially enrolled showed increased visual acuity after treatment, and the subretinal injection did not cause any detectable damage at 6 months post-treatment, the latest time point assessed so far (57). Clinical trials for Leber hereditary optic atrophy (LHOA) caused by mutations in the mitochondrial ND4 gene (NCT02161380) (58) and for autosomal-recessive RP caused by mutations in the RPE-specific MERTK gene (NCT01482195) (59) have just been started and are currently recruiting patients. Both trials use AAV2 to subretinally deliver the wildtype version of their respective mutated genes, and results will be eagerly anticipated. AAVs are also being used in clinical trials for nonmonogenic disease. There are currently at least two open trials for the treatment of exudative age-related macular degeneration with AAV carrying a VEGF scavenger receptor (NCT01024998/NCT014948050) as an attempt to replace the costly anti-VEGF antibody injections which are currently in use.

Although AAV has been the viral vector of choice for retinal gene therapy clinical trials thus far due to its high transfection rate and its tropism toward RPE and photoreceptor cells (60), it cannot efficiently package more than 5 kb (61), a limitation when considering large disease genes. In contrast, lentiviruses have a packing capability between 8 to 10 kb (62), and have also been demonstrated to transfect the RPE cell layer with high efficiency after subretinal injection (63), though most serotypes do not transfect photoreceptors cells well (64). However, the equine infectious anemia virus (EIAV) shows modest transgene expression in rods and cones especially with the addition of photoreceptor-specific promoters (65). Two mouse models of retinal degeneration were treated effectively with EIAV-delivered transgenes: an Usher syndrome model showed rescue of light-induced photoreceptor cell death (66) and a Stargardt's disease model demonstrated a reversal in the degeneration (67). The former led to development of a drug named UshStat which is presently under investigation in a phase I/II clinical trial currently recruiting study subjects (NCT01505062). The latter showed a favorable safety profile in extensive studies in rabbits and macaques (68) and is currently in a phase I/II clinical trial in patients with Stargardt's disease under the brand name StarGen (NCT01367444) and results are pending.

1.2. Therapeutic benefits of non-viral ocular gene therapies in pre-clinical testing

As clinical trials with viral vectors are progressing, there are still concerns regarding their safety and efficacy in human patients sparking interest in non-viral gene delivery approaches. Presently, nonviral gene therapy vectorsare not part of any active clinical trials for retinal degeneration but have been tested for other diseases. For example delivery of the p53 gene with liposomal nanoparticles (NPs) showed a favorable safety profile and appreciable transgene expression in solid tumors (NCT00470613) (69). Similarly, several different types of non-viral gene delivery, including cationic liposomes(NCT00470613) (70) and polylysine compacted NPs (71) have been tested for their ability to mediate improvements in cystic fibrosis patients.

Although no ocular gene therapy trials have yet used non-viral vectors, their therapeutic benefits have been achieved in animal models. For example, polyethylene glycol-substituted polylysine (CK30PEG) NPs (further discussed below) have been used in several disease models (for review see (72)). These NPs are non-toxic, non-immunogenic and have no adverse effects on the retina after subretinal injection in wildtype mice or mice with retinal degeneration (73, 74), and importantly have a large carrying capacity (tested up to 14 kbp in the eye (75) and up to 20 kbp in the lung (76)). When subretinally delivered to neonatal or adult mice, CK30PEG NPs were capable of mediating structural and functional improvements in several different mouse models carrying mutations in photoreceptor specific genes, the *rds*$^{+/-}$ model of RP(74, 77), the *Abca4*$^{-/-}$ model of Stargardt disease (75), and the *rho*$^{-/-}$ and P23H models of rhodopsin-associated RP ((78) and Naash MI, Nanoparticle-based gene therapy for ocular diseases: an update, ARVO 2015 Abstract #3185). They have also been used to mediate improvements in the *rpe65*$^{-/-}$ LCA model (79, 80), indicating that they are suitable for both photoreceptors and RPE targeting. In each case, the improvement persisted anywhere from 8 months to 2.5 years (depending on the ages assessed). In spite of these positive outcomes, levels of gene expression from subretinal injection of NPs have yet to meet wildtype levels and drop over time. As a result they have provided incomplete rescue, highlighting the need for further refinements in nanoparticle formulation, vector content and delivery approach.

In addition to CK30PEG NPs, various other non-viral ocular gene delivery strategies have been explored. One of the latest developments in this field, and one of the few non-viral vectors to extend testing into therapeutic animal models as opposed to just expression of reporter constructs, is the use of liposome-protamine-DNA complexes (LPD) to deliver the RPE65 gene(81). These liposomes incorporate cell penetrating and nuclear targeting peptides to improve gene expression/delivery compared to untargeted liposomes and carry an RPE-specific promoter to limit ectopic expression(81). Subretinal injection of LPDs into RPE65 knockout mice at five days of age (P5) led to improvements in cone ERG function and increased cone survival. Though extensive safety studies will have to follow before a clinical trial (81), this technology represents an additional promising approach.

In the remainder of this review, we will focus on strategies that are being employed to improve the efficacy of non-viral gene therapy in the eye, including delivery approaches, vector modifications, and non-viral packaging methods.

As comparison of clinical trials applying viral versus non-viral vectors clearly shows, non-viral gene therapy vectors are still far behind their viral counterparts. Their improved safety profile gives them a huge advantage however for future applications. As the efforts into the non-viral DNA compaction methods are intensified, we can be sure that these will soon catch up in terms of transgene expression levels and transfection efficiency with the AAV vectors currently in clinical trials.

2. Delivery strategies for non-viral gene therapy in the retina

The delivery route for non-viral gene therapy vectors is crucial for the success of the treatment and should lead to the highest transfection rate for the targeted cell type and the least risk for severe adverse effects (for a summary see **Table 1**).

2.1. Topical administration

Topical administration of therapeutics is by far the least invasive form of delivery, and is routinely used for pharmacological treatment of anterior segment diseases such as glaucoma. However, it is also the least efficient for retinal targeting due to the many barriers surrounding the eye. After corneal application of liposome-compacted DNA, transgene expression is scarce and only detectable in the iris, limbus and conjunctiva (82). Internalization of drugs into the corneal epithelium can be achieved (83) as well as transfection of corneal cells with AVV with therapeutic transgenes (84), but gene delivery to the retina via the topical administration route is not currently effective.

2.2 Intravenous injection

The delivery of therapeutic compounds directly into the eye is always associated with the risk of damaging the fragile structure of the retina or inducing a detrimental inflammatory reaction. To avoid any unnecessary adverse effects caused by the delivery route, compounds can be administered directly to the bloodstream. The main barrier for drugs to reach the retina after intravascular delivery is the blood-retina barrier, a structure much like the blood-brain barrier (99) which prevents diffusion of most large compounds into the retina (86). While the non-specific transport across the blood retina barrier is rather inefficient in general, particle size seems to be of minor importance, at least for diameters up to 460 nm (100). To overcome this and allow non-viral NPs to penetrate the retina after systemic administration, particles have been targeted with an antibody against the transferrin receptor present in retinal vascular cells. This strategy led to widespread expression of the transgene in ocular tissues, namely in the inner retina and the RPE (87). Though this approach is less invasive, one of the main disadvantages of delivery of genetic therapies in the blood stream is that serum proteins tend to cause opsonization and swift removal of particles from circulation (85). This massively decreases bioavailability, but can be ameliorated by chemical modification of the particle as discussed in section 5. Another concern is the volume of therapeutic needed to achieve efficacious concentrations in the eye (given the volume of the systemic circulation and potential unavoidable uptake into other tissues), but with proper targeting and vector specificity (to prevent ectopic expression) intravenous delivery could be considered.

2.3 Periocular injections

In addition to the more invasive but more common subretinal and intravitreal injections (discussed below), researchers have also developed other routes for injection of drugs into the eye. The injection of non-viral RNA particles in the subconjunctival space showed appreciable transfection of cells in the retina, but the majority of the particles migrated towards the cornea (88). It is therefore not surprising, that this method has been mainly used in the delivery of gene therapy vectors for the cornea (101, 102), especially as only very small particles (20 nm diameter) seem to be able to successfully penetrate into the inner ocular tissue (103).

2.4 Intravitreal injection

Though intraocular (intravitreal and subretinal) injections are significantly more invasive than other delivery methods, they are currently the most widely used for delivery of genetic therapies, largely due to the increased efficacy with which they are associated. The main barrier for therapeutic compounds after intravitreal injection is the vitreous itself. Despite its clear appearance, the vitreous contains a 3D meshwork of connected fibers of collagen, hyaluronan and other proteoglycans like heparin and chondroitin sulfate which gives it its jelly-like texture (104-106). This structure caninterfere with diffusion of particles both by steric hindrance and also because charged delivery vehicles can interact with the charged proteins in the vitreous. It has been shown that polystyrene nanospheres are completely immobilized in the vitreous humor and DNA-compacting lipoplexes aggregate immediately upon contact with the vitreal meshwork (91). The charge of a NP is crucial for this process, as experiments have demonstrated that cationic serum albumin particles do not show any vitreal penetration, while anionic particles of the same kind penetrate well (107). Penetration through the vitreous can be altered by coating the particles, a topic which will be discussed later.

In addition to charge, particle size also has a large influence on diffusion rates. Studies with fluorescently labelled polystyrene nanoparticles showed an average pore size in the vitreal mesh of around 550 nm, allowing particles of up to 500 nm in diameter almost uninhibited diffusion (108). Rare pores can show sizes up to 1000 nm, which also explains the ability of larger, uncharged particles to diffuse through the vitreous(94, 108). The diffusion paths and rates of various particles in the vitreous have been extensively studied with single-particle tracking (SPT) microscopy. This method allows the tracking of single particles and therefore also the description of specific paths instead of overall distribution (109). By teaming up SPT with an ex vivo model of intact bovine vitreous, the behavior of nanoparticles can be described in detail in relation to size and charge which is demonstrated with polystyrene beads (94). In addition, DNA compacted with polylysine or synthetic polymers like poly(amide amine) (CBA-ABOL) into nanoparticles analyzed with the same method showed unhindered passage through the vitreal meshwork when their charge was neutralized by the addition of PEG (see section 5 below) (94, 108). Intravitreal delivery of NPscan be effective for gene delivery in the retina with many different particles including liposomes or PLGA particles (110, 111). Successful diffusion of particles from 350 to 600 nm in diameter through the whole retina with subsequent transfection of photoreceptors and RPE cells was observed after intravitreal application(112, 113).However, studies show a much higher

transfection rate for cells in the inner retina after intravitreal delivery, with only inefficient transfection of outer retinal cells using this method (90, 92). Delivery to the inner retina, can be beneficial, for example for the treatment of glaucomatous optic neuropathy (82), but the lack of penetration to the outer retina means that intravitreal injection has largely been avoided in favor of subretinal injection for photoreceptor and RPE delivery. Since intravitreal injections are already routinely used for ocular drug delivery and are less invasive than subretinal delivery, there is great interest in improving outer retinal targeting of genetic therapies after intravitreal delivery. Nevertheless, risks are associated with the intravitreal application including possible retinal detachment and endophthalmitis which occur, though with low incidence (93, 114).

2.5 Subretinal injection

While the risk of retinal detachment after intravitreal injection is low, detachment is a routine feature after subretinal injections, as the introduction of therapeutic liquid induces the separation of the photoreceptor from the RPE cell layer (95, 98). This process is damaging to the retina, as the photoreceptors rely on the structural support and the nutrients from the RPE cell layer especially for regeneration of photopigments (115). It is therefore not surprising that detachment of the retina is associated with photoreceptor cell death and loss of visual function (96-98), a pathology which can also be observed in patients with retinal detachment due to other reasons (116). Though the retina typically reattaches, this detachment is a critical limitation and one that is a great concern clinically. In addition to detachment, subretinal injection can also be associated with inflammatory response and recruitment of immune cells into the subretinal space. For example, one of the viral vectors currently in use in gene therapy trials, StarGen, causes a cellular inflammatory response after subretinal injections in macaque and a certain amount of retinal degeneration and atrophy is detectable (68). The first data about the safety of subretinal injections in the RPE65 clinical trials show that retinal detachment occurred in the study participants and was associated with some minor adverse effects. Nevertheless, results thus far from the clinical trials (choroideremia NCT01461213 and LCA NCT00999609) indicate thatthe improvements in function in the treated eyes seem to outweigh the detrimental effects of the delivery in most patients (57, 117). There are currently no data available on the impact of repeated injections in human eyes, which may have more severe consequences, thus underscoring the need for long-lasting therapeutics. Studies in mice have shown that subretinal delivery is by far the most efficacious for the transfection of photoreceptor and RPE cells with non-viral vectors and yields the highest transgene expression in the outer retina (74, 90). This route of application is therefore chosen for most applications in inherited retinal disease in spite of its invasiveness.

2.6 Suprachoroidal injection

An additional option is delivery to the suprachoroidal space, between choroid and sclera, which prevents the detrimental detachment seen in subretinal injections while still delivering the therapeutic compound close to the RPE. With this method, widespread transfection of RPE cells with naked DNA can be achieved, but only with the aid of electroporation (89). Suprachoroidal injections have a very beneficial safety profile, as they do not induce any retinal detachment or bleeding and with proper DNA packaging, it might be possible to

overcome the scleral barrier without electroporation to make this delivery method more clinically viable (118). However, the transcleral diffusion rate of nanoparticles is very low even for small particles up to 20 nm in diameter, while larger particles (200 nm) do not diffuse across the sclera at all (119).

From a clinical point of view, the only currently feasible application methods for future ocular gene therapy in human patients are the intravitreal and the intravenous approach. The other applications are either too invasive or have been shown to be little effective. While intravitreal injections still bear a certain risk of adverse effects, intravenous applications generally have a higher toxicity profile due to the widespread distribution of the therapeutic compound and the larger doses needed to reach a significant concentration of drugs in the target tissue. Nevertheless, if the efforts for the search of tissue-specific, targeted particles (see section 3) are successful, this issuewill be reduced and intravenous injections may become the method of choice.

3. Improvement of cellular and nuclear uptake of non-viral vectors

Efficient uptake of therapeutic vectors in the target cell is crucial for the success of the treatment. The requirements in this process depend very much on the nature of the vector, but in this case, we solely focus on DNA plasmids for the delivery of transgenes. Once the particle has reached the cell membrane, there are several obstacles it has to overcome to reach the nucleus and achieve expression. Modifications on the particle and the vector described in this section are designed to facilitate this process (see **Figure 1**).

3.1. Cellular uptake

Once delivered to the site of interest, the plasma membrane is the first barrier for effective gene therapy. RPE cells have a high rate of phagocytosis due to their involvement in photoreceptor disc renewal and can readily take up particles in their environment (120). Due to this activity, RPE cells can be easily transfected, even with naked (i.e. uncompacted) plasmid DNA(80), which partially explains the success of RPE-targeting gene therapy trials. Photoreceptors on the other hand, the main site of expression of many retinal disease genes, do not exhibit phagocytosis and are notoriously hard to transfect. Nevertheless, even non-phagocytic cells take in many different proteins to maintain their function and these endocytic systems can be taken advantage of. The first system discovered for receptor-mediated endocytosis involves clathrin-coated pits which form at the plasma membrane upon binding of the ligand to its receptor. The receptor-ligand complex is then internalized and ultimately fuses with protein-processing organelles in the cytoplasm (121). Subsequently, many clathrin-independent endocytic pathways have been described (122, 123), and this knowledge has been used to increase specificity and uptake of non-viral vectors by modifying particle envelopes to target receptors on the cell surface.

Significant progress in this direction has been made by coating drug carriers with antibodies specific for receptors on target cells. Cancer cells, for example, are known to express high levels of certain integrins and by covalently linking an anti-integrin antibody to NPs, cytotoxic compounds can be delivered specifically to target cells thus minimizing adverse effects (124). This method has also proven valuable for the delivery of therapeutic DNA in

hard to transfect neurons. An antibody specific for the neurotrophin receptor p75NTR (125) was coupled to polyethylenimine NPs carrying a reporter plasmid. Neonatal rats were injected with these particles and GFP expression was reported exclusively in motor neurons expressing p75NTR while non-targeted particles yielded very little expression. These are exciting results, as the p75NTR receptors becomes highly upregulated in disease damaged motor neurons and could therefore be used as a feasible therapeutic vector (125). Incorporating such antibody-targeting into ocular therapeutics may help increase cellular uptake in hard to transfect cells like photoreceptors.

In addition to antibodies, small receptor ligands have also been shown to enhance the uptake rate of NPs. Many tumor cells express large amounts of growth factor receptors such as the EGF receptor (126). Delivering chemotherapeutic drugs like cisplatin (127) or doxorubicin (128) in EGF targeted NPs largely decreases their toxic side effects. Similarly, folate and transferrin may be useful for NP targeting to malignant tumors even after systemic administration (129, 130). Transferrin-mediated uptake has also been explored as a way to facilitate uptake from the vasculature into the retina (87). As mentioned above, intravenous delivery of transferrin-targeted particles led to transgene expression in RPE cells, as well as in neurons in the inner retina (87). An alternative approach is the use of hyaluronic acid in NPs which targets the CD44 receptor widely expressed in retinal glia cells (131) and the RPE cell layer (110). The incorporated hyaluronic acid increases the transfection efficiency in RPE cells in vitro (132) as well as in vivo in the rat eye (110). The use of the CD44 pathway not only significantly increases the efficiency of the uptake of the particles, but also decreases the intracellular degradation rate of the particles which is associated with other uptake pathways (133).

Instead of using cell-surface receptors to increase uptake rates, cell-penetrating peptides (CPP) can also be used. These peptides are coated on the outer shell of the particles and originally take their sequence from various viruses. One of the earliest CPP's identified is the Tat protein of human immunodeficiency virus 1 (HIV-1), which mediates entry into the cell without interacting with a specific receptor (134). Similarly, the structural VP22 protein from the herpes simplex virus is able to enter cells without any external aid (135). The Tat protein has been successfully incorporated into lipid NPs and used for the delivery of transgenes to retinal cells (81). However, not all of the CPPs work in vivo in the retina (136, 137), so recent studies have used synthetic peptides which were specifically engineered for ocular tissue and yielded much higher success rates (138).

3.2. Nuclear entry

After successfully entering the cell, the transgenic DNA has to reach the nucleus to escape cytoplasmic degradation and to be transcribed effectively. Nuclear delivery in actively dividing cells is straightforward, as the nuclear envelope breaks down during mitosis allowing plasmids to enter (139). However, in post-mitotic cells like photoreceptors the nucleus is only accessible via the nuclear pores, which have an opening of 9 nm in diameter allowing ions and smaller proteins to diffuse freely, but excluding molecules larger than ~50 kDa (140). Larger molecules can enter the nucleus by active transport, which depends on the presence of a nuclear localization signal (NLS) (140). Many viruses have evolved these

targeting sequences, and the minimal sequence was identified from SV40 as a single seven amino acid stretch (PKKKKRKV) (141). These NLS sequences can interact with different proteins inside the cell like nuclear pore transport proteins or transcription factors which lead to active translocation into the nucleus (140, 142). Inclusion of NLSs on the envelope of the particle can be used to directly target non-viral vectors to the nucleus, an approach that has been tested successfully in the eye(81). Studies have shown that the inclusion of such a sequence can increase nuclear accumulation tremendously, while plasmids without such a signal barely enter the nucleus at all (142-144). The peptide protamine also contains a NLS and its addition to compacted DNA increases the transgene expression in cultured ARPE-19 cells by more than 6-fold (145). Another way to target non-virally delivered DNA or NPs to the nucleus is to take advantage of other cytoplasmic proteins that have the tendency to translocate to the nucleus. For example, CK30PEG NPs have been shown to traffic to the nucleus of airway epithelial cells by associating with nucleolin and glucocorticoid receptor, and stimulation of nuclear translocation of the glucocorticoid receptor with cortisone improves transfection efficiency (146).

In addition to the peptide/protein based approaches, early research with plasmids based on SV40 indicated a region of the viral genome around the origin of replication enhancingnuclear uptake of the plasmid in non-dividing cells (147). Further investigation narrowed down the necessary sequence for this process to a 72 bp repeat (148). Introduction of this sequenceinto a CMV-driven expression vector showed a 20-fold increase in transgene expression after delivery to skeletal muscles of mice (149). In addition, some plasmids can interact with transcription factors via binding sites in their promoters leading to increased rates of movement across the cytoplasm to the nucleus. This often takes advantage of the microtubule cytoskeleton and can be beneficial for effective gene transfer (142).

Another approach to increasing nuclear localization is to decrease the size of the delivered vector. Minicircle DNA (see description below) was developed to prevent gene silencing caused by the bacterial backbone, but experiments in vitro revealed that the smaller size resulting from the excision of the backbone also increases the transfection efficiency (150). To decrease the size of the DNA plasmid even further, it has been suggested that type II topoisomerase can be used to tighten the DNA into a so-called miniknot which would be unwound inside the nucleus by the host's topoisomerase enzymes (151). Similarly, the ability of CK30PEG NPs to transfect non-dividing cells has been in part attributed to their small size; CK30PEG NPs compacted with acetate as the lysine counterion are rod shaped with a minor diameter of 8-11 nm (76, 152). In the meantime, CK30PEG particles have been successfully used to transfect photoreceptor cells in the retina (75, 153).

As mentioned at the beginning of this section, we mainly discuss DNA plasmid vectors in this review.For vectors containing mRNA instead of the corresponding DNA sequence, nuclear entry is not necessary and therefore not an issue. Successful transgene expression with compacted RNA nanoparticles has been shown with high expression levels, but due to the unstable nature of RNA only for short time periods(154, 155).

Recent research has clearly shown that targeted nanoparticles have a clear advantage over their non-targeted counterparts with higher transfections efficiencies and a far better safety

profile. The ability to target one specific set of cells or maybe even only diseased cells is likely to reduce the issue of off-target toxicity and will also decrease the costs of treatment by limiting the amount of therapeutic particles needed. Nuclear entry is a topic that has been addressed only minimally in retinal gene therapy so far. It is however immensely important to do so in the future, as the post-mitotic status of photoreceptors naturally has a negative effect on the transgene expression efficiency in the retina.

4. Vector engineering

Non-viral gene therapy is a desirable approach because of its safety profile, ease of production, and large payload capabilities. However, even after reaching the nucleus effective therapy requires persistent, elevated transgene expression. Coupled with insufficient transfection rates, weak transgene expression and rapid downregulation have thus far impeded successful clinical application of non-viral therapies in the eye. To improve the expression levels and longevity of these vectors, proper design of the expression vector is extremely important and has been anactive research field in recent years (see **Figure 1B,C**). Luckily, the availability of non-viral packaging methods which can deliver larger DNA payloads (76) has made inclusion and testing of additional DNA regulatory elements easier.

4.1. S/MARs

A number of different factors can lead to loss of gene expression over time including loss of vector DNA and silencing of the vector due to epigenetic or transcriptional changes. Transgene loss can occur due to the non-integrated state of non-virally delivered DNA. Some viruses like SV40 have the ability to replicate episomally inside the nucleus of an infected cell without integration into the host genome (156). The virus achieves this by producing the T antigen, which has the ability to transform and immortalize infected cells, which unfortunately makes it unfit for the use in human gene therapy (157). Piechaczek et al. overcame this limitation by replacing the sequence for the T antigen in the vector with the sequence for a scaffold/matrix attached region (S/MAR) of the human interferon β-gene to create the now extensively used pEPI plasmid vector (158). The S/MAR sequence has been shown to interact with proteins in the nuclear matrix thereby stabilizing the vector inside the nucleus for episomal replication in dividing cells (159). Vectors with this S/MAR sequence do not show any signs of integration, but can stay inside dividing cells for more than 100 generations (158). S/MARs can also promote increased transgene expression (80, 160, 161) apart from benefits conferred by self-replication, indicating that they can also be useful in post-mitotic cells.However, this increased expression depends on the vector and system used and is not always detectable (162). In vivo studies using the pEPI vector have shown transgene expression in the RPE cell layer of the retina up to two years after injection(80), even though decreased expression over time is still a problem(75, 80).

4.2 Epigenetic regulation

In addition to vector loss, gene silencing is often associated with the methylation of CpG islands (cluster of C and G nucleotide pairs) in the promoter region (163), and with the presence of heterochromatin. In recent years, several options have been investigated to

improve the transcription rate in plasmids after transfection. The discovery of the high activity of the cytomegalovirus (CMV) promoter in mammalian cells raised hopes for swift development of effective gene therapy vectors (164), but this promoter is silenced quickly in vivo due to extensive methylation (165). Bacterial backbones are necessary for the efficient production of plasmids, however, delivering bacterial backbones in vivo can lead to silencing, both due to methylation and due to heterochromatin associations(166, 167). Several options are available to limit the adverse effects of the backbone. One is to deliver linearized DNA fragments that have been removed from the backbone. An alternative is to place phage-integrase specific recombination sites on both sides of the eukaryotic expression cassette in a plasmid which also carries the integrase gene. When expression of the integrase is induced in bacteria, the bacterial backbone is excised leaving behind a circular miniplasmid ready for delivery (150). Recent work has shown that minicircles can lead to sustained gene expression, are less susceptible to methylation, and retain an active chromatin conformation (168).The production of minicircle DNA is rather inefficient and contamination with parental plasmids and circular backbone have prevented large-scale use of this technology, but improvements like the inclusion of restriction sites leading to the degradation of the bacterial backbone have helped make the approach more practical(169). Recently, minicircles have been used to successfully deliver shRNA and rescue the phenotype in a myocardial infarct model(170). A final option for minimizing methylation is to construct CpG depleted vectors. For example, a newer version of pEPI was constructed with a CpG-depleted backbone, called pEPito (171). Experiments have shown that pEPito can drive stable transgene expression at higher levels and for a longer time period than its successor pEPI in vitro and in vivo especially in the retina (171, 172). This has the added benefit of a minimized inflammatory response (173), which is often associated with typical CpG patterns in bacterial backbones (174).

In addition to methylation, other factors can influence chromatin structure. Chromatin structure is extremely important for transcription initiation, as the proteins in the transcription complex need access to specific sites in the DNA. Studies have shown that the curvature of the DNA helix plays a major role in the accessibility of the promoter and that left-handedly curved DNA is more efficiently transcribed than right-handedly curved DNA (175). This phenomenon can be partially explained by the fact that curved DNA allows nucleosomes, which organize the chromatin into functional sections, to slide more easily along the helix and therefore can open up access to specific binding sites like the TATA box (176). The sequences causing the helix to bend are mostly A- and T-rich and have been tightly conserved among species (177), but in recent years, artificial versions have also been used to enhance vector expression in vitro and in vivo. The shortest effective sequence has been identified as a quadruple repeat of five thymidines connected by a four base pair linker aptly named T4 (178). The addition of T4 in front of the promoter sequence increases transcription rates up to ten-fold in vitro (175), but even higher rates can be achieved by increasing the repeat number (178). The magnitude of the effect is less in vivo, nevertheless, a T36 repeat addition was able to increase the transgene expression levels after transfection of a mouse liver by eight times (179). These curved sequences are fairly short even with a higher number of repeats, and the fact that the expression levels can be modulated by

changing the number of repeats, makes the curved DNA sequence an excellent candidate for vector engineering strategies to enhance gene expression.

Other sequences, termed insulators, can also help prevent the plasmid DNA from adopting the detrimental heterochromatin structure(180). These sequences keep heterochromatin from forming around adjacent expression cassettes by recruiting histone acetyltransferases which prevent the methylation of lysine 9 on histone 3, a hallmark process in heterochromatin formation (181). An insulator sequence identified in the chicken genome has demonstrated great potential in inhibiting vector silencing in lentiviral transfection, but occupies a considerable amount of space in the viral genome with its 400 bp (182). However, a recent study identified several very potent and short (between 100 and 300 bp) insulators in a genome-wide search, providing potential candidates for future investigations into this topic (183) and inclusion of these sequences into ocular gene delivery vehicles may be beneficial.

4.3 Addition of intronic sequences

Due to capacity constraints for the packaging approach (e.g. ~5kb for AAV), many gene therapy vectors only contain the coding sequence of the transgene without any regulatory sequences or introns. This can have detrimental effects on the protein level of the expressed gene as research has shown that inclusion of regulatory elements in the native gene can have positive effects on expression.Introns in particular can improve gene expression by multiple mechanisms including altering transcription, translational yield, polyadenylation, etc. (184-186). These effects are highly variable, dependent on the gene, intron content, and intron position (185). The inclusion of intron A of CMV increases the expression levels of transgenic proteins in cultured mammalian cells when placed inside the coding sequence, a phenomenon named intron-mediated enhancement which was originally described in plants (187). The ability to enhance the expression can be independent of the actual intron sequence, as introns can sometimes be substituted between genes, even though there are differences in the magnitude of expression enhancement from gene to gene(188). The presence of untranslated sequences in the mRNA seems to stabilize it (189) and enhance the rate of maturation, for example by augmenting the polyadenylation of the transcript (190). Several different introns have been tested for intron mediated enhancement in gene therapy vectors so far and the results show a clear benefit from the additional sequences. Transgenic mice have shown an almost 5-fold increase in the production of human thrombopoietin upon inclusion of only a single intron of the same gene (191) and introns of mouse, rabbit and human β-globin have been shown to be potent enhancer elements (192, 193). Several viral introns including SV40 (194) or CMV (195) have also been tested in vitro and in vivo more or less successfully.

Since some non-viral NPs can deliver large genetic sequences, it has recently become possible to directly compare the expression efficiency of genomic DNA (i.e. including introns) with that of the cDNA. We have shown that in one case at least, that of NP-mediated rhodopsin gene delivery, inclusion of genomic sequences significantly improves expression levels and phenotypic rescue of the *rho*$^{-/-}$ mouse model of RP. Though the mechanism of this improvement is unknown, mRNA levels were the same for genomic and cDNA, while protein levels were improved in genomic vs. cDNA suggesting the benefits

were on protein translation(153). Introns have demonstrated their ability to increase mRNA stability and enhance translation, but in many cases, even large capacity packaging methods cannot deliver the full genomic sequence in any practically applicable way as the genes are just too large (e.g. the Usher syndrome type 2A gene that spans 800 kbp). As a result, consideration must be given to inclusion of heterologous introns, or only a select number of native introns, and the relative contributions of any given intron to the overall expression level can be evaluated.

4.4 Other Enhancer Elements

Though the CMV promoter is rapidly silenced and therefore not clinically useful, in 1985 it was discovered that a small part of the promoter acts as an enhancer (196) and when added to almost any promoter, greatly increases the transcription rate (194, 197). Inclusion of this enhancer can be used to improve the expression of normally weak but tissue-specific promoters without compromising the specificity of the promoter (198), and has been implemented in the eye with the RPE65 promoter (172). This enhancer effect has also been demonstrated for regions from other promoters such as the photoreceptor-specific interphotoreceptor retinoid binding protein (IRBP), even though the effect is much less potent than in the case of CMV (65). Subsequently, a genome-wide search for enhancing elements via in vitro analysis yielded thousands of possible candidates for enhancement (199). One 37-bp leader sequence was analyzed in-depth and demonstrated an ability to increase mRNA levels of the transgene by more than ten-fold (200). Sequences of this sort are valuable because of their small size and high activity, but will have to be tested in vivo extensively to assess their capabilities. In addition, the effects of enhancer sequences can vary based on target tissue and gene/promoter so individual testing for any given application will likely be required.

Viruses like herpes simplex have evolved short sequences to boost the translation of their intronless genes in the host cell, which can be utilized in plasmids in lieu of introns(201). One of those sequences was identified in the human hepatitis B virus (HBV) and aptly named the post transcriptional regulatory element (PRE) (202). The PRE sequence does not increase the transcription rate of the gene nor does it have an influence on the mRNA stability in the cytoplasm, but rather enhances the nuclear export of the mRNA which in turn greatly improves translation rates (202). It was later discovered that a close relative of HBV, the woodchuck hepatitis virus, contains an even more potent PRE labelled WPRE (203). The HBV PRE consists of only two subelements, while the WPRE is a tripartite enhancer element which explains the difference in strength (203). The full WPRE sequence is only 600 bp in size, but in vitro transfection experiments in neurons showed that it can be shortened to 247 bp without impeding its translation enhancing ability (204). Since its discovery the WPRE sequence has been demonstrated to be one of the most successful enhancer elements and is currently under investigation for in vitro production of therapeutic proteins (205) and for gene therapy in AAV vectors ((206, 207) as examples) because of its small size and its ability to significantly increase transgene expression. The WPRE sequence has also been used as part of an AAV vector containing the cDNA for brain-derived neurotrophic factor (BDNF) which induced transgene expression and phenotypic improvement in a rodent model of glaucoma (208).

4.5 Influence of polyadenylation site on protein expression

Efficient protein translation is not possible without a proper polyadenylation signal added to the mRNA and mutations in the poly A signal sequence quickly lead to aberrant gene expression and disease (209). A short sequence (AATAAA) is the consensus signal for the polyadenylation of mRNAs, but deletion experiments have shown that additional recognition sites are necessary for the successful addition of poly A but are not conserved between genes (210). Different poly A signals can influence protein levels of transgenes very differently, as shown by the direct comparison of the signals of SV40 and the bovine growth hormone (BGH) gene. Expression of luciferase was increased two- to ten-fold with the BGH poly A sequence over the SV40 in different cell lines in vitro and in multiple tissues in vivo including kidney, lung or heart (194, 195). The differences in efficiency of the poly A signal between different genes may be an evolved mechanism to control the level of innate gene expression. Varying the polyA signal may be used as an advantage in gene therapy vectors, though such alternate polyA signals have not yet been tested in ocular gene delivery plasmids.

The discovery of countless DNA and RNA regulatory mechanisms in the last several years shows how little we actually know about regulation of gene expression in mammalian cells. These new regulatory elements will certainly pave the way for better transgenic expression vectors, even though the process is must still be evaluated using a largely trial and error approach untilwe gain a deeper understanding of the processes involved. The lack of appropriate transgene expression is one of the main issues in non-viral gene therapy and can hopefully be resolved by the insertion of a combination of any of the enhancers mentioned above.

5. NP formulations for gene therapy

Non-viral gene packaging is an active research topic, as it is considered a safer alternative to viral gene delivery. The concerns about the safety of viral vectors are based on two major incidents: in 1999, a participant of a gene therapy study died of an acute immune response after the injection of a therapeutic adenovirus (211, 212) and in 2002, children treated for severe inherited immunodeficiency with a retrovirus developed leukemia (213, 214). These two cases illustrate the two main issues with viral gene therapy: recognition of the vector by the immune system and random insertion of retroviral vectors causing mutagenesis. The use of non-integrating AAV has so far not caused any severe adverse effects; nevertheless, the development of an alternative is desirable particularly because traditional AVV cannot deliver DNA molecules larger than 5 kb (61). In addition, though AAVs are safer than older viral vectors such as adenoviruses, they can induce an immune response, in particular the production of neutralizing antibodies against the viral capsid (215, 216). This is particularly true after intravitreal delivery (217, 218), but can also occur after subretinal injection (219). Non-viral vectors show generally a very low immunogenicity, especially when DNA is compacted with innate proteins like human albumin (see below). Plasmids delivered via non-viral gene therapy can be maintained episomally (158) which removes the risk of insertional mutagenesis, but can also lead to transient instead of stable transfection. In direct comparison with AAV, most non-viral gene delivery vectors show inferior transfection

efficiency and do not lead to sufficient transgene expression (220). Improvements can be achieved on the DNA level as described previously, but the formulation of the particle or packaging method itself can also be optimized.

Non-viral particles for gene therapy can be broadly divided into two groups, lipoplex particles containing lipid molecules and polyplex particles, which are based on polycations from chemical groups such as polysaccharides and proteins. Particles from both groups have been shown to benefit in some way from the modification with polyethylene glycol (PEG), therefore this very common modification and its consequences will be discussed separately (for a summary of this chapter see **Table 2**).

5.1. Lipoplex NPs

Lipid-based delivery systems have been widely utilized as a non-viral method to carry DNA molecules across the hydrophobic lipid bilayer of the plasma membrane and therefore increase the transfection efficiency immensely compared to naked DNA (233). The compaction is mediated by the interaction of the positively charged cationic head groups of the lipids and the negatively charged base pairs of the DNA leading to a small particle with a diameter of around 50nmor larger with a slightly positive charge (234). Early clinical trials in cancer patients showed no toxic adverse effects and transgene expression in tumor cells, demonstrating the viability of the approach (235). Different lipid formulations have been used for the compaction of DNA into liposomes allowing adaption of the particle to specific requirements. Phospholipids like DOPE, DOTAP and DSPE are widely used andparticles made with combinations of these lipids show no toxicity and moderate transfection efficiency in vivo (236).

Solid lipid particles consisting of DNA complexed with protamine and dextran and compacted into cationic lipids with DOTAP to a size of around 250nm showed high transfection rates in vitro in transformed RPE cells (ARPE-19),and also the ability to transfect retinal cells after intravitreal or subretinal delivery and corneal cells after topical application (90). The addition of protamine, which is a mixture of small basic peptides, can produce particles with higher transfection efficiency (237) and dextran inhibits the interaction of the particles with erythrocytes, a beneficial characteristic for intravenous applications, as it prevents fatal blood clots (238). These particles show potentialfor the efficient transfection of retinal cells and are currently under investigation for gene therapy in a mouse model of retinoschisis(223). Protamine was also used as a transfection enhancer in modified targeted liposomes formulated with a mixture of DOTAP, DOPE and cholesterol (81). The introduction of cholesterol into the outer layer of the liposomes has been shown to increase transfections rates in vitro (239). These particles were able to successfully transfect RPE and photoreceptor cells and to mediate appreciable rescue in a mouse model of LCA (81). Though several liposome formulations are effective in vitro, (e.g (132, 222)), the in vivo environment is very different making in vivo testing of potential formulations essential. One of the major drawbacks of liposome formulations is their tendency to aggregate when in contact with serum proteins which largely decreases bioavailability after intravenous administration (240). Nevertheless, with the appropriate modifications, liposomes can be delivered intravenously and lead to successful transgene expression in retinal cells (87).

5.2. Polyplex NPs

5.2.1. Peptide and protein-based NPs—Proteins and peptides can be used as an alternative to phospholipids for compaction of DNA and some formulations have shown promising results. Human serum albumin can compact DNA into particles of 150 to 280 nm via a cost-effective and highly reproducible process (241). Human albumin as a major serum component is non-immunogenic, biodegradable and easily prepared in large amounts as an ultrapure recombinant protein (242) and the safety of albumin in the retina after intravitreal application has been established (243). Compaction with albumin effectively protects the plasmid against degradation and imparts a transfection rate higher than lipofectamine in vitro in the ARPE-19 cell line (224). Cytotoxicity was detected neither in vitro nor in vivo after intravitreal injection in the mouse eye and protein expression was detectable in retinal extracts even though the exact location of expression was not established (224). Further investigations into the mobility of these particles in the vitreous revealed aggregation of positively charged formulations probably due to interaction with abundant negatively charged proteins in the vitreous humor, but altering the surface charge of the particles can resolve this issue (107). However, the influence of altering the charge on the transfection efficiency of the particles will need to be determined. Gelatin-compaction is another attempt at using innate proteins as a nonimmunogenic drug delivery vector which shows no adverse effects in the eye after intravitreal injection (232). Gelatin can also compact DNA effectively (244, 245), but it has so far not been tested in retinal gene therapy despite its potential.

NP compaction can also be achieved by peptides instead of whole proteins. The positively charged amino acid lysine is a perfect candidate for this and early experiments demonstrated high transfection efficiency for DNA compacted with polylysine (246). Since then, the compaction procedure has been refined and used in different tissues for transgene delivery(247-249). With the addition of PEG (see discussion below), polylysine NPs can effectively transfect lung epithelial cells and induce minimal immune response even when administered at high dosage (250). CK30PEG NPs can also transfect postmitotic cells, something which has historically proven difficult with non-viral delivery methods (74). These particles have been widely used in the eye to provide therapeutic improvement as described above and do not show any adverse effects after delivery to the eye even after repeated treatment (225), nor any extraocular gene expression after subretinal delivery (251). The efficiency of polylysine particles can be altered by adjusting the lysine counterion used during the compaction process. For example, switching from acetate to trifluoroacetate can change the shape of the particle form rod to sphere and influence the transfection rate both in the eye and in the lung (76, 252). Likewise, the addition of histidines to the polylysine compaction agent can increase the efficiency of the gene transfer up to 20-fold (tested in vitro). This ‘proton sponge’ effect is due to the amino nitrogens in the histidine which are able to accept protons (253). This immense buffering capability prevents the acidic degradation of the particle in the endosome after uptake and leads to rupture of the organelle and escape of the DNA into the cytoplasm (247). Polylysine can also be engineered into branched dendrimers before compaction, increasing its buffering capacity and thereby its gene delivery capabilities (254). This type of particle is still completely biodegradable and therefore exhibits much less toxicity than other dendrimer

polymers like PAMAM described below. These examples show how versatile the polylysine particles are and a change of certain amino acids or the content of lysine could have immense influence on the transfection efficiency of the final particle.

5.2.2. Polysaccharide-based particles—Polysaccharides are well suited for use as drug delivery agents due to their non-immunogenic and biodegradable properties, inexpensive production and ease of modification. Hyaluronic acid can be used to target lipid NPs to the CD44 receptor as described above, but can also be used to deliver drugs on its own. Hyaluronic acid is present in large amounts in the interphotoreceptor matrix of the retina and is highly biocompatible and non-immunogenic (255). Compaction of chemotherapy drugs with hyaluronic acid has been successfully achieved (256), however, for gene therapy, a mixture of hyaluronic acid and chitosan is more generally in use as the overall negative charge of hyaluronic acid interferes with productive DNA compaction. In combination with chitosan, a cationic polysaccharide harvested from shellfish, hyaluronic acid can form small particles (100-200 nm in size) which protect DNA from degradation and can effectively transfect cells in vitro (257). These particles are also able to successfully transfect corneal epithelium cells, become internalized efficiently,and induce transgene expression (258). During production, the size and surface charge of the particles can be easily manipulated by changing the ratio of chitosan to hyaluronic acid which has a distinct influence on transfection efficiency and transgene persistence (257). Chitosan can also be used without hyaluronic acid to compact DNA and deliver NPs, either unmodified or with various alternate functional groups. The interaction of chitosan with plasma membrane proteins has been discussed as a factor for the transfection abilities of chitosanNPs though the precise uptake mechanisms are not clear (259). Whatever the mechanism, chitosanparticles can deliver plasmid DNA to a variety of different cells including intestinal and corneal epithelial cells (227, 260). In vitro transfection is inefficient without additives, but in vivo, transgene expression was observed in RPE cells (92, 226) and photoreceptors (92) after subretinal injections without any notable cytotoxicity. Together with the highly efficient compaction process (261), these results show the potential of chitosan as a non-viral gene packaging method, but no therapeutic efficacy studies have been published as yet.

Other polysaccharides like cyclodextrin or dextran have also been shown to facilitate cellular uptake of nucleic acids(262). Dextran has been tested as a carrier for siRNA and exhibits considerable transfection efficiency in vivo in lung tissue when formulated as a nanogel (263). For plasmid transfection, dextran is mostly used in combination with other polymers, but not as a compaction agent alone. Dextran can increase the transfection rate of lipid NPs after intravenous delivery (238)and decrease the cytotoxicity associated with some polymer-based particles (264). For the use in ocular gene therapy, a combination of dextran, gelatin and chondroitin has been used to condense DNA and transfect corneal cells in vitro. This approach could be developed into a topical application of therapeutic DNA for the cornea (228) but has not yet been evaluated in the retina.

5.2.3. Polymer-based NPs—There are many different compounds neither peptide- nor polysaccharide-based which have been tested for compaction of drugs, siRNA and DNA into nanoscale particles for delivery and here we briefly discuss some of the most successful

ones.One of the most efficient transfection agents, polyethylenimine (PEI), was discovered in 1995 and has since been tested in many applications. The transfective quality of PEI is linked to the high amount of amino nitrogens in the macromolecule which confer the abovementioned ‘proton sponge’ effect(253) and promote endosomal release. PEI condenses into small particles (100-1000 nm) upon combination with DNA, a process which is highly dependent on pH and ionic strength of the reaction solution (253). PEI-compacted DNA has emerged as a powerful tool for non-viral transfection in notoriously hardtotransfect neurons, especially when coupled with a cell-specific receptor ligand like mannose (265, 266). The feasibility of this approach has been shown in the retina; PEI NPs successfully transfectedRPE and Müller glia cells in vitro and in vivo (229, 230), but these experiments also showed fairly high acute cytotoxicity of native PEI in vitro in differentiated and undifferentiated epithelium cells(231, 267) and ARPE-19 cells (231). This toxicity can be alleviated by the addition of PEG to the PEI particles, as described below (268), and PEI remains of great interest as a non-viral delivery strategy.

Another ‘proton sponge’ compound is polyamidoamine (PAMAM), which is synthesized as a repetitively branched dendrimer and can compact DNA into small particles (around 130 nm) (269). Successful transgene expression has been shown after in vivo deliveryof the PAMAM-DNA particles to tumors and cardiac grafts (270, 271), but the compound was associated with relatively high toxicity levels in vitro in Y79 cells and moderate pathology in mice 30 days after systemic administration from the beginning, which explains why it is not more broadly in use for gene therapy applications (272). Again, studies as recent as 2014 with PEGylated dendrimers have demonstrated reduced induction of cell death in vitro in HEK293T cells compared to unmodified PAMAM, however in this case, the PEGylation led to loss of transfection efficiency, a phenomenon described in more detail below (273). The ‘proton sponge’ effect is naturally of interest for the development of better non-viral vectors and is therefore subject of serious research. The imidazole ring of the amino acid histidine can serve the same purpose and actually shows appreciable gene transfer in vitro without associated toxicity (274).

Other polymers have also had noteworthy success for gene transfer. The use of poly(lactic acid) (PLA) as a DNA-compacting agent is quite common, as the compound is simple to synthesize and releases its cargo easily (275). The particles features very low short-term toxicity levels in vitro in liver carcinoma cells(276) and high stability in the presence of serum when PEGylated (277). Poly(D,L-lactide-co-glycolide) (PLGA), a derivative of PLA, forms stable nanospheres of less than 200 nm together with DNA (278). The particles achieve rapid endosomal escape (279) and can efficiently induce transgene expression in RPE cells and ganglion cells in vivo upon intravitreal administration(111, 112). Cationic hyperbranched poly(amino ester) (PAGA) was described as a possible non-toxic DNA carrier as well (280), but the transfection efficiency is clearly inferior to other particles compacted with PAMAM or PEI (281).

It is conceivable due to their large variety that there are many more possible synthetic, degradable polymers which could provide non-toxic, highly efficient non-viral gene delivery. In an early study, a high-throughput approach used to test the transfection qualities

of a library of polymers yielded 140 possible candidates (282) and experiments like this could lead to the next-generation of polymer vectors.

5.3. Modification of NPs with polyethylene glycol (PEG)

All the NP formulations described above have distinct transfection efficiency, toxicity and bioavailability profiles and they have virtually all been conjugated with PEG to help overcome some of their deficiencies. Hydrophobic and electrostatic interactions of NPs with serum proteins in the blood, a process called opsonization, leads to a swift removal of particles from the circulation by phagocytic cells (85, 283). For intravenous delivery this is an issue, as it decreases the availability of the therapeutic compound at the target location. One way to decrease the opsonization rate of particles by serum proteins is the addition of PEG to the outer shell. The PEG molecules form a hydrophilic, neutral cover around the particle which creates a protein-rejecting buffer, also called stealth coating, preventing opsonization, increasing the half-life of the particles in the bloodstream, and decreasing macrophage uptake significantly (284-286). Plasmid DNA compacted with polylysine showed an increased stability in the presence of serum proteins when the particles were PEGylated (287) and siRNA compacted into liposomes demonstrated much better tissue delivery with the addition of PEG after intravenous application (288). This can lead to significantly higher transgene expression rates in the target tissue as shown with PEGylated polylysine particles in the lung (250). The modification of gelatin-based nanovectors with PEG also showed a significant increase of tissue accumulation of plasmid DNA after systemic administration which is due to the increased stability of the particles in the blood stream (289). The effect of PEGylation on macrophage uptake can be quite dramatic, a decrease up to three-fold, as research on drug-carriers formulated with PLGA has shown (290). This leads to a prolonged circulation time of up to 72 hours while non-conjugated drugs are cleared within 6 hours (290). There is also evidence of increased stability of PLA-PEG particles in the digestive tract after oral administration (291) and reduced aggregation of PEGylated liposomes in the vitreous (222).Another issue for many NP formulations is their considerable cytotoxicity. The addition of PEG can be beneficial in this respect as well, decreasing the negative effect of compounds like the PAMAM dendrimer (292), dextran (293), and PEI (268) and therefore increasing their potential feasibility for clinical application.

Despite all the positive results on the use of PEG for NP formulations, there is a huge disadvantage. PEGylation masks the positive charge on NPs, so it can also decrease the transfection efficiency since that depends on the same cationic charge (294). The added stability of PEGylated particles can also interfere with the endosomal escape of particles after uptake and can therefore lead to rapid degradation of the carrier and its cargo in the endosome (295). This issue has been described by many researchers in the field and is commonly called the ‘PEG dilemma’, as the increased stability of the particles is desirable for delivery, but comes at the cost of hugely decreased transfection rates (222, 293, 296). Different solutions to this problem have been proposed, one suggestion is the post-PEGylation of particles with PEG-ceramide which would dissociate from the inner particle upon contact with the target cell membrane or in the endosomal compartment. This would increase the mobility of the particles in the vitreous just like covalently linked PEG without

hampering the transfection (222). Another approach takes advantage of the specific environment found in target tissues, in one case cancer cells. Tumors have a slightly acidic pH due to their high metabolic rate which can be used to cleave off the PEG coating after delivery to the site of interest if pH-sensitive hydrazine bonds were used to link the PEG to the particle (297). As an alternative to PEG, particles can also be stealth coated with peptides changing charge from negative to positive upon encountering low pH values (298). Another approach which may be more valuable for gene therapy vectors in the eye is the use of chemical bonds cleavable by matrix-metalloproteinases (MMP) abundant in the extracellular matrix of certain cells (299). This is particularly applicable since the retinal detachment that occurs during subretinal injection leads to upregulation of MMP-12 and MMP-13 (300). In any case, the “PEG dilemma”remains to be fully resolved and remains a topic of interest to researchers.

Liposome-mediated transfection of cells was initially described over 30 years ago and researchers have discovered many other compounds suitable for compaction of DNA in small particles capable of penetrating the cell membrane in the meantime. Unfortunately for the whole field of non-viral gene therapy, compounds with very high transfection efficiency like PEI are also the ones with the highest toxicity levels and are therefore unsuitable for application in patients. The toxicity issue is partially alleviated with the addition of PEG, but that also reduces the transfection efficiency to a level comparable with the non-toxic compounds. This predicament needs to be resolved before successful non-viral gene therapy in patients can become a reality, but high-throughput synthesis and testing of new compounds for example described by Lynn et al. 2001 will certainly accelerate the process.

6. Conclusions

Since the development of liposomes in the 1980 as transfection agents, non-viral vectors have come a long way. Recent breakthroughs in particle compaction, surface coating and vector engineering have shown that these vectors have large potential in the future of gene therapy. The introduction of peptides like polylysine which are more biocompatible and less toxic (75) make the delivery of larger, therapeutically relevant doses possible. The advent of intravitreal injections of anti-angiogenic drugs in the past decades has also led to improved surgical techniques and reduced the probability of injection-related adverse effects (114). The intravitreal method of delivery is also gaining traction with the invention of stealth coating with PEG or hyaluronic acid as the bioavailability of intravitreally delivered particles in the retina increases (222, 301). When coupled with receptor-targeting of particles, this may lead to vectors which can be injected intravenously or intravitreally with high efficacy and with minimal risk of off-target adverse effects (87). Together with the advances in vector engineering, non-viral vectors have the capability to be a clinically relevant complement to viral vectors in gene therapy, particularly for the delivery of large or precisely engineered genes.

Since viral vectors have already been used in clinical trials in the eye, there arenow data available on human efficacy and limitations for ocular gene therapy, which are informing the next generation of viral trials. Though much of what has been learned from early ocular viral gene therapy trials can be applied to non-viral gene delivery as well (namely the

dangers associated with subretinal injection, the need for higher levels of expression and improved distribution), non-viral gene delivery still has a long way to go reach to wide application in patients.However, with the innovations seen in the last couple of years and the fast evolution of gene therapy vectors, both viral and non-viral, there is hope that a viable treatment optionat least for some patients with retinal degeneration will be available in the next decade.

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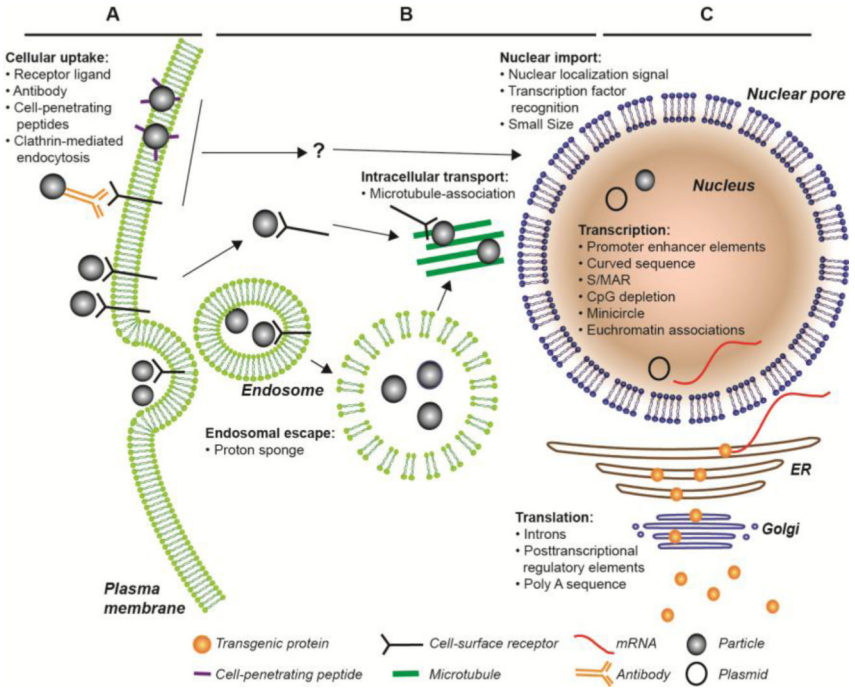

Figure 1.

Diagram illustrating some of the ways that non-viral therapies can be taken up and expressed in cells. Bulleted lists indicate places/methods where modifications to the particle or vector can be used to improve transfection efficiency or expression. Question marks indicate trafficking pathways that remain to be elucidated.

Table 1

Non-viral therapeutic delivery methods can dictate the transduction efficiency of different ocular cell types

Delivery Method	Target cells	Pro	Con	Ref.
Topical	Cornea	No injection	Inefficient penetration	(82-84)
Intravenous	Mostly inner retina	No adverse effects due to injection at the eye	Opsonization and phagocytosis of particles Off-target delivery to other tissues	(85-87)
Periocular	Corneal epithelium Photoreceptors RPE	Non-invasive	Low transfection rates for retina and RPE	(88, 89)
Intravitreal	Inner retina	Non-invasive Adverse effects rare	Vitreous humour aggregates particles. Ocular infections and retinal detachment can occur	(90-94)
Subretinal	Photoreceptors RPE	Highest transfection rate	Invasive Retinal detachment can cause cell death	(95-98)
Suprachoroidal	Photoreceptors RPE	No retinal detachment	Very inefficient transcleral transport	(89)

Table 2

Packaging methods.

Compaction agents	Target cells	Retinal model	Ref.
DOTAP	Neural retina/RPE/cornea	Wistar rats ARPE-19 (in vitro)	(221-223)
DOTAP/DOPE	RPE RPE	$RPE65^{-/-}$ mouse (LCA) ARPE-19 (in vitro)	(81) (132)
DSPE	Inner retina/RPE/Cornea	Balb/c albino mouse	(87)
Albumin	RPE	ARPE-19 (in vitro)	(107, 224)
Polylysine	RPE	Balb/c albino mouse $RPE65^{-/-}$ (LCA)	(80, 225)
	Outer retina Outer retina	$Rho^{-/-}$ mouse (RP) $ABCA4^{-/-}$ mouse(Stargardt's disease)	(153) (75)
Chitosan	Inner retina/outer retina RPE	Sprague Dawley rat Balb/c albino mouse	(92) (226)
Chitosan/hyaluronic acid	Cornea	Human corneal epithelium (in vitro)	(227)
Dextran/chondroitin sulfate	Cornea	Human corneal epithelium (in vitro)	(228)
PEI	Retinal Muller Glia Glia and ganglion cells Retina	Lewis rat Human retinal cells (in vitro) White Japanese rabbit	(229) (230) (231)
PLGA	Inner retina/RPE	Lewis rat	(112)
Gelatin*	Outer nuclear layer	RCS Rats	(232)

* Thus far these particles have only been used to deliver drugs, not genetic material, to the eye.