Gene delivery to the retina: focus on non-viral approaches

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For more than two decades, gene therapy has sought to treat diseases with a genetic component. The eye is a promising target organ for gene therapy because of its unique features like easy accessibility and convenient methods of direct assessment of visual function as an effect of therapy. Several retinal diseases have been linked to specific genes in combination with environmental factors and hence gene therapy offers hope for a long-term cure for them. Developing novel non-viral routes for delivering therapeutic genes to the retina is emerging as an important area of drug delivery research. In this review, we focus on different non-viral vectors for gene delivery to the retina, the barriers that such delivery systems face and methods to overcome them.

Introduction

Gene therapy, which involves intracellular delivery of DNA either to block a dysfunctional gene or to deliver a gene as a therapeutic, has huge potential for treating diseases with a genetic component [1]. Since the first gene therapy clinical trial in 1990 [1], the field has opened new prospects as more information on genetic basis of diseases unfurl. In addition to fatal diseases, those that compromise the lifestyle of patients are also being investigated for potential cure through gene therapy. Blinding disorders, like glaucoma, retinitis pigmentosa (RP) and age-related macular degeneration (AMD), have genetic components, either exclusively or in association with environmental factors [2]. These diseases, which together contribute to blindness in more than 25% of the eye-disease-affected population globally [3], are promising gene therapy candidates and current pharmacological management is ineffective for many of them. Management of such diseases also imposes a significant financial burden on the society [4].

The success of gene therapy relies on the efficient delivery of DNA to target cells and achieving optimum long-term gene expression. The eye is an excellent target in this respect because of its unique location as an organ that allows easy accessibility of drugs [5,6]. This allows localized delivery; reducing the requirement of systemic administration of the gene-based drugs and, in turn, the barriers faced by the therapeutic en route to its site of action. Moreover, small targeted tissue volume and low diffusion into systemic circulation ensure that the amount of the therapeutic required is low. Besides, the success of gene transfer can be determined in a non-invasive manner by directly assessing visual function [5].

Gene delivery to different ocular tissues has principally used viral vectors that package DNA efficiently and give high levels of transgene expression. Toxicity, immunogenicity, possible genomic integration, limited size of inserted DNA and difficult preparative procedures can limit the application of viral vectors [7]. Non-viral vectors offer a promising alternative since they are less toxic, less immunogenic and are relatively easy to produce and handle. Moreover, the size of inserted DNA is theoretically unlimited, and it can also be targeted to specific tissues or cells through ligands. Several of them have been shown to be sufficiently successful in gene delivery in different organs and few have been tested in clinical trials [8].

In this review, we discuss the advances in non-viral systems used for gene delivery to the retina. The barriers that these delivery systems face with different methods of administration in the eye are presented. Strategies to overcome these barriers and new non-viral delivery systems being formulated for retinal gene delivery are also discussed.

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1359-6446/06/$ - see front matter doi:10.1016/j.drudis.2008.09.012

Retinal structure, diseases and targets

The retina is the sensory tissue that lines the back of the eye. It consists mainly of three cell types: the photoreceptors, different nerve cells and retinal pigment epithelium (RPE). The ganglion cells, which are the output neurons, lie innermost in the retina, whereas the photoreceptors (rods and cones) are in the outermost layer against the pigment epithelium and choroid (Fig. 1). The cones are concentrated in the macula, the portion of the retina responsible for central and colour vision. The rods, by contrast, are spread throughout the peripheral retina. Different cell types of the retina are affected in different ocular diseases and hence can be suitable targets for therapy (Fig. 1). The retinal ganglion cells, photoreceptors and RPE are the targets in some of the common retinal diseases like glaucoma, RP and AMD [5], as discussed below. The gene therapy approaches for these different ocular disorders would however differ on the basis of the disease aetiology.

Age-related macular degeneration (AMD)

AMD affects the central regions of the retina and choroid with progressive degeneration of RPE eventually affecting the photoreceptors leading to irreversible blindness. Abnormalities are seen in four functionally interrelated tissues: RPE, photoreceptors, Bruch's membrane and choriocapillaries [9]. Interactions among multiple genes and environmental factors seem to contribute to disease pathogenesis. Anti-angiogenesis approaches have been used to treat choroidal neovascularization, a hallmark of one form of AMD in which there is proliferation and growth of choroidal blood vessels into the subpigment epithelial and/or subretinal space. Some attempts at disease intervention target the vascular endothelial growth factor (VEGF) and block its expression. These therapies aim to avoid further vision loss rather than reverse the course of the disease [9].

Retinitis pigmentosa (RP)

RP is a genetically heterogeneous retinal disease that causes progressive degeneration of the photoreceptors [10]. With a world wide prevalence of 1 in 3000 [11], at least 50 different genes have been identified in which mutations can cause different forms of the disease. These genes encode proteins involved in the rod phototransduction cascade, cytoskeletal and structural proteins integral to photoreceptors, signaling and intracellular trafficking proteins [10]. Gene therapy approaches for treatment are dependent on the type of mutation. Loss-of-function mutations typical

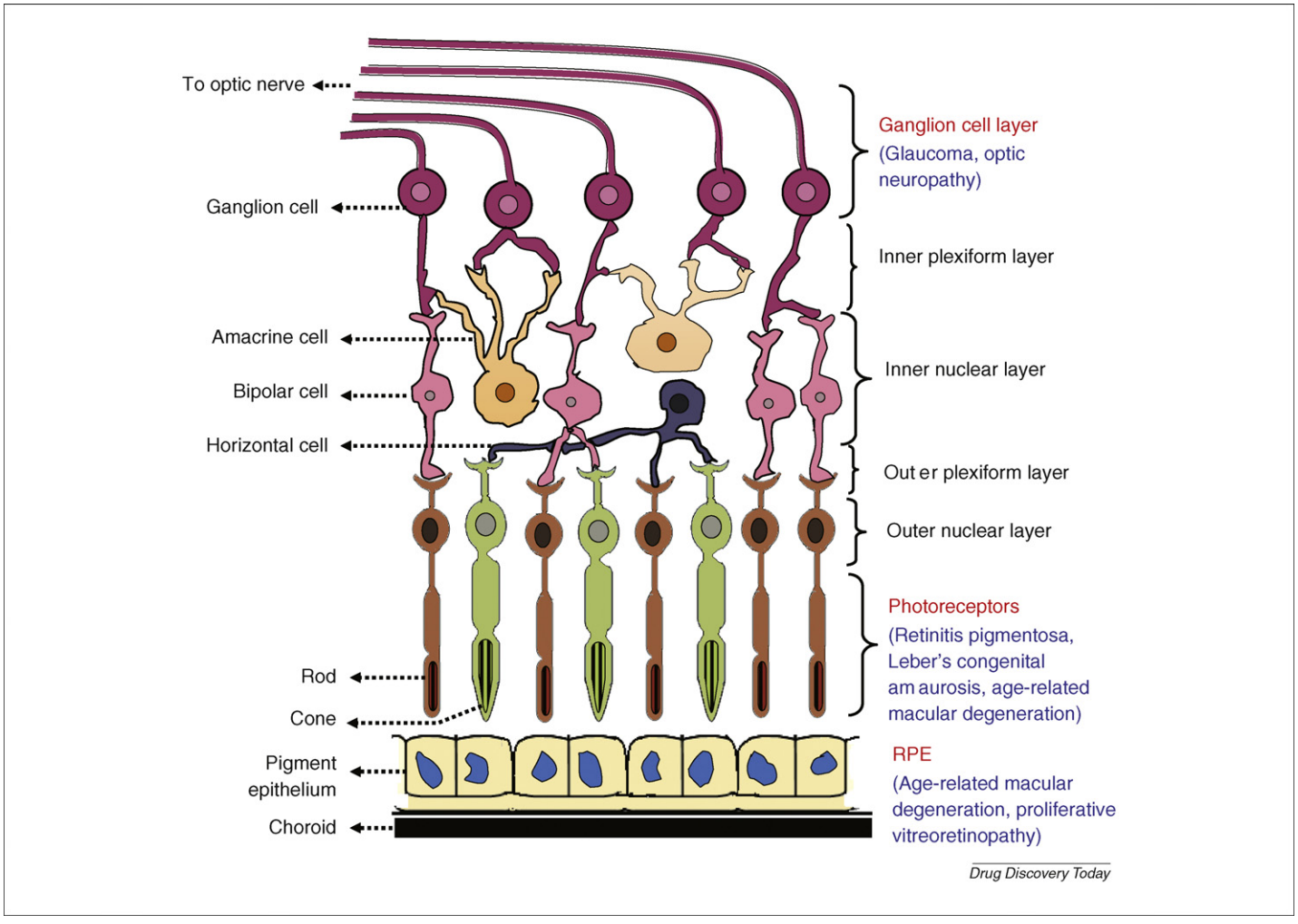

FIGURE 1

Different cell types present in the layers of the retina. The cell types marked in red are targets for gene delivery. The diseases that affect these cell types are marked in blue.

of recessively inherited diseases require gene-replacement strategies, whereas diseases with dominant negative effect require gene silencing [10].

Glaucoma

Glaucoma is a group of diseases associated with progressive optic neuropathy in which the final common pathologic event is retinal ganglion cell death leading to irreversible visual impairment [12]. Common treatment strategies are aimed at reducing the intraocular pressure, but topical anti-glaucoma medications can cause serious side effects [13]. Additionally, for a large number of patients, these topical drugs fail to maintain lower intraocular pressure over a period of time, leaving filtration surgery (trabeculectomy) as the only choice of treatment. Thus, identification of alternative routes for gene therapy in glaucoma has a major impact on disease management. Targets for glaucoma gene therapy comprise the trabecular meshwork (which maintains the physiological pressure inside the eye), ciliary epithelium, ciliary muscle, retinal ganglion cells and Müller cells [12]. Very few drugs target the trabecular meshwork, hence gene therapy to this tissue is a promising strategy. Even though a number of currently available drugs target the ciliary body, no current gene transfer studies addressing this tissue are available [5].

Vectors for gene delivery

Viral vectors

Gene delivery to different retinal cell types *in vitro* and in animal studies *in vivo* has been attempted mostly using viral vectors (Table 1A). In a significant successful case, recombinant adeno-associated virus (rAAV) was used to transfer RPE65 cDNA to the

TABLE 1

Vectors used to deliver genes to the retina.

Vector	Disease	Gene	Route	Species/Cell type	Reference
(A) **Viruses**					
Adenovirus	Retinitis pigmentosa	PDEβ	Subretinal	Mouse	[52]
		RPE65	Subretinal	Mouse	[53]
		Mertk	Subretinal	Rat	[54]
	AMD	PEDF	Intravitreal or subretinal	Mouse	[55]
			Intravitreal	Human phase I clinical trial	[56]
		Endostatin	Intravenous (tail vein)	Mouse	[55]
	Glaucoma	Stromelysin	Intracameral	Rat	[5]
		RhoA		Perfused human anterior segment culture	[5]
		BDNF	Intravitreal	Rat	[57]
		XIAP	Axotomized optic nerve stump	Rat	[58]
	Diabetic retinopathy	Endostatin	Intravitreal	Mouse	[59]
		sFlt-1	Intravitreal	Rat	[60]
Adeno-associated virus	Retinitis pigmentosa	Peripherin/RDS (prph2)	Subretinal	Mouse	[61]
		prph2 + GDNF	Subretinal	Mouse	[62]
		RPE65	Subretinal	Dog	[14]
				Human phase I clinical trials	[17]
		XIAP	Subretinal	Rat	[63]
		Channelopsin2	Intravitreal	Rat	[64]
		Lrat	Subretinal	Mouse	[65]
		Ribozyme targeting rhodopsin	Subretinal	Rat	[66]
	AMD	PEDF	Intravitreal or subretinal	Mouse	[67]
		Angiostatin	Subretinal	Rat	[68]
		sFlt-1	Subretinal	Rat	[55]
	Glaucoma	BIRC4	Intravitreal	Rat	[69]
		MAP2K1	Intravitreal	Rat	[69]
		CNTF	Intravitreal	Rat	[69]
		BDNF + TrkB	Intravitreal	Rat	[70]
	Diabetic retinopathy	Sflt-1	Subretinal	Rat	[71]
		Angiostatin	Intravitreal	Rat	[72]
Lentivirus	Retinitis pigmentosa	PDEβ	Subretinal	Mouse	[73]
		RPE65	Subretinal	Mouse	[74]
Retrovirus	Proliferative vitreoretinopathy	HSV thymidine kinase	Intravitreal	Rabbit	[5]
		αPDGFR	Intravitreal	Rabbit	[75]

TABLE 1 (*Continued*)

Vector	Disease	Gene	Route	Species/Cell type	Reference
(B) Liposomes					
DOTMA/DOPE DOTMA/Cholesterol	–	Luciferase	Intravitreal	Rabbit	[23]
DOTAP/DOPE/Protamine sulfate	–	Secreted alkaline phosphatase	Transfection	ARPE-19	[26]
Pegylated immunoliposome	–	β-galactosidase with GFAP promoter	Intravenous	Mouse	[47]
		β-galactosidase/luciferase with opsin promoter	Intravenous	Rhesus monkey	[45]
Phospholipid/Cholesterol/PEG-DSPE	–	ODN	Intravitreal	Rabbit	[46]
DOTAP/DOPE/PEG ceramide	–	Luciferase	Transfection	D407	[49]
Artificial viral envelope liposome	–	β-galactosidase, ODN	Intravitreal	Rat	[27]
(C) Polymers					
Lipid-lysine dendrimers	AMD	Anti-VEGF ODN	Intravitreal	Rat	[34]
Human serum albumin nanoparticles	AMD	SOD1	Intravitreal	Mouse	[76]
PLL, PEG-PLL, PLL dendrimers	–	Luciferase	Transfection	D407	[28]
Polyamidoamine dendrimer	–	GFP	Transfection	Human primary RPE cells	[29]
Polyethylenimine	–	shRNA targeting melanopsin	Intravitreal	Mouse	[30]
	Proliferative vitreoretinopathy	Anti-TGFβ-2 ODN	Transfection	Rodent primary retinal Müller glial cells	[33]
			Intravitreal	Rat	
Poly(lactic-co-glycolic acid)	–	GFP, RNFP	Transfection	Bovine primary RPE cells, ARPE-19	[31]
			Intravitreal	Rat	
	–	Anti-VEGF RNA aptamer	Trans-scleral	Rabbit	[32]
Vectosome	–	ODN	Transfection	OCM-1, ARPE-19	[77]
			Intravitreal	Rat	
(D) Peptides					
CK30PEG10K	–	GFP	Intravitreal, subretinal	Mouse	[50]
$GGG(ARKKAAKA)_4$	–	siRNA, GFP	Transfection intravitreal, subretinal	HER 911 Mouse	[51]

(A) Different viral vectors used for gene delivery to the retina ***in vivo*** in animal models. The genes that have been chosen are important in the retinal diseases listed alongside. In addition to genes associated with retinitis pigmentosa, age-related macular degeneration and glaucoma, those associated with other retinal diseases like diabetic retinopathy and proliferative vitreoretinopathy have also been used for *in vivo* delivery. Adenovirus, adeno-associated virus, lentivirus and retrovirus are the most common viral vectors.
(B–D) The major chemical non-viral vectors used for gene delivery to retinal cells *in vitro* and animals *in vivo*. Liposomes (B), polymers (C) and peptides (D) are the most common classes of non-viral delivery systems. Most of them have only been used for reporter gene delivery in different retinal cell lines.
Abbreviations: PDEβ, rod photoreceptor cGMP phosphodiesterase β subunit; RPE65, retinal pigment epithelium-specific protein 65 kDa encoding an isomerase essential for the synthesis of 11-*cis*-retinal; PEDF, pigment epithelium derived factor; BDNF, brain derived neurotrophic factor; XIAP, X-linked inhibitor of apoptosis; sFlt-1, soluble form of VEGF receptor Flt1; GDNF, glial derived neurotrophic factor; Lrat, lecithin:retinal acyl transferase; BIRC4, human baculoviral IAP repeat containing protein 4 (caspase inhibitor); TrkB, receptor tyrosine kinase B; CNTF, ciliary neurotrophic factor; αPDGFR, platelet derived growth factor α receptor; DOTMA, *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride; DOPE, 1,2-dioleoyl-3-phosphatidylethanolamine; DOTAP, 1,2-Dioleoyl-3-trimethyl ammonium propane; GFAP, glial fibrillary acidic protein; PEG-DSPE, 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-[poly(ethyleneglycol)-2000]; ODN, oligodeoxynucleotide; VEGF, vascular endothelial growth factor; SOD, superoxide dismutase; PLL, poly-L-lysine; GFP, green fluorescent protein; shRNA, short hairpin RNA; TGFβ-2, transforming growth factor beta 2; RNFP, red nuclear fluorescent protein; siRNA, small interfering RNA.

RPE65-deficient eyes of Briard dogs. A single subretinal injection led to partial rescue of retinal function and the gene expression was stable for longer than three years without adverse effects [14,15]. This success was translated into three clinical trials in humans [16]. In one of these, three young adults with Leber's Congenital Amaurosis (LCA) were administered subretinal injections of rAAV expressing RPE65 cDNA under the control of a human RPE65 promoter. There were no adverse effects in any of the patients and one patient showed significant improvement in visual function [17].

In spite of significant successes with viral vectors in retinal gene delivery and therapy, particularly in case of retinitis pigmentosa, several chemical non-viral methods are currently being explored. This is because viral vectors suffer from crucial limitations like risk of insertional mutagenesis and adverse immune effects [7]. Non-viral vectors, with the additional advantage of controlled synthesis, offer the scope for improvement in design for more efficient delivery. The most common chemical methods involve formation of liposome–DNA complexes (lipoplexes) and cationic polymer–DNA condensates (polyplexes). Considerable progress has also been achieved in non-viral delivery using physical methods like electroporation [18,19], iontophoresis [19] and ultrasound-mediated gene transfer [20]. Here, we will limit ourselves to only the chemical non-viral methods for retinal gene delivery (see Table 1B–D for a summary of some of the common systems used, details described below).

Liposomes

Liposomes are vesicular lipid systems generated by the self-assembly of amphiphilic molecules like phospholipids and cholesterol.

Liposomes are attractive as sustained delivery vehicles as they are biodegradable and remain localized at the site of administration [21]. Hydrophilic drugs can be encapsulated in the inner aqueous cavity and lipophilic drugs are incorporated in the membrane [22]. Cationic lipids, such as TMAG (N-(α-trimethylammonioacetyl)-didodecyl-D-glutamate), DC-cholesterol (3-β[N-(N′,N′-dimethylaminoethane)-carbamoyl] cholesterol) and DOTAP (1,2-Dioleoyl-3-trimethyl ammonium propane), are often used in liposomes as they bind DNA electrostatically to form stable lipoplexes for gene delivery. In addition, DOPE (1,2-dioleoyl-3-phosphatidylethanolamine) is often used as the neutral lipid in cationic liposomes because of its pH-sensitive ability to destabilize the lysosomal membranes after cellular entry of the lipoplex through endocytosis [23].

Liposomes constitute the most common non-viral method of successfully delivering plasmids and oligonucleotides (ODNs) to the inner retinal layers and the RPE cells *in vitro* and *in vivo*. *In vitro* studies involve understanding the role of lipid formulation in dictating transfection efficiency. For example, transfection efficiency in human primary RPE cells with liposomes is dependent on the type of lipid used. Multivalent cationic lipids with increased hydrophilic character show the best efficiency [24]. Liposomes have also been used for the delivery of antisense oligonucletotides for gene silencing. Jääskeläinen *et al*. [25] investigated the efficiency of delivery of antisense phosphorothioate oligonucleotides into an RPE cell line using different cationic polymeric and lipidic carriers. The antisense effect was seen only with small sized lipid-based carriers with a membrane active component such as DOPE or an added peptide JTS-1 (membrane-active pH-dependent peptide) that releases the ODN through membrane fusion. *In vivo*, intravitreal administration of plasmid DNA complexed with DOTMA (*N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium chloride)/Cholesterol liposomes in rabbits produced transient transgene expression throughout the eye, with a peak level at three days [23]. Further efforts in this area concentrate on improved formulations of liposomes for maximum transfection efficiency along with increased duration of expression.

Other agents have also been used in conjunction with liposomes to improve their transfection efficiency. Protamine sulfate has been shown to enhance liposomal gene transfer *in vitro* and *in vivo*. In differentiated RPE cells *in vitro* lipoplexes of DOTAP/DOPE with protamine sulfate gave higher and more sustained gene transfer than the parent DOTAP/DOPE complexes [26]. In the HVJ liposome method for gene transfer, plasmid DNA and high-mobility group 1 (HMG1) non-histone nuclear protein are co-encapsulated in liposomes that are then coated with the envelope of inactivated hemagglutinating virus of Japan (HVJ). The viral envelope proteins mediate fusion of the liposomes with the cell membrane, and the packaged plasmid DNA is introduced directly into the cytoplasm, bypassing the lysosomal degradation. HMG1 then facilitates the transport of the plasmid DNA into the nucleus. Plasmid DNA and ODN delivery through such artificial viral envelope (AVE)-liposome system to rat eyes with choroidal neovascularization (CNV) by intravitreal injections showed sustained expression of the DNA specifically in the CNV [27].

Polymers

Amine-containing cationic polymers such as poly-L-lysine (PLL), polyethylenimine (PEI) and polyamidoamine dendrimers are capable of condensing DNA and also interact with the negatively charged cell surface molecules to facilitate cell uptake. These systems can also be conjugated with ligands to achieve cell-specific DNA delivery through receptor-mediated uptake.

PLL is the most common polymer and has been used for ocular delivery of plasmid DNA *in vitro*. Shape, molecular weight and architecture of poly-L-lysines control DNA delivery properties in RPE cells *in vitro* [28]. The cellular uptake of PLL is much higher than DOTAP, but other intracellular barriers cause lower transfection efficiency. Polyplex-mediated gene transfer into RPE using the commercially available starburst polyamidoamine dendrimer Superfect has also been successful [29].

PEI has been used to deliver a plasmid encoding an shRNA and a reporter red fluorescent protein to retinal ganglion cells *in vivo* through intravitreal injections. The shRNA was targeted to melanopsin, a photopigment mediating the light response of the chronobiologically relevant ganglion cells. The melanopsin expression in the transfected area was reduced to an undetectable level by the shRNA [30] demonstrating the applicability of PEI for such purpose. Poly(lactic) acid (PLA), poly(glycolic) acid (PGA) and their co-polymer poly(lactic-co-glycolic acid) (PLGA) have also been used for retinal DNA delivery. These nanoparticles can escape the early endo-lysosomal formation during endocytotic delivery and enter the cytosolic compartment by a mechanism of surface charge reversal, thereby improving their delivery efficiency. PLGA nanoparticles have been used to deliver green fluorescent protein (GFP) or red nuclear fluorescent protein (RNFP) encoding plasmids to RPE cells *in vitro* and *in vivo* in rats by intravitreal injection [31]. In bovine and human RPE cells, the transgene expression was seen in 10–20% of the cells, without any toxic effects up to eight days in culture. *In vivo*, a preferential RNFP expression within the RPE cell layer was detected. In addition, PLGA microspheres encapsulating anti-VEGF RNA aptamer were used as a trans-scleral device. The aptamer was delivered in a sustained manner *in vitro*, over a period of 20 days, with retained activity [32].

Polymers have also been used for the delivery of antisense oligonucleotides. Complexes composed of PEI and anti-TGFβ-2 ODN showed higher transfection efficiency in rat retinal Müller glial cells (RMG) when prepared in HEPES buffered saline as compared with complexes prepared in water because of formation of a smaller, non-aggregated core-shell structure in the former. Localization of the complexes was also seen in the inner limiting membrane and the RMG until 3 days after intravitreal administration in rats, demonstrating that the vitreous does not present a barrier to these nanosized complexes [33].

Amphiphilic lipid-lysine dendrimers with a tail of lipidic α-aminocarboxylic acids and a poly-lysine head have also been used to deliver ODN-1 (anti-VEGF) into cultured D407 retinal pigment epithelium cell line [34]. They combine the structural features of both lipids and amino acids and, thus, facilitate transmembrane transportation, act as lipid solubilizers and protect DNA from nuclease digestion. Dendrimers with more number of positive charges were able to achieve higher levels of transfection. Intravitreal injections of the dendrimer–ODN-1 complex in rats were found to significantly inhibit development of choroidal neovascularization for 4–6 months by up to 95% in the initial stages. The ODN was also able to penetrate the entire retinal cell layers to reach the RPE [35].

Barriers for retinal gene delivery using non-viral vectors

Method of administration

Although non-viral formulations for gene delivery to the retina hold a lot of promise, the major impediment of practical application comes from the low concentrations of the formulations in the site of action. This is because the ocular barriers required for the protection and maintenance of ocular functions tend to block entry of pharmaceuticals. The first barrier arises from the method of administration (see Fig. 2).

1. Delivery of gene-based drugs through intravenous administration is associated with poor pharmacokinetics arising out of multiple barriers during passage from the blood. This involves blood ocular barriers like the blood-aqueous barrier (in the anterior part of the eye) and the blood-retinal barrier (in the posterior part) [36]. Hence high doses of the drug need to be administered since the amount of drug finally reaching the retina is a small fraction of the administered amount.
2. Topical administration suffers from limited permeation of the complexes from the ocular surface to the retina, since it involves penetration of the cornea and diffusion through the vitreous to reach the retina against the normal flow of the aqueous. Also, a large fraction of the drug dosage is absorbed into the systemic blood circulation via the conjunctival and nasal blood vessels [37].
3. Intravitreal and subretinal injection have been more commonly used for *in vivo* retinal gene delivery. Direct injection into the subretinal space, although technically challenging, allows for increased contact time between the injected DNA and the posterior retinal layers, but area of contact is restricted only to the injection site. Moreover, there are chances of induction of lesions in RPE cells, limiting its suitability in

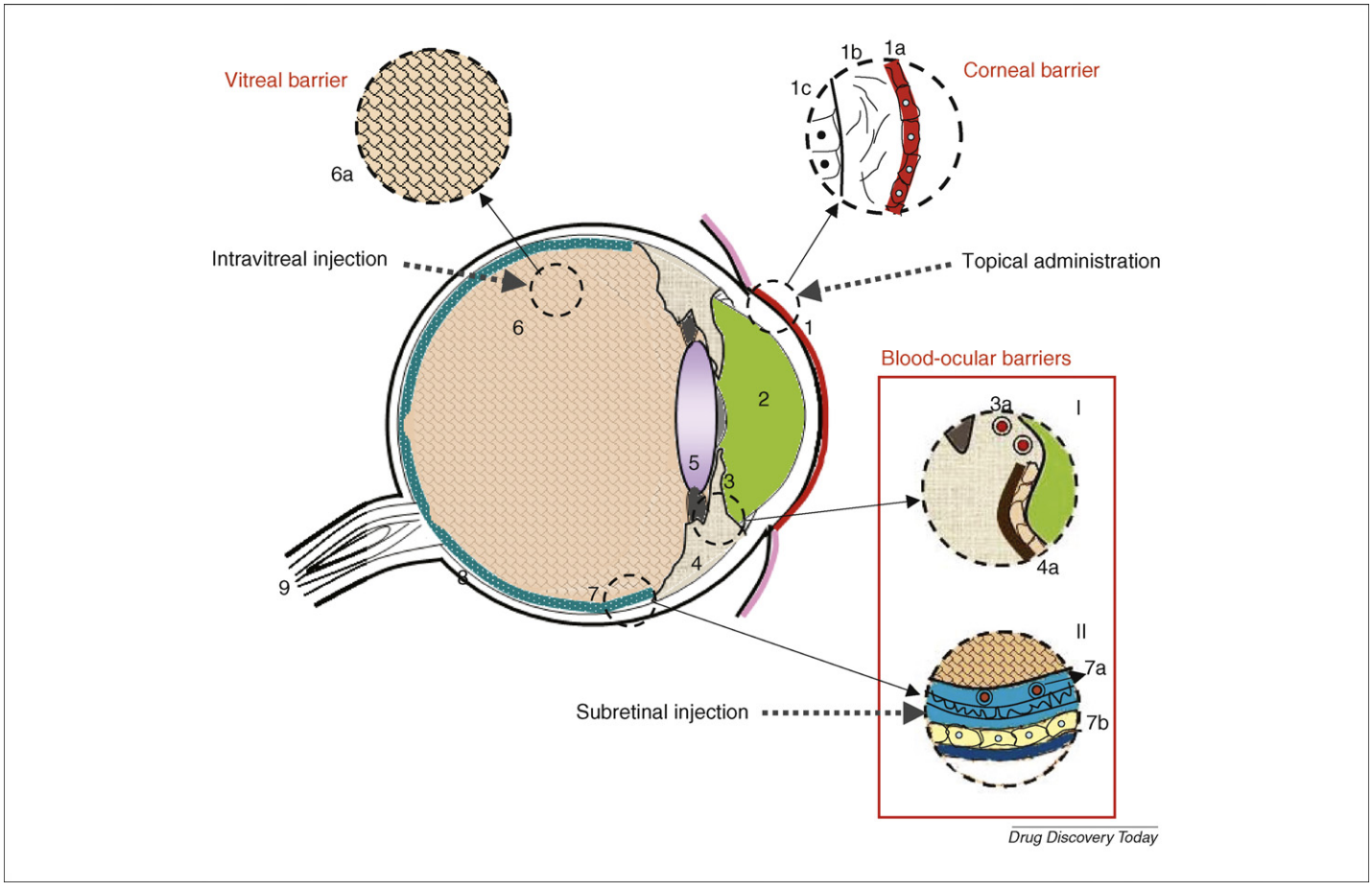

FIGURE 2

Barriers to retinal gene delivery with different routes of administration. The different parts of the eye shown here are: (1) Cornea: 1(a) corneal epithelium, 1(b) Stroma and 1(c) Endothelium; (2) Aqueous humor; (3) Iris; (4) Ciliary body; (5) Lens; (6) Vitreous humor; (7) Retina; (8) Choroid and (9) Optic nerve. The barriers involved with each route of administration are:

- Intravenous administration: This involves blood-ocular barrier composed of the blood-aqueous barrier (I) and the blood-retinal barrier (II). Blood-aqueous barrier is formed by the endothelial cells of the blood vessels in the iris (3a) and the non-pigmented cell layer of the ciliary epithelium (4a). Blood-retinal barrier is composed of the endothelial cells of retinal blood vessels (7a) (inner blood-retinal barrier) and retinal pigment epithelium (7b) (outer blood-retinal barrier).
- Topical administration: Tight junctions in corneal epithelium (1a) impede the permeability of DNA-carrier complex. The passage from the anterior chamber to the posterior chamber is also hindered by the vitreous (6).
- Intravitreal injection: Vitreous is the most important barrier for this route. The three-dimensional network of collagen bridged by proteoglycan filaments and hyaluronan in the vitreous (6a) prevents mobility of positively charged and high molecular weight lipoplexes and polyplexes.
- Subretinal injection: Diffusion across the neural retina and entry into the RPE is affected by the extracellular and membrane associated glycosaminoglycans.

clinical settings. Intravitreal injection is more clinically acceptable since it is comparatively less invasive. By contrast, nucleic acids have very low intravitreal half-lives thereby necessitating repeated intraocular administrations to achieve a continuous presence in the retina, which might lead to lens damage and retinal detachment.

Choosing a mode of administration would also depend on the target cell type. For example, in case of lipoplexes made with DC-cholesterol and TMAG, topical application induced gene expression in the retinal ganglion cells up to 1 month after application, whereas expression in the RPE was observed only with intravitreal or subretinal injection [38].

Vitreous as a barrier

Although intravitreal route of administration is often a preferred mode of drug administration, the passage through the vitreous itself constitutes a barrier in gene delivery. Vitreous is a gel-like material made of an ordered three-dimensional mesh of collagen fibrils bridged by proteoglycan filaments (which contain the negatively charged glycosaminoglycans), several non-collagenous structural proteins and serum components. Collagen (40–120 μg/ml) and hyaluronic acid (100–400 μg/ml) are the two major structural components. The interfibrillar space is filled by a dense network of negatively charged hyaluronan [39] and proteoglycans containing chondroitin sulphate, and possibly heparan sulfate. These glycosaminoglycans (GAGs) are known to interact with polymeric and liposomal DNA complexes (Fig. 3).

The vitreous presents a barrier to gene delivery by: (a) immobilizing the nanocarriers in the proteoglycan filament network [40]; (b) binding of the nanocarriers to the negatively charged GAGs [41], thereby decreasing their zeta potential and inducing aggregation and (c) destabilizing the nanocarriers through GAG interaction that can impede cellular uptake and/or intracellular trafficking.

Other glycosaminoglycans as barriers

In addition to the vitreous, the retina contains a profuse spread of GAGs linked to proteoglycans. The interphotoreceptor matrix (IPM) and the apical surface of RPE cells, along with the nerve fibres, contain chondroitin sulfate proteoglycans. By contrast, heparan sulfate proteoglycans are abundant in the basement membranes of retinal capillaries, Bruch's membrane and the inner limiting membrane. Keratan sulfate proteoglycans are localized in the nerve fibre layer and optic nerve. Similar to the vitreous, extracellular GAGs in the retina can modify the uptake and trafficking of lipoplexes and polyplexes (Fig. 3). This phenomenon is dependent on the nature of both the carrier and the GAG [42]. Sulfated GAGs are more likely to hinder the uptake and gene expression of cationic complexes. Additionally, cell surface GAGs and proteoglycans can act as receptors for lipoplexes and polyplexes but, in the process, they are internalized through different endocytotic pathways leading to variable gene expression [43].

The blood-retinal barrier

The blood retinal barrier (BRB) is formed by the inner barrier constituted by the endothelia of retinal capillaries and the outer barrier composed of the retinal pigment epithelium. Tight junctions in these cell layers impede the penetration of the molecules from systemic blood circulation to the retina and from the vitreous to the blood stream (Fig. 2). RPE and the retinal vessel walls are poorly permeable to small hydrophilic compounds whereas lipophilic substances can permeate more easily.

Many substances are eliminated from the vitreous either by active transport or passive diffusion across the BRB. High lipophilicity of a drug or the presence of an active transport mechanism can lead to rapid transport from the vitreous across the retina into the systemic blood circulation [36]. Therefore, high molecular weight and water solubility tend to prolong the half-life in the vitreous and minimize transport across the BRB [44].

Approaches for improved delivery

PEGylation of the vectors

It has been observed that shielding of the surface of non-viral vectors by polyethyleneglycol (PEG) chains enhances their stability and transport in serum and cystic fibrosis (CF) sputum. This strategy has been adopted for retinal DNA delivery as it can partly overcome the barriers of the vitreous and the cell surface GAGs (Fig. 3). PEGylation has been explored in delivery of lipoplexes to increase the stability in vitreous and systemic circulation, protect the liposome from early degradation and DNA release, minimize reticulo-endothelial uptake and reduce toxicity [45]. ODNs encapsulated in PEGylated liposomes also show increased half-life in the vitreous and decreased release in non-targeted tissues [46]. Varying amounts of PEG attached to cationic liposomes prevented aggregation of the lipoplexes in bovine vitreous and also circumvented binding to the fibrillar structures [40]. A single intravenous administration of plasmid DNA encapsulated in an 85 nm PEGylated immunoliposome (PIL) that is targeted to the photoreceptor layer can produce gene expression in the entire eye in Rhesus monkey up to 14 days [45]. PILs are targeted across the blood-retinal barrier and into ocular cells with a monoclonal antibody against receptors of transferrin or insulin present on retinal cells. The expression of reporter genes is also restricted by the use of retina-specific promoters [47]. PEGylation has even been attempted in the oligonucleotide itself. Pegaptanib (Macugen®), an anti-VEGF aptamer linked to PEG, was the first aptamer therapeutic approved for use in humans for the treatment of choroidal neovascularization associated with AMD [48]. The two monomethoxy-PEG chains protect the 28-mer oligonucleotide from rapid degradation after intravitreal injection.

Nevertheless, the major disadvantage of PEGylation is the lower transfection efficiency of the lipoplexes. PEGylation either decreases the cellular interaction and uptake of the lipoplexes or interferes with their endosomal release. In lipoplexes, the structural transition from a lamellar phase to an inverted hexagonal phase strongly assists in endosomal escape. Incorporation of PEG stabilizes the lamellar phase thereby preventing endosomal escape and reducing transfection efficiency. Reversible PEGylation, that is, exchangeable PEG chains attached to the delivery vehicle, has been utilized to circumvent this problem. For this purpose, PEG-ceramides have been used that easily dissociate from the lipoplex when they come in contact with the cell membrane. They are incorporated by 'post-PEGylation' of lipoplexes and have been shown to give better transfection efficiency than even non-PEGylated lipoplexes in RPE cells [49].

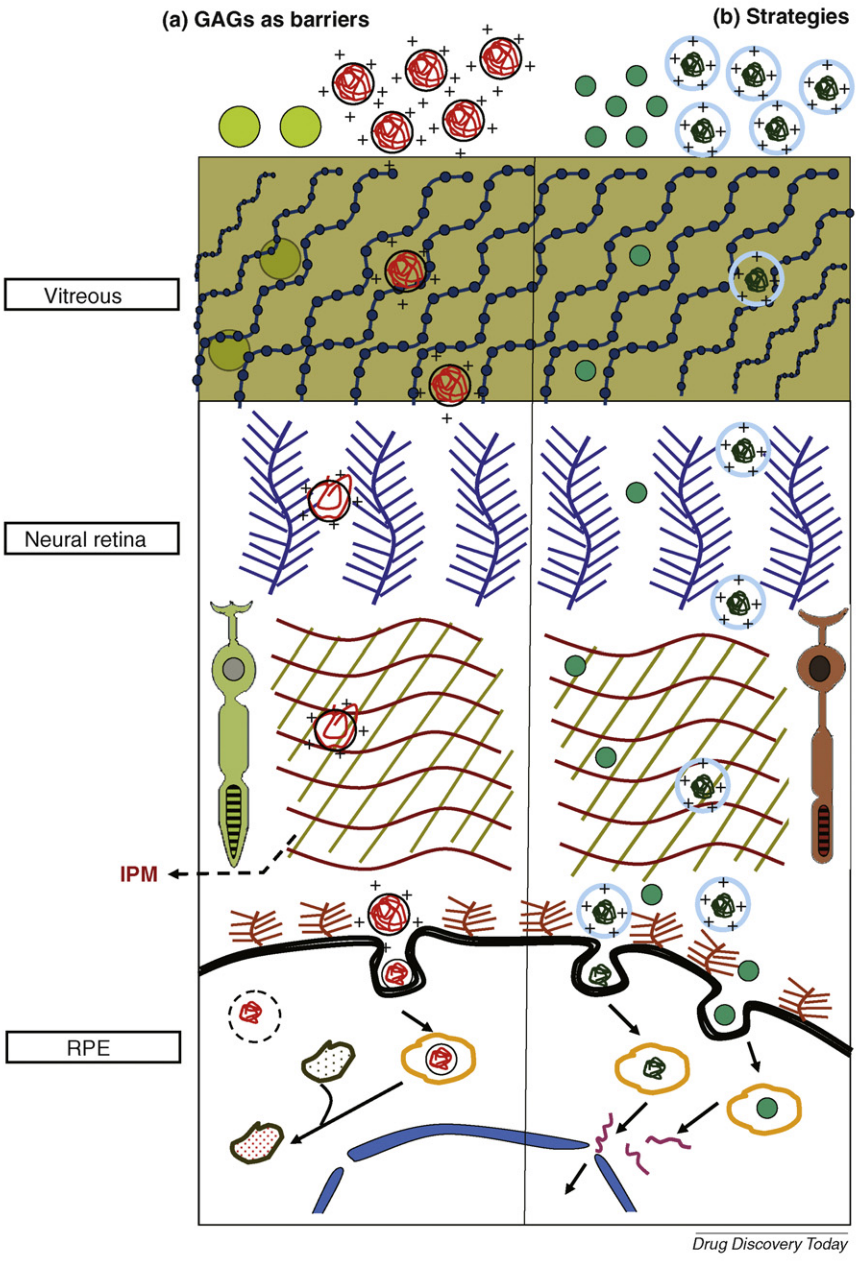

FIGURE 3

Glycosaminoglycans as barriers and strategies to overcome the barriers. **(a)** Glycosaminoglycans (GAGs) in the vitreous and the retina are barriers to gene delivery to the retina. Large molecular weight complexes (●) are immobilized in the vitreous. Complexes with positive surface charge (●) are either immobilized in the vitreous or loosened by interaction with GAGs. If they reach the surface of RPE cells, the interaction with cell surface GAGs leads to internalization by endocytosis but are entrapped within endosomes or degraded by lysosomes. **(b)** Strategies to overcome the barrier of GAGs include low molecular weight complexes (●) which can easily diffuse through the vitreous and masking the surface charge with PEG (●). These complexes can diffuse through the neural retina to reach the surface of RPE. Proteins are not to scale. IPM, interphotoreceptor matrix; RPE, retinal pigment epithelium. (〰) Collagen; (≡, ≈) Chondroitin sulfate proteoglycan; (≡) Keratan sulfate proteoglycan; (///) Hyaluronan; (○) Endosome; (𝄞) DNA.

Peptides for ocular gene delivery

Recent studies show that cationic peptides hold some promise in retinal gene delivery. Compacted nanoparticles of plasmid DNA with PEGylated lysine peptides have been used to transfect post-mitotic cells efficiently *in vitro* and *in vivo* [8]. Small size (10–20 nm), allowing easy diffusion through the vitreous (Fig. 3), makes these systems very attractive for future applications. CK_{30}PEG10K, a 30-mer lysine peptide with an N-terminal cysteine conjugated to 10 kDa polyethylene glycol has been used for plasmid DNA delivery to different ocular tissues in mice using acetate or trifluoroacetate as the lysine amine counterion [50]. Intravitreal injections caused strong gene expression in the inner retina and in ocular tissues near the vitreous, but the levels of expression varied among different sites depending on the chemical nature of the

nanoparticles. Similarly, subretinal injection resulted in substantial transfection of the photoreceptor layer and RPE for selected formulations. Moreover, delivery of these compacted nanoparticles did not show any toxicity or decrease in retinal function.

Cell penetrating peptides (CPPs) that show high translocation across the cell membrane can also be fine-tuned for efficient gene delivery in the retina. Such novel peptides developed specifically for ocular delivery are likely to add to the repertoire. Termed as PODS (peptide for ocular delivery), these molecules show rapid cellular entry in a temperature dependent manner and get localized to RPE, photoreceptor and ganglion cells [51]. These can compact DNA into nanocarriers and achieve transgene expression in human embryonic retinoblasts. The choice of a peptide for optimal delivery, however, still remains a challenge. The varied chemical nature of the CPPs (from strongly positive to highly amphipathic) makes peptide design difficult. Peptides containing positive residues like arginine have been stated to enhance delivery across plasma membrane through hydrogen bond formation between the guanidine group and the surface molecules but this might get masked when the CPP is already complexed with DNA. Moreover, the nanoparticles formed at high peptide:DNA charge ratios are likely to interact strongly with the GAGs leading to their destabilization in the vitreous or on the retinal surface. A possible alternative might be the use of peptides that undergo a change in their secondary structure on binding to the GAGs for facile movement through the vitreous leading to unhindered cellular entry.

Modification of promoters for increasing duration of expression

Temporary expression of genes owing to silencing of the promoter has been reported in many instances [50]. Increasing the duration of expression by using minicircle plasmids in which all bacterial sequences are eliminated has shown great promise in gene delivery to the liver, where expression persisted for several years. Such approaches are being followed in nanoparticle-mediated retinal gene delivery also with high transfection efficiency [8].

Future

While recent trends in the literature show that non-viral chemical routes for retinal gene delivery are being developed, most of the systems chosen for delivery are not specially designed to overcome the retinal barriers. Since barriers for retinal gene delivery are unique, novel strategies that can circumvent those barriers need to be explored. Such systems should be characterized *in vitro* in conditions that can simulate the barriers and also in specific cell lines for different retinal cell types. These efforts will help in modulating the chemistry of the carrier and in developing vectors fine-tuned for retinal delivery.

Acknowledgements

The authors would like to thank Council of Scientific and Industrial Research (CSIR), India for research support. RN acknowledges research fellowship from the Department of Biotechnology, India.

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