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# Nanoparticle motivated gene delivery for ophthalmic application

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## Abstract

Ophthalmic gene therapy is an intellectual and intentional manipulation of desired gene expression into the specific cells of an eye for the treatment of ophthalmic (ocular) genetic dystrophies and pathological conditions. Exogenous nucleic acids such as DNA, small interfering RNA (siRNA), micro RNA (miRNA), etc., are used for the purpose of managing expression of the desired therapeutic proteins in ocular tissues. The delivery of unprotected nucleic acids into the cells is limited due to exogenous and endogenous degradation modalities. Nanotechnology, a promising and sophisticated cutting edge tool, works as a protective shelter for these therapeutic nucleic acids. They are able to be safely delivered to the required cells in order to modulate anticipated protein expression. To this end, nanotechnology is seen as a potential and promising strategy in the field of ocular gene delivery. This review focused on current nanotechnology modalities and other promising non-viral strategies being used to deliver therapeutic genes in order to treat various devastating ocular diseases.

### Keywords

Ocular disorder; nanotechnology; nanoparticle; non-viral gene therapy

## 1. Introduction

Over the last few decades, gene based therapeutic clinical strategies have been extensively explored for the treatment of ocular diseases[1–8]. Gene therapy, from its inception, aims to repair or replace the disease-causing mutations by delivering the suitable therapeutic genetic materials along with the rational regulatory elements into the desired cells to express the deficient protein at normal levels. For therapeutic purposes, it has already been established that the genomic and the coding and/or non-coding RNA sequences can be modulated by introducing nucleic acids into the ocular tissues (Fig. 1). The effectiveness of inserting therapeutic genes into the desired cell is not only manipulated by DNA designing strategies,

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but also significantly depends on the efficiency and safety of its delivery vehicle(s). This review will summarize details on several ocular dystrophies of the leading causes of blindness in the world. In addition, major focus will be on the existing preclinical studies on nanotechnology-motivated synthetic non-viral gene delivery vehicles. These vehicles have already resulted in the improvements of structural and functional recovery of several major retinal disorders in animal models, and might have promising clinical significance in future human ocular gene therapy.

## 2. Ocular disorders

### 2.1. Retinitis pigmentosa

Retinitis pigmentosa (RP) is a group of clinically and genetically heterogeneous disorders[3, 9]. RP has a worldwide prevalence of 1 in 3000 to 7000 people[10]. This is characterized by initial night blindness (tunnel vision) due to the loss of rod cells in the periphery of the retina. As the disease progresses, cone cells start to degenerate, which leads to complete physical blindness of the patient via loss of central vision. The reason behind the loss of cone cells in RP cases is not yet clear, although rod cells possess defective genes. In this context, it is assumed that these cone cell dystrophies might be due to loss of rod cell-based supporting factors[11]. Multiple genetic inheritance patterns were found in RP patients. Among these patients, 15–25% of cases were autosomal dominant (adRP), 5–25% of cases showed autosomal recessive (arRP), and 5–15% showed X-linked traits[10, 12]. In general, RP is divided into two categories: one is syndromic (40%), and the other one is non-syndromic (or simple, 60%). The non-syndromic, or simple RP, is limited to the eye. The syndromic RP is beyond the eye, which also affects other organs and tissues in the body. The most frequent and studied syndromic disease is Usher syndrome (hearing loss followed by RP)[10].

**2.1.1. Rhodopsin (Rho) mutations—**RP is associated with more than 100 mutations[12] in the different regions of the rhodopsin (Rho) gene that accounts for 30–40% of adRP[10], and thus is genetically heterogeneous[3, 5, 10]. Rhodopsin protein helps to keep the structural integrity of the rod outer segments, as it was observed that $Rho^{-/-}$ mice were not able to extend rod outer segments. Rhodopsin protein is covalently associated with the 11-*cis* retinal chromophore to form a visual pigment, G protein-coupled receptor (GPCR), and plays a vital role in the conversion of electromagnetic radiation (light) to electrical signal in the retina, which is further processed in the brain as an image[10]. Upon absorption of a photon, the chromophore (11-*cis* retinal) is isomerized to all-*trans* (Fig. 2a) and causes conformational changes in the visual pigment (GPCR), which triggers a signal transduction cascade. This cascade results in the closure of nonselective cyclic guanosine 3'-5'-monophosphate (cGMP)-gated cation channels in the photoreceptor (PR) outer segment and hyperpolarization of the PR plasma membrane that generates receptor potential at PR synapse. Rod cells are responsible for night vision and remain sensitive as they are activated by single photons.

Mutation in the Rho gene can affect the rod cell functions at different severity levels. The same RP phenotype is observed from mutations in different regions of the rhodopsin gene that can cause different amino acid substitutions, and therefore leads to different rates of progression of this disorder. When the interdiscal protein mutates, it remains less severe than

that in the cytoplasmic or retinal binding site domains. Mutated rhodopsin protein in the cytoplasm possesses inappropriate transport properties towards the outer segment of rod cells.

The adRP currently does not have any therapeutic support. Rho mutations are classified as Class I and Class II based on tissue culture studies[13–15]. In Class I mutation, opsin expresses wild type level and binds to 11-*cis* retinal to form retinal photopigment (Fig. 2a) along with proper folding.

Class II mutation is the major cause of RP in North America[15]. Class II mutants accumulate and remain misfolded in the endoplasmic reticulum (ER). The misfolded protein causes activation of unfolded protein response (UPR), and leads to the cellular dystrophies associated with various ocular diseases. The first identified, and so far the most important Class II, RP is associated with the single missense mutation in the codon 23 in the human opsin gene. This mutation results in one proline substitution by histidine (RhoP23H), and is responsible for the most frequent cause of adRP[15]. The cause related to the RhoP23H toxicity remains unclear. Due to the dominant nature of the disorder, suppression and replacement of the gene are considered a useful and logical therapeutic approach in the treatment of the mutational heterogeneity of these adRP cases.

**2.1.2. Phosphodiesterase 6b (PDE6b)—**Rod cyclic guanosine 3'-5'-monophosphate (cGMP)-specific phosphodiesterase-6 (PDE6) enzyme is responsible for the hydrolysis of cytoplasmic cGMP in photoreceptor cells of the retina. PDE6 is a peripheral membrane heterotetramer enzyme that is composed of two inhibitory gamma (γ) subunits and two catalytic homologous α and β subunits in rods (PDE6αβγγ), and related α' subunits in cones (PDE6 α'α'γγ). PDE6 plays a key role in the photo-transduction cascade by regulating cGMP levels in the photoreceptor cells. Guanylate cyclase synthesizes cGMP from GTP and regulates the ion channel in the plasma membrane. During dark adaptation, cGMP keeps the ion channels open, thus allowing the influx of calcium ions into the outer segment of photoreceptors that causes overall depolarization of these cells. In light adaptation, PDE6 hydrolyses the cGMP and thus the cGMP amount is reduced, which leads to closing of cGMP gated ion channels and overall hyperpolarization of the photoreceptor cells. The beta subunits of PDE6 are encoded by the PDE6B gene, and therefore mutations in this PDE6B gene lead to malfunctioning of the PDE6 enzyme activity. In the light and dark adapted mechanism, the non-functional PDE6 enzyme is not able to hydrolyse cGMP, which results in accumulation of cGMP without closing the ion channels. This leads to the accumulation of excess calcium in cytoplasm that finally induces the degeneration of photoreceptor cells, resulting in blindness[16]. The mutation in the PDE6B gene is responsible for the arRP in humans. This earliest and most severe form of the disease is contributing to 5% of all arRP cases.

## 2.2. Usher syndrome

Usher syndrome (USH) is a genetically heterogeneous group of autosomal recessive genes that affects both hearing and vision (related to inner ear and retina, the most sensitive neurosensory organs in mammals), along with occasional loss of balance. This syndrome

was first discovered by Scottish ophthalmologist Charles Usher in 1914, upon examination of over 69 patients who were dealing with deafness with RP. There is a predictable occurrence in which 3–6 per 100,000 human patients are observed with both blindness and deafness[17, 18]. Clinically, there are three types of Usher syndrome (USH1, USH2 and USH3)[3, 19, 20]. Regarding the onset of hearing loss, USH1 is the most severe[19]. In USH1, a child is born with severe to profound deafness. The vision problem starts within 10 years of age, and progresses quickly to complete blindness. The child with USH1 might also have more balance problems associated with sitting and walking than normal. USH2 is comparatively less severe than USH1. The child is born with moderate to severe hearing loss and normal balance. Night vision problems appear in late childhood or teens with slow progressive loss of vision[19]. In USH3 cases, the child is born with normal hearing and balance, but there is an advanced loss of hearing by adolescence[19]. Vision loss varies in severity, and night vision problems develop during the teenage years. Night blindness and loss of peripheral vision are the early symptoms of RP due to the loss of rod photoreceptor cells. As the disease progresses, the cone photoreceptor cells also start to degenerate, leading to loss in the central vision that finally results in blindness of USH patients.

Significant amounts of research are currently underway to identify the genes related to USH syndrome. There are six genes that have been found so far to be associated with USH1, and these are MYO7A (myosin VIIA), USH1C (harmonin), CDH23 (cadherin 23), PCDH15 (protocadherin 15), SANS (scaffold protein containing ankyrin repeats and sam domain) and CIB2 (calcium- and integrin-binding protein 2) [18]. The proteins, encoded by these genes, are expressed in the cochlear hair cells in the inner ear, as well as in the photoreceptor cells of the retina. These USH proteins are helping the inner ear hair cell bundles in their development and maintenance of their function and stability. Mutations in these genes can result in the loss of protein functions that finally lead to the prevention of hair cell developments and loss in hearing. In mice, MYO7A has also been found in the RPE (retinal pigmented epithelium) cells, along with different sections of photoreceptor cells like connecting cilia, inner segment, and synapse. The USH2 (usherin, VLGR1 and whirlin) and USH3 (clarin-1) proteins are also found in the same sections of photoreceptor cells in the mouse retina. Literature shows that MYO7A functions the same way in both human and mouse RPE cells with respect to melanosome mortality[21].

### 2.3. Stargardt's disease

Stargardt's is an autosomal recessive juvenile disorder[22] which mainly occurs in children between the ages of 6–16 years, with a prevalence of 1 in 8000 to 10000 patients[23]. This common genetic macular disorder presents throughout the world, due to the mutation in a gene that encodes a photoreceptor ATP binding cassette (ABC) lipid transporter protein (more commonly known as ABCA4)[1, 24, 25]. ABCA4 is mainly expressed in photoreceptor cells and remains in the outer segment disc membrane[25]. In dark adaptation, opsin protein remains covalently conjugated with the 11-*cis* retinal chromophore. During light activation, this 11-*cis* retinal is isomerized to the all-*trans* retinal (atRAL) form that isomerizes back to 11-*cis* retinal in RPE cells to reconstitute the photopigment in maintaining the visual cycle (Fig. 2b). In this process, atRAL is converted to all-*trans* retinol (atROL) by all-*trans* retinol dehydrogenase 8 (atRDH8), and is transported to the RPE cells by interphotoreceptor

retinoid-binding proteins (IRBP). Through some enzymatic pathways, atROL is now converted back to 11-*cis* retinal in RPE, and is immediately shuttled back to the photoreceptor outer segment by IRBP to reconstitute rhodopsin photopigment. During this process, there is another pathway where a fraction of atRAL is conjugated with phosphatidylethanolamine (PE) to form N-retinylidenephosphatidylethanolamine (N-ret-PE) in the disc membrane lumen side[24]. The flipase ABCA4 protein binds with N-ret-PE and flips it from the lumen side to the cytoplasmic side of the disc membrane using ATP energy source[24]. Once this N-ret-PE is brought to the cytoplasmic side, it is hydrolysed by all-*trans* retinol dehydrogenase 8 (RDH8) to atROL and PE. Therefore, ABCA4 makes sure the disc membrane atRAL is converted to atROL in cytoplasm, and is shuttled towards RPE to regenerate 11-*cis* retinal.

Mutation in the ABCA4 gene can cause the accumulation of N-ret-PE in the lumen side of the disc membrane, which facilitates further possible reaction with another molecule of atRAL in the disc membrane to form a phosphatidyl pyridinium diretinoid derivative A2PE (2: 1 ratio)[25]. Under normal phagocytocis of photoreceptor cells, A2PE is engulfed by RPE and degraded to A2E by lysomal enzymes. RPE cells are not able to metabolize the A2E that leads to a high amount of A2E accumulation as fluorescent lipofusin[25]. This A2E accumulation causes excessive generation of reactive oxygen species (ROS) and leads to degeneration of RPE cells. The macula also has a high density of photoreceptor cells, and therefore causes high levels of A2E accumulation. RPE, just underlying the macula (which possesses the highest density of cone photoreceptor cells), plays a key role in providing the structural and functional integrity of photoreceptor cells. The loss of RPE cells causes damage to the photoreceptor cells in the macula, which finally leads to Stargardt's disease.

### 2.4. Leber congenital amaurosis (LCA)

Leber congenital amaurosis (LCA) is an autosomal recessive inheritance pattern which was found by Theodor Leber in 1869. So far, there are 14 genes evaluated which are responsible for this severe congenital blindness that presents in early childhood[26]. The worldwide prevalence of this disease is 1 out of 30,000 cases [27], 20% of all congenital blindness, and 5% of all inherited retinal dystrophies. One of the most important mutations is the RPE65 gene that encodes RPE65 (RPE-specific 65 KDa) protein in RPE cells [6, 8, 28, 29]. This protein works as a retinoid isomerohydrolase in RPE cells to convert all-*trans* retinoid to 11-*cis* retinal in the reconstruction process of photopigments in the photoreceptor cells, which completes the visual cycle[6] (Fig. 2b). Therefore, mutation in the RPE65 gene can cause a generation of non-functional RPE65 protein that will not be able to regenerate 11-*cis* retinal, and causes genetically heterogeneous and severe visual impairment at birth or during the first month of life. The RPE65 thus causes the disturbances in the visual cycle at this early stage of life.

Another form of severe LCA gene is the retina specific guanylate cyclase-1 (GUCY2D), which encodes guanylate cyclase-1 (GC-1) in the photoreceptor cells that convert GTP to cGMP and control the cyclic nucleotide-gated (CNG) ion channels in the photoreceptor cell plasma membrane[28, 30]. Therefore, GC-1 plays a vital role in the visual photo-transduction process and conversion of electromagnetic radiation to chemical signals. The mutation in

this gene causes malfunctioning of GC-1 protein, which impairs the production of cGMP that leads to a permanent closure of the cGMP gated channel.

Proto-oncogene tyrosine-protein kinase (MER)[31] is a transmembrane protein that is encoded by the MERTK gene in humans. This protein has an intracellular tyrosine kinase domain. The extracellular signaling domain of this protein remains on the apical portion of RPE cells[32]. MERTK signaling plays an important role in the daily clearance of shed photoreceptor outer segment debris by RPE phagocytosis and survival of photoreceptor cells of the retina[32]. Therefore, it helps to inhibit the intracellular antigen-induced inflammation and autoimmune responses. It is observed that 0.6% of LCA is caused by the mutation of this MERTK gene, which encodes non-functional MER protein. Mutation in this gene causes the reduction of phagocytic activity of RPE cells that results in the accumulation of photoreceptors shed in the subretinal space of the retina. As a consequence, the subretinal debris generates inflammation and autoimmune diseases and blocks the oxygen and nutrition supply to photoreceptor cells. These detrimental effects lead to rapid loss of vision due to photoreceptor degenerations.

### 2.5. Choroideremia

Choroideremia (CHM), an X-linked inheritance, was found in 1871 by Mauthner, and is the leading rare inheritance with a prevalence of 1 in 50,000 patients[33]. This disease causes a progressive degeneration of the choroid, retinal pigment epithelium, and retina that leads to loss of peripheral vision (night blindness) followed by loss of central vision in middle age. Female carriers have a rare progression of degeneration, but the males are affected by severe damage of RPE cells and choriocapillaries that develop night blindness during adolescence and complete loss of visual acuity by late adulthood. Choroideremia is caused by a mutation in the CHM gene that encodes ubiquitously expressed Rab-escort protein-1 (REP-1)[33]. REP-1 plays a key role in intracellular trafficking of vesicles to the cellular compartments. The mutation in the CHM gene remains null most of the time, and absence of this protein results in the severe effect of trafficking of the opsin protein to photoreceptor outer segment, migration of melanosome to the apical part of RPE cells, and phagocytosis activity of RPE cells.

### 2.6. Diabetic retinopathy (DR)

There are 93 million people suffering from diabetic retinopathy (DR) worldwide[34]. Diabetes can cause damage to the retinal blood vessels that feed the retina. The leaky blood and other fluids can cause thickening and swelling of the retina[35]. Fluid is accumulated by chronic high blood sugar levels, which cause blurred vision. Hyperglycemia is assumed to be the main reason for microvascular complications in DR, where generation of reactive oxygen species, vascular growth factor, and increase in vascular permeability play a vital role. Retinal vasculature plays a supporting role in the health of retinal neuronal and glial cells, and they are degenerated as the microvascular complications begin. DR can sometimes be controlled if the blood glucose level is stabilized, but not in all cases. For example, DR associated with gene alteration may not always be controlled by the long-term management of blood sugar by using insulin therapy. Currently, multidisciplinary strategies are being evaluated, such as laser photocoagulation, anti-vascular endothelial growth factor (anti-

VEGF), and intravitreal steroid therapies. With the progression of DR, proliferative DR generates as the retinal blood vessels proliferate towards the retinal cells and vitreous. This leads to the generation of new blood vessels and results in vitreous hemorrhage, retinal detachments, and increase in the permeability of retinal blood vessels.

### 2.7. Age-related macular degeneration (AMD)

Symptoms of age-related macular degeneration generally present around age 60, and are caused by damage to the macula, according to a National Eye Institute (NEI, USA) report 15 million Americans and millions of people around the world are affected by this devastating retinal disorder. Macular degeneration does not affect the majority of patients until old age, and it is therefore difficult to study the sequential pattern in family members. There are two forms of AMD; one is “DRY” (non-nonvascular) and the other is “WET” (neovascular) AMD. The majority of AMD presents in dry form. Dry AMD is associated with the deposition of yellowish lipid proteins known as drusen under the retina that develop slowly and lead to gradual loss in central vision. Dry AMD can progress to geographic atrophy or the more devastating wet form. In wet AMD, an abnormal angiogenesis quickly leads to the choroidal neovascularization (CNV) within the retina and degenerates photoreceptor cells in the macula. This is responsible for 90% of AMD related blindness. There are some treatments available for wet AMD that involve inhibiting the growth of new blood vessels. Several delivery approaches of anti-angiogenic drugs like bevacizumab (trade name: Avastin) or ranibizumab (trade name: Lucentis) have been studied to inhibit vascular endothelial growth factor A (VEGF-A), which is responsible for the proliferation and growth of new blood vessels [36]. There is no specific gene candidate established for AMD, and thus gene therapy remains an unpredictable therapeutic approach so far.

### 2.8. Glaucoma

Glaucoma is the second leading cause of blindness in the world. Overall, 9% to 12% of blindness is attributed to glaucoma. Damage to the optic nerve causes irreversible dystrophy in the eye, leading to blindness[37]. Increased intraocular pressure (IOP) is a key risk factor associated with this disease. However, this is not a guaranteed cause of glaucoma, as it is observed that 25% of glaucoma patients do not have elevation in their IOP[37]. In the anterior chamber, the aqueous humor forms by the ciliary body and is removed by the trabecular meshwork outflow pathways. IOP is based on the rate of removal of this aqueous humor from the interior chamber, which under normal conditions remains balanced with the rate of formation. The loss of retinal ganglion cells (RGCs) leads to damage in the ganglion cell axons, which finally degenerates the optic nerve. There are several therapeutic approaches developed so far to reduce the IOP by using drugs or surgery. The neuroprotection of these RGC cells is established as another well-studied therapeutic approach using different neuroprotective agents.

## 3. Ocular gene delivery

The success of retinal gene therapy primarily depends on the efficiency of the delivery vehicle to the targeted cells. The monogenic nature of retinal diseases is the basis for using the gene replacement therapeutic strategies. Two main approaches have been shown to be

promising for the delivery of therapeutic genes to the targeted cells. One approach is viral vector, and the other is a non-viral synthetic cargo-based gene delivery vehicle.

The targeting and delivery efficiency of the viral vectors depends on the vector serotype and nature of targeting tissues. Different types of viral vectors, including adenovirus, adeno-associated virus (AAV, a non-pathogenic), and lentivirus, were demonstrated to be efficient for the retinal tissues in *in vivo* animal models and *in vitro* tissue cultures. To date, viral vectors are among the most commonly used vectors for gene therapy. Among these, AAVs (Fig. 3) are currently the most frequently used viral vectors and AAV2 is the most widely used AAV serotype[38]. Significant progress has been made using viral vector for gene targeting. For example, in 2001, the successful viral gene replacement therapy using AAV was carried out in three Briard dogs with RPE65 mutation[39]. Delivery of the MYO7A gene (~9kb), packaged in AAV2 and AAV5, was injected into the subretinal space of the *shaker 1* mice of a USH1 model[40]. By splitting into two separate AAV virions that contained splice donor and splice acceptor independently, they were able to efficiently express large MYO7A cDNA (~6.7 kb) *in vitro* and *in vivo* [40]. The use of EIVA (equine infectious anemia virus) lentivirals mediated delivery of large wild type ABCR genes (~6.8kb) into the subretinal space of $ABCR^{-/-}$ mice, which increased the transduction efficiency of both rod and cone photoreceptor cells and decreased the toxic A2E levels in RPE cells[41, 42], etc. However, the packaging capacities of AAV and lentivirus vectors are limited to ~5 and 9 kb, respectively. This size capacity restricts their delivery efficiency for large therapeutic genes. Therefore, this can also limit the delivery of large genes which might be composed of non-coding elements (e.g. intron) required for the improvement of *in vivo* gene expression[43]. In addition, the major drawbacks are their potential immunogenicity, carcinogenesis, broad tissue tropism, and genomic insertional mutagenesis that generate severe patient outcomes.

Based on these disadvantages of viral vectors, synthetic non-viral gene delivery and replacement methods have been evaluated as promising gene delivery alternatives. This synthetic field has several important advantages, which include nonimmunogenecity, ease and low production costs, simplicity in manipulating molecular structure according to requirements, and most importantly, safety to the genome. Therefore, this area of research has been growing as an attractive and opportunistic field for the development of promising synthetic lipid based liposomes, polymers (linear and branched dendrimers and polysaccharides), and polypeptide based gene carriers. Next, we will review recent progress for using non-viral nanoparticles (NPs) to carry out gene targeting in ocular cells.

### 3.1 Lipid based liposomes vehicles

Lipid based liposomes are widely applied as non-viral gene delivery vectors. It was first discovered in the 1980s when phosphatidylserine phospholipid was utilized to compact and deliver SV40 DNA to the monkey kidney cells[44]. The constituent lipid molecules are composed of a cationic or neutral or zwitterionic head group, a hydrophobic tail group, and a linker group in between them. When these lipid molecules come into contact with negatively charged DNA, they form a complex (lipoplex, schematically presented in Fig. 5a) with the dimension of 100–300 nm. The shape and overall charge of the complex depends on the structure of the lipid and conditional adjustments. The overall charge of the lipoplex remains

positive, which helps it to interact with the cell surface, followed by the fusion with cell membrane and internalization into the endosomal compartment[45]. To enhance the NP stability and transfection efficiency, a neutral/zwitterionic lipid such as fusogenic phospholipid DOPE (1, 2-dioleoyl-3-phosphatidylethanolamine) or membrane component cholesterol are introduced in the lipoplex formulation (Fig. 4a). DNA gets released into the cytosol generally via an endosomal escape pathway.

For the first time in 1996, the beta galactosidase reporter gene, under the control of cytomegalovirus (CMV) promoter, was administered in the adult Wistar rats using three cationic liposomes via topical, anterior chamber, vitreous, and subretinal space routes[46]. The three liposomes were TMAG (N-(triethylamminoacetyl)-didodecyl-D-gluamate), DDAB (dimethyldioctadecylammonium bromide), and DC-Cholesterol (3-beta [N-(N'-N'-dimethylaminoethane)-carbamyl] cholesterol) (Fig. 4a). The gene was expressed in the RGCs on topical administration using TMAG and DC-cholesterol, but not by using DDAB up to 1 month. The TMAG liposome directed delivery of the gene and exhibited its highest level of expression among all three liposomes, irrespective of administrative route. The major problem associated with this technique was that the expression pattern was seen only in RGCs and RPE cells, whereas no expression was found in photoreceptor cells.

In 2005, Kachi and colleagues showed that the LacZ gene under the control of vitelliform macular dystrophy 2 (VMD2) promoter transduced to the RPE cells by using commercial 40% lipofectamine 2000 (Lf)[47]. Use of another lipid carrier, NeuroPorter, was tolerated well[47]. At two weeks, the scotopic a- and b- wave electroretinography (ERG) values were reduced by 40% and 8%, respectively, after subretinal injection of Lf in adult bulb/c mice. On the other hand, after 10% of NeuroPorter injection in the subretinal space, normal retinal morphology and functions were revealed for up to 14 days, and expressed in the RPE cells without any noticeable toxicity to retinal cells. Due to phagocytic activity of RPE cells, the transfer of reporter or therapeutic genes using lipid complex (lipoplex) is feasible (Fig. 5a), and may be a future therapeutic approach for RPE cell-related human ocular diseases.

More recently in 2014, Puras and colleague showed that a novel noisome composed of cationic lipid 2, 3-di (tetradecyloxy) propan-1-amine with polysorbate 80 additive, could deliver pCMS-EGFP plasmid DNA into the rat retina[48]. Injection into the subretinal space transduced both photoreceptor and RPE cells. Intravitreal injection expressed a uniform distribution of the reporter EGFP gene throughout the inner retina. Also in 2014, a novel strategy was discovered when liposome-proteamine decorated with cell-penetrating nuclear localizing signal peptides entrapped functional DNA and expression in the RPE cells[49]. So far, all of these ocular gene therapeutic approaches are limited to proof-of-principle steps and in the near future we can expect more novel lipid based formulations for human use.

### 3.2 Polymer based vehicles

A cationic polymer combines with negatively charged anionic DNA and forms a polyplex (Fig. 4b) of different surface charges[45]. The well-known polymers used for ocular gene delivery are composed of biopolymers (proteins), dendrimers, polysaccharides, polylysin, polyethyleneimines and small organic biocompatible lactic acid (e.g. PLA) and/or glutamic acid (e.g. poly (lactic-co-glycolic acid) molecules (Fig. 4b). In 2013, Puras and colleagues

evaluated the gene delivery efficiency of low molecular weight oligochitosan (Fig. 4b) and pCMS-EGFP polyplex *in vitro* and *in vivo*[50]. This polyplex protected plasmid DNA from nuclease degradation and transfected well to the ARPE 19 cells at pH 7.1. Subretinal injection expressed EGFP to the RPE cells, while intravitreal injections exhibited transfection to the inner nuclear layer, plexiform layer, and primarily to RGCs in rat retina.

In 2014, Mitra and colleagues synthesized and characterized an ethylene glycol modified chitosan (glycol chitosan, GCS, Fig. 4b) and pCBA-EGFP polyplex that protected plasmid DNA from nuclease, which expressed reporter EGFP gene in the RPE cells when injected into the subretinal space of adult Balb/c mice without affecting the morphology and function (using ERG) of retinal cells[51]. In 2012, Klausner and colleagues evaluated the enhancement of transgene expression using chitosan DNA nanoformulation on topical administration on the rat cornea[52]. At 1 day post injection of chitosan DNA complex, it was observed that expression of CpG free pCpG-Luc plasmid DNA enhanced by 7.1, 116.8, and 76.8 folds, compared to commercially available gWiz-CMV-Luc, pPEI-CMV, and pPEI-UbC plasmid DNAs respectively, and demonstrated the development of effective vectors for corneal gene therapy[52]. Another biopolymer gelatin has also been investigated to deliver and significantly express mucin MUC5AC (responsible for dry eye syndrome) transgene into the cornea and conjunctiva *in vivo*[53]. In 2012, Delgado and colleagues reported the ocular gene therapy using solid lipid NPs (SLN) composed of biocompatible dextran, protamine, and a plasmid DNA (pCMS-EGFP or pCEP4-RS1)[54]. Dextran is a neutral nonionic polysaccharide and combines with cationic polymer to entrap, stabilize, and protect negatively charged DNA. On topical administration of SLN to the rat eye, reporter EGFP gene was expressed in the cornea and provided a strategic opportunity to deal with various ocular surface diseases[54].

In 2014, Alqawlaq and colleagues demonstrated the localization of Cy5 labelled pCMV-GFP plasmid DNA into the nerve fiber layer of the retina by intravitreal administration using Gemini surfactant, whereas the same happened to anterior chamber tissues including limbus, iris, and conjunctiva on topical administration to the 4 week old C57BL/6 mice[55]. Intravitreal route of injection is promising, as this reduces the induction of photoreceptor cell degeneration seen during subretinal injections. However, this route possesses huge viscosity, and thus provides resistance to the mobility of cationic polyplexes (Fig. 4b). Therefore, when the polyplexes are strategically coated with the hyaluronic acid (HA, component of vitreous matrix), they are less aggregated and able to flow more easily through the vitreous space. Most recently in 2015, Martens and colleagues have demonstrated that in an *ex vivo* experiment where cationic N, N′-cystaminebisacrylamide-4-aminobutanol (p (CBA-ABOL) vector was coated with the HA, there was a significant amount of enhancement in the gene expression in the retina via intravitreal route of administration [56].

Albumin, a widely used protein carrier, is retained with a high percentage (60–70%) among all the proteins in the vitreous compartment [57]. Therefore, this biocompatible and biodegradable component is safe to use for *in vivo* ocular gene therapy. In 2007, Mo and colleagues found enhanced Cu, Zn superoxide dismutase (SOD1) gene expression by 5 fold, compared to the untreated control via intravitreal injection in the mouse eye when entrapped in human serum albumin NP (HSA NP)[58]. In another separate study in 2009, Kim and

colleagues evaluated the potential of anionic HSA NP as a promising gene delivery tool for RPE cells via the subretinal space[59]. A category of highly branched nanodimension molecules made of central core, interior branches, and exterior surface functional groups, known as dendrimers, were also evaluated as potential gene delivery vehicles for *in vitro* and *in vivo* models. When dendrimer is composed of a high number of surface amine functional groups, it condenses negatively charged DNA and protects it from external nuclease degradation. Polyethylenimine (PEI) and polyamidoamine (PAMAM) are commonly used dendrimers for retinal gene delivery applications due to their "proton sponge" mediated endosomal escape attitude. Oligonucleotide polyethylenimin (ODN/PEI) complex has been observed to be efficient in transfecting retinal glial cells at 72 hours after intravitreal injection in normal rat eyes without any detectable toxicity [60]. In another attempt, PEI condensed shRNA plasmid DNA to target melanopsin in Balb/c mice, and was able to knock down melanopsin in RGC cells at 16 hrs of intravitreal injection, which lasted for 2 months[61]. In 2015, Mastorakos and colleagues showed the combined effect of hydroxyl-terminated PAMAM and triamcinolone acetonide (TA) in enhancing transfection of dendrimer-gene complex into the most challenging human RPE cells *in vitro*[62]. In 2012, Sunshine and colleagues synthesized a novel poly (β-amino ester, Fig. 4b), which exhibited the expression of reporter GFP gene into the RPE and choroid at post injection of 72 hrs via subretinal delivery[63]. In 2004, Marano and colleagues evaluated for the first time the intravitreal delivery of anti-vascular endothelial growth factor (VEGF) oligonucleotide (ODN-1) into the rat eyes using the lipophilic amino-acid dendrimer, which significantly inhibited laser-induced choroidal neovascularization (CNV) for 4–6 months by 95% in the initial stage of CNV development, thereby paving the way for the treatment of angiogenic eye disorders [64].

Polycationic compounds (Fig. 4b) are generally limited by their high positive charge and significant toxicities. These toxicities are strategically eliminated by encapsulating them in conjunction with DNA into the neutrally charged (at physiological pH) biocompatible and biodegradable poly (lactic-co-glycolic acid) (PLGA) molecules. PLGA has been widely used for drug delivery and approved by both the US Food and Drug Administration (FDA) and the European Medicine Agency. In 2010, Zhang and colleagues developed hypoxia-inducible factor 1α (HIF-1α) shRNA and GFP co-expressed plasmid DNA-loaded PLGA NPs (pshHIF-1α NPs) [65]. The result showed that the intravitreal injection of these NPs reduced the mean area of CNV in the rat laser photocoagulation model without any deleterious effects on the retina. Another study by Bejjani and colleagues showed the expression of reporter RNFP (red nuclear fluorescence) in RPE cells up to 14 days post intraocular injection without any apparent structural damage or toxicity[66].

### 3.3 Polypeptide based vehicles

In the pipeline of developing gene delivery vehicles, polypeptide based systems are evaluated as a promising tool for ocular gene delivery. A novel cell penetrating peptide, peptide for ocular delivery (POD, $CGGG(ARKKAAKA)_4$), was able to transduce GFP protein under the control of CMV promoter, which was expressed into the RPE and photoreceptor cells via subretinal injection in C57BL6/J mice[67]. On intravitreal injection, the POD compacted GFP plasmid DNA (POD-GFP) transduced retinal ganglion cells, with

limited expression in the inner nuclear layers and lens capsule[68]. The topical administration of POD-GFP expressed GFP protein in the corneal epithelium cells. In 2014, Read and colleagues demonstrated that the pegylation of POD peptide was able to increase pCAGLuc expression by 215-fold, compared to naked plasmid DNA in the RPE cells by subretinal injection in Balb/c mice[69]. These PEG-POD NPs were also able to protect photoreceptor cells by delivering glial cell line-derived neurotrophic factor (GDNF) into the subretinal space of the blue light-induced photoreceptor apoptosis adult murine model at the 14$^{th}$ day post light treatment[70]. Based on polylysin and DNA compaction strategy, a novel cationic CK30-PEG polymer[71] was developed to compact plasmid DNA to form an NP formulation (Fig. 5b) that could transfect therapeutic genes to the retinal cells in different mice models. The shape of these NPs determines the targets in retinal tissues[71]. The rod shaped NPs transfect the RPE and photoreceptor cells, while ellipsoidal NPs transfect only RPE cells *in vivo*. The major advantage of these NPs is the compaction efficiency of plasmid DNA with long molecular range (from 5.3 kb to 20.2Kb), while in all cases the charge of the NPs remains neutral, minimizing toxicity[72]. Subretinal injection of these NPs didn't leave the eye, compared to the AAV mediated ocular gene delivery approach. No apparent toxicity to the retinal cells was seen, which makes this NP a promising alternative as a non-viral gene delivery vehicle to ocular tissues[72]. The CK30-PEG compacted GFP reporter gene (driven by CMV promoter) was able to highly express to RPE and photoreceptor cells by subretinal injection, while intravitreal route of injection exhibited GFP expression mainly in ganglion cells along with less amounts in the cornea, trabecular meshwork, and lens. The CK30-PEG compacted NPs highly expressed human RPE65 gene driven by VMD2 promoter (RPE cell specific) in the RPE cells of RPE65$^{-/-}$ mice model of LCA disease, with long term phenotypic rescue (up to 2 years)[73, 74]. Human photoreceptor cell specific ATP-binding cassette transporter gene (ABCA4) gene was expressed by subretinal injection of this compacted NP (by CK30-PEG polypeptide), under the control of IRBP promoter (photoreceptor cells specific), into the retina of the ABCA4$^{-/-}$- knockout mice model of Stargardt's disease, with a persistent expression of up to 8 months post injection[75]. A significant amount of retinal degeneration slow (RDS) gene expression with partial rescue of photoreceptor cell function was observed when compacted with CK30-PEG and delivered into the subretinal space of Rds$^{+/-}$ mice [76]. This CK30-PEG has not shown any apparent toxicity towards retinal cells, even with a second set of subretinal injection of NPs[77], and can drive gene expression levels on the same scale and duration as AAV[78]. These NPs have so far been evaluated as a promising ocular gene delivery vehicle, and can be the potential tool for ocular gene delivery with some modifications in their chemical structures to target primary and secondary retinal neuronal cells.

## Conclusion

Despite this promising non-viral delivery vehicle development for ocular tissues, achievements primarily have been limited to transfection efficiency into RPE cells, but not towards photoreceptor and other neuronal tissues in the eye, which is the origin of major retinal disorders. Polypeptide-based vehicles have demonstrated some success, but rational chemical modifications of these compounds might develop smart gene delivery cargos as a

promising alternative to viral vectors and are very appealing for human ocular gene therapy in the near future.

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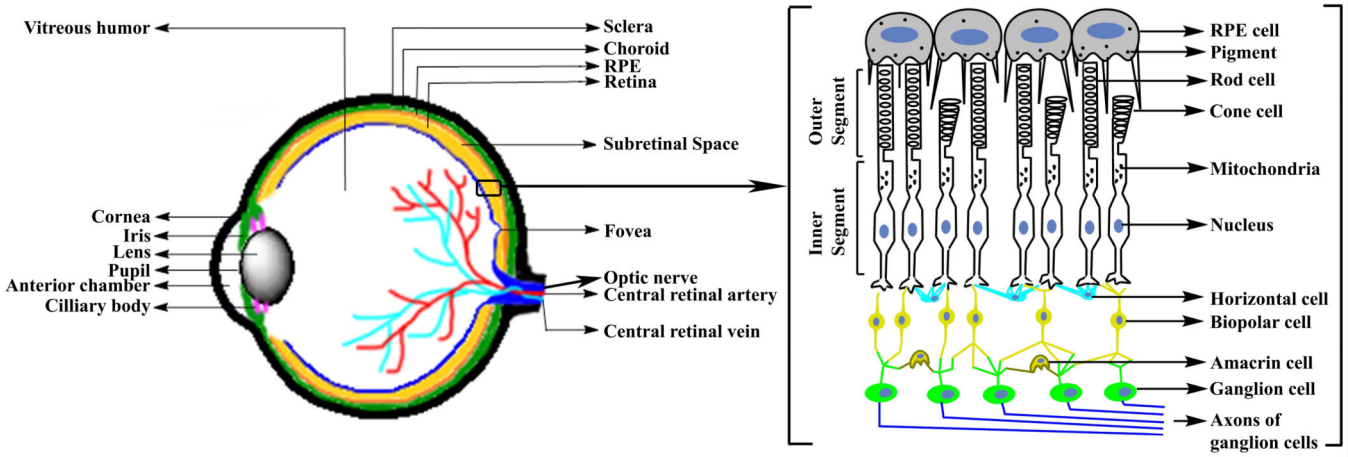


**Fig. 1.**
Diagrams of vertebrate eye (left) and the retina (right).

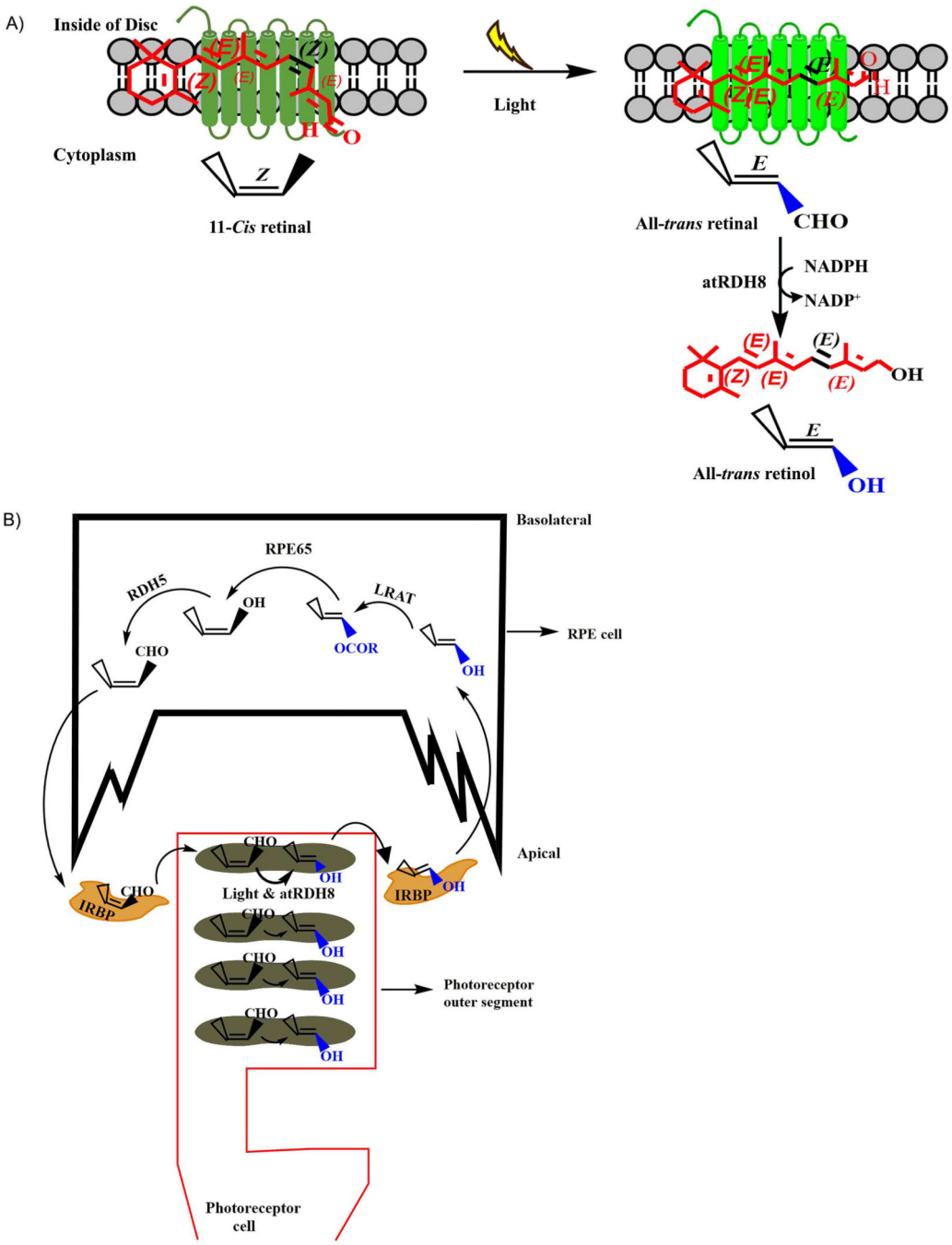


**Fig. 2.**

**A)** Photo activation leads to an isomerization of 11-c*is* retinal molecule to all-*trans* retinal (atRAL) in rhodopsin (conjugation of rod opsin + 11-c*is* retinal) pigment in outer segment disc. The atRAL is now converted to all-trans retinol (atROL) by all-*trans* retinol dehydrogenase (atRDH8) and initiates visual photo transduction processes. **B)** Simple representation of the visual cycle in vertebrate eye. In rod cell, retinal chromophore (11-*cis* retinal) binds to the rod opsin protein and forms GPCR. Absorption of light causes activation of this photopigment, and leads to isomerization of the 11-*cis* retinal to atRAL

that is subsequently reduced by atRDH8 to atROL in photoreceptor outer segment. This atROL is now transferred to RPE cells via IRBP carrier enzymes, where it is esterified to long-chain fatty acids (all-*trans* retinyl esters) by Lecithin retinol acyltransferase (LRAT). All-*trans* retinyl esters are then enzymatically isomerized and hydrolysed to the 11-*cis* retinol by retinal pigment epithelium-specific 65 kDa (RPE65) isomerohydrolase. This 11-*cis* retinol is then finally converted to 11-*cis* retinal, a universal chromophore for visual pigment, by 11-*cis* retinol dehydrogenase (RDH5), and is consequently shuttled back to photoreceptor cells by IRBP to reconstitute rhodopsin pigment in photoreceptor disc, where it completes the visual cycle.

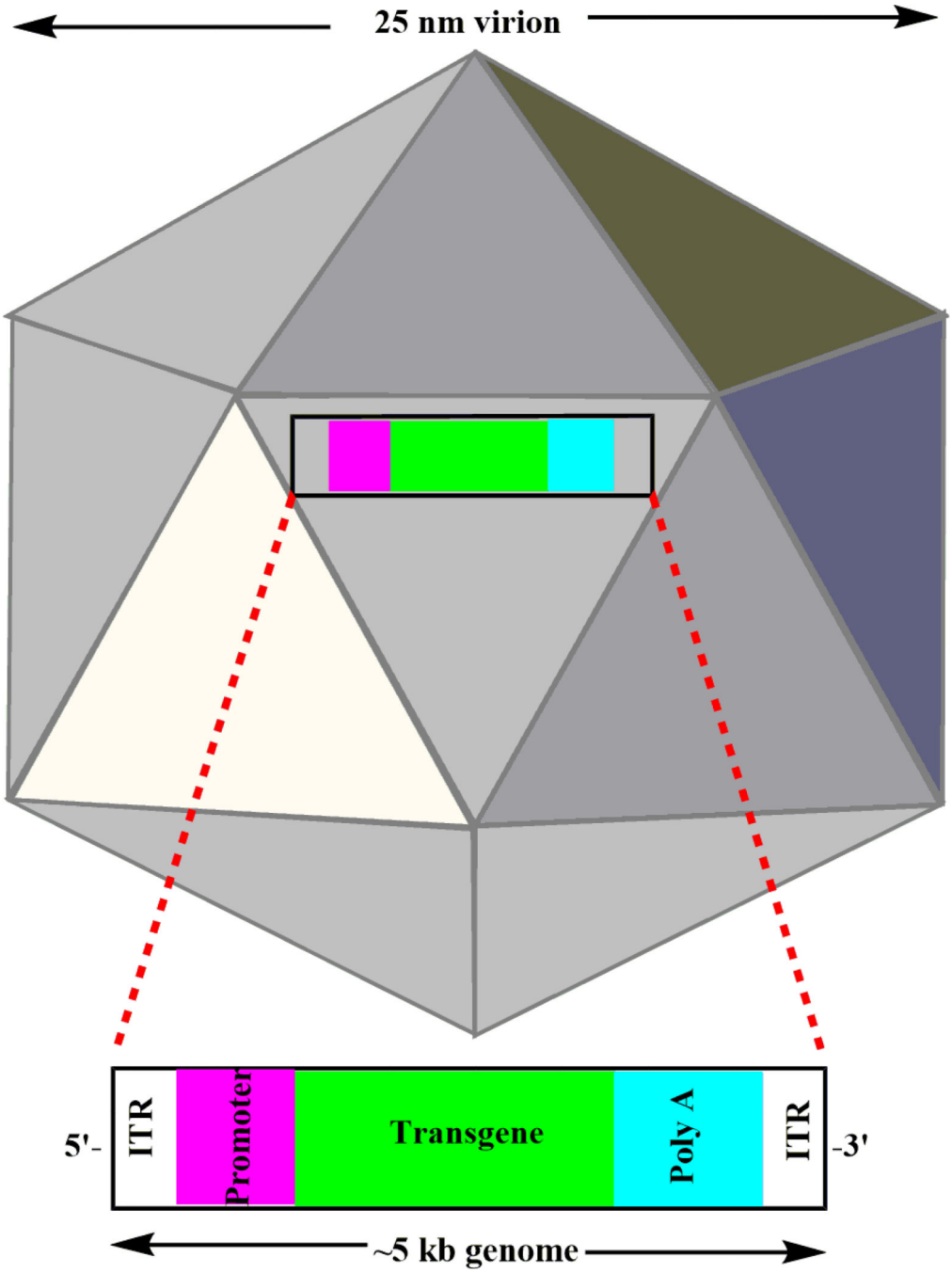


**Fig. 3.**

Representative 25 nm icosahedral capsid of AAV virion. The ~5 kb AAV genome is packaged within the non-enveloped capsid. A gene of interest is inserted between the ITRs under the control of promoter at upstream. ITR: Inverted Terminal Repeat.

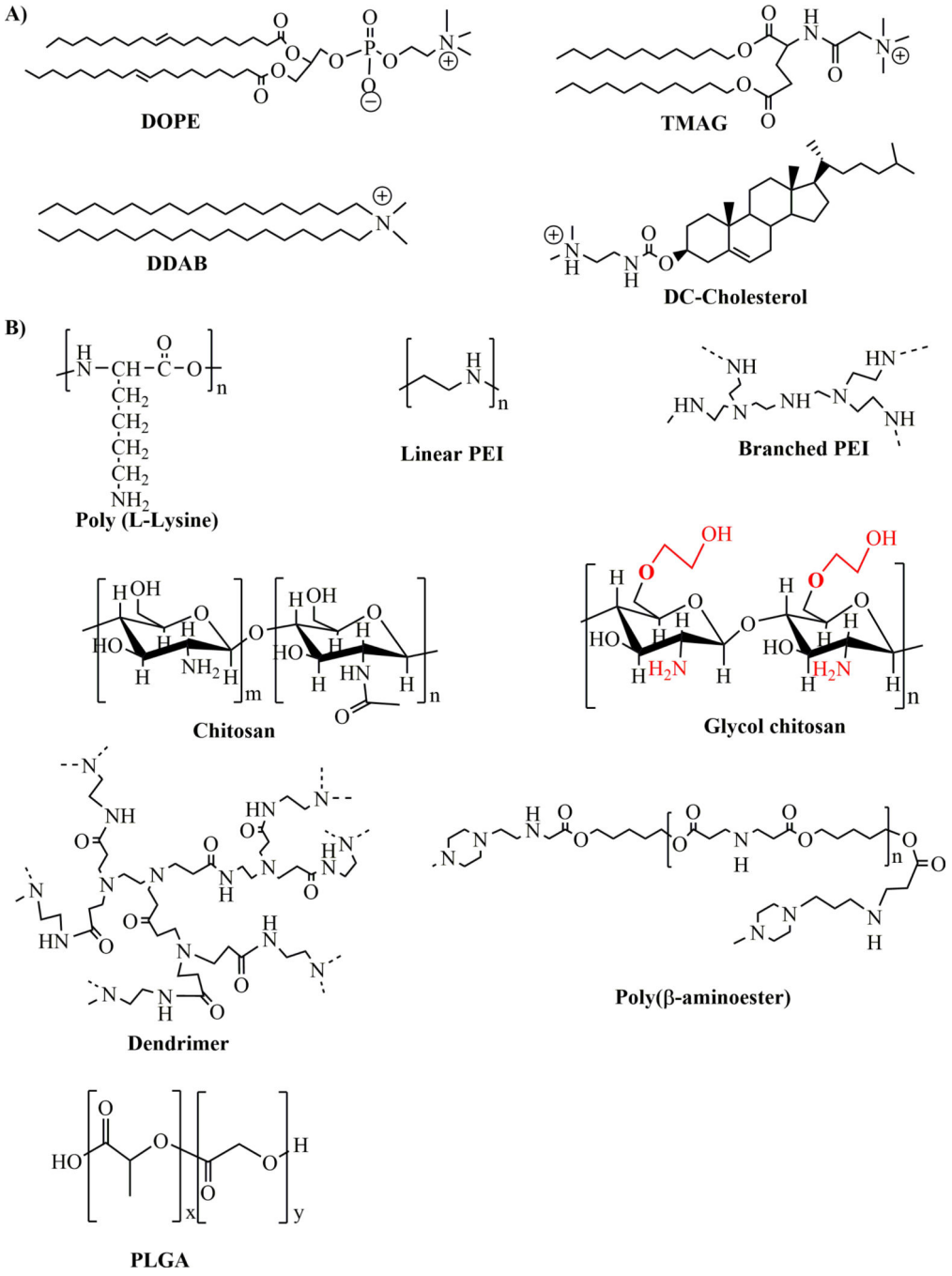


**Fig. 4.**

Chemical structures of commonly used non-viral compounds for ocular gene delivery. **A)** Chemical structures of lipid based compounds and **B)** chemical structures of some frequently used polymeric compounds.

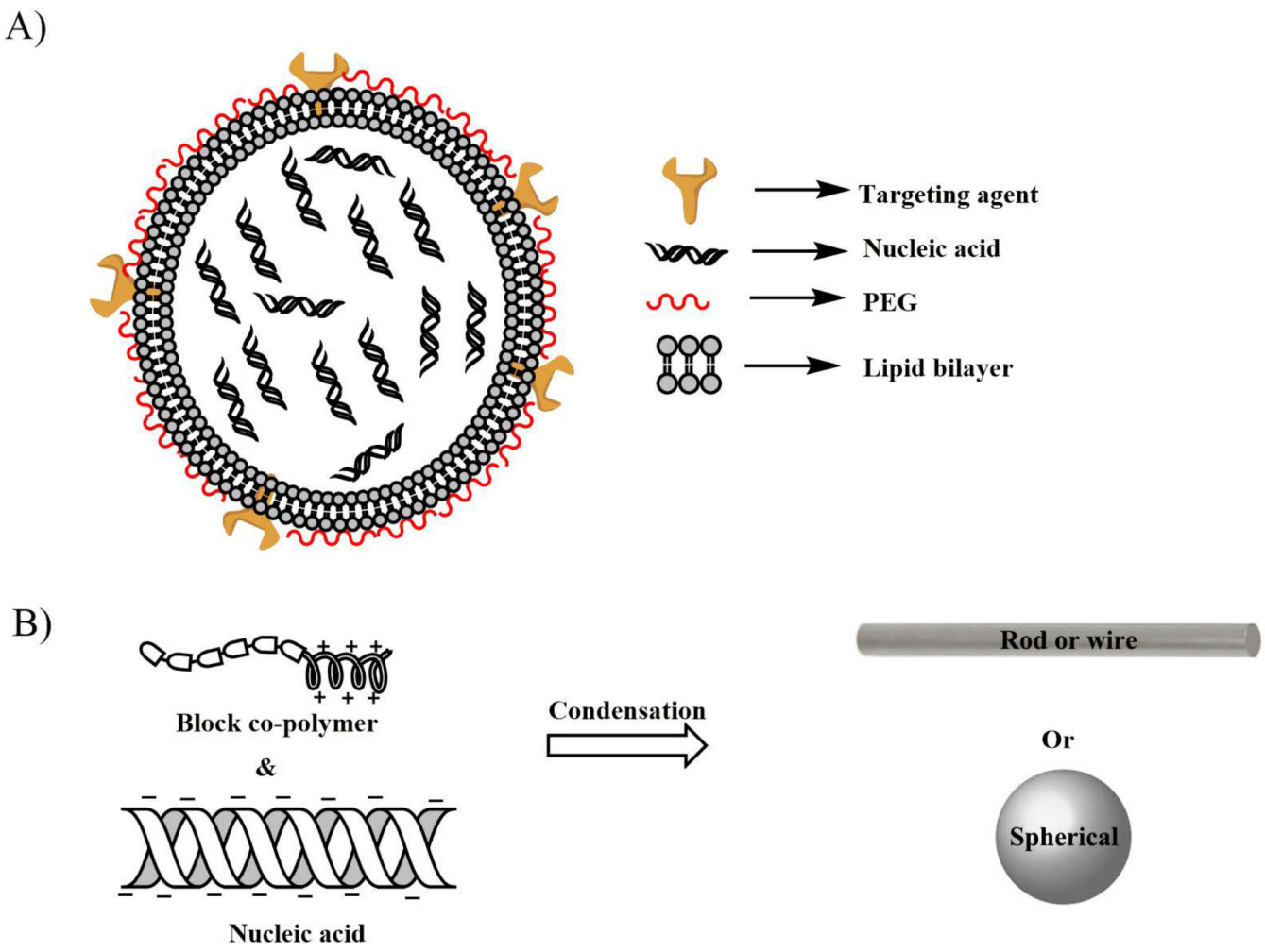


**Fig. 5.**

Simple representation of **A)** lipoplex, where lipid molecules can form bilayer structures and are thus able to encapsulate hydrophilic nucleic acids inside the nanoparticle core. The lipid coating can be used with different targeting agents. The polyethylene glycol (PEG) can also work as a shielding element to protect the nucleic acids from harsh extracellular and intracellular nuclease, as well as lysomal environments, and **B)** polyplex, where morphology of the nano-composites depend primarily on the chemical structure and charge of the constituent polymer compound (s). The negatively charged nucleic acids and positively charged polymers (via electrostatic interactions) constitute the compacted charge-neutral DNA nanoparticles. The PEG block shields the nanocompactions and protects it from nuclease and other degradative pathways.