Molecular Vision 2006; 12:1334-47 <http://www.molvis.org/molvis/v12/a150/>
Received 12 October 2005 | Accepted 13 September 2006 | Published 30 October 2006

Review

Ocular gene therapy: A review of nonviral strategies

Charlotte Andrieu-Soler,[1] Riad-Antoine Bejjani,[1] Thérèse de Bizemont,[1] Nadia Normand,[1] David BenEzra,[2] Francine Behar-Cohen[1,3]

(The first two authors contributed equally to this publication)

[1]INSERM, U598, Paris, [2]Hadassah Hebrew University Hospital, Jerusalem, Israel, [3]Rothschild Ophthalmic Foundation, Paris, France

Along with viral vectors, non-viral strategies have been developed in order to efficiently deliver nucleic acids to ocular cells. During the last decade, we have observed that the outcome of these non-viral delivery systems depends on the genetic material used, the targeted tissue or cells, the expected effect duration, and the routes of administration. Assessment of efficiency has been evaluated in normal eyes or in animal models of ocular diseases. The chemical and physical methods that have been adapted for the delivery of nucleic acids to ocular tissues are highlighted and discussed in this review. Also, the results obtained with different non-viral strategies from their initial conception to their present development are summarized. At the present, selective targeting of ocular tissues and cells can be achieved using the most yielding route of administration to the eye in combination with an appropriate drug delivery technique.

The eye is a choice organ for local gene therapy. The tissue volume to be treated is small, the needed therapeutic concentration of drug relatively low and the diffusion of active products from the eye into the circulation minimal. Furthermore, the eye benefits from a relative immune privilege, minimizing the potential immune and inflammatory reactions that may follow the intraocular injection of foreign antigens.

Viral vectors such as adenovirus, adeno-associated virus (AAV), and retroviruses have been used for ocular tissues transfection of DNA and have yielded high levels of therapeutic protein expression in ocular cells and tissues [1-5]. Recently, visual function was restored to RPE65$^{-/-}$ dogs, a model for Leber's congenital amaurosis (LCA), using recombinant AAV carrying wild-type RPE65 gene [2]. Following these outstanding and encouraging results, clinical trials for the treatment of humans using adenoviral vectors are being carried out [6]. However, the potential dangers of viral vectors may hamper their further development for ocular gene therapy in humans [7]. Most blinding ocular diseases are not life-threatening. Therefore, the use of potentially harmful viral vectors is questionable.

Non-viral vectors have potential advantages. Most of them are safe and non-toxic to ocular tissues. The major drawback of non-viral vectors is their low transfection rate and relatively short life. These shortcomings are being overcome by the use of slow release devices and repeated treatments as needed.

Correspondence to: Francine Behar-Cohen, INSERM U598, Institut Biomédical des Cordeliers, 15 rue de l'Ecole de Médecine, 75006 Paris; Phone: 331 40 46 78 46; FAX: 331 40 46 78 55; email: behar@idf.inserm.fr

Many methods of nucleic acids delivery to ocular cells have been investigated. To enhance nucleic acids stability, favor their intracellular internalization and allow their slow release lipids, peptides, and various polymers (poly-L-lysine, poly-ethylenimine, polylactides chitosan, gelatin, dendrimers, alginate and cyanoacrylates) have been used as "carriers." Liposomes, lipoplexes, polyplexes, nanoparticles, microparticles, or a combination of these have been preferentially investigated. To enhance nucleic acid delivery to cells, adjunct techniques such as electric current (iontophoresis and electrotransfer), high hydrostatic pressure, or ultrasound (sonophoresis) have been applied also.

The particular characteristics of the target tissue (or cell), the specific therapeutic material to be used, and the expected beneficial outcome, determine the selection of the most appropriate route of delivery. For the eye, topical instillation, periocular or intraocular (intravitreal or subretinal) injections are the most widely used. Systemic administration, on the other hand, is seldom considered due to the presence of blood-ocular barriers and the potential secondary side effects.

For a more practical approach, we have reviewed the various nonviral gene delivery strategies and their outcome according to the route of nucleic acids administration to the eye.

Stability of oligonucleotides in different ocular tissues and media: The biodistribution of oligonucleotides (ODNs) after their delivery to ocular tissues has been previously investigated in vivo by various groups, but a few studies have evaluated the stability of ODNs in ocular tissues and media [8-10]. Using a heterogeneous competitive hybridization assay, Bochot et al. showed that, in the vitreous, free 16 mers phosphodiester ODNs were not stable. Only 0.7% of the injected ODNs re-

mained intact 24 h after administration [9]. Chemical modification of ODNs, encapsulation, or formulation in various compounds (lipids, polymers, peptides) can increase their stability in vivo [8-10]. After intravitreous injection, the pharmacokinetic distribution of anti-CMV (cytomegalovirus) phosphorothioate (PS) ODNs has been asssessed in the monkey before its clinical application [10]. The findings of this study are detailed in the intravitreous section of this review. It has to be stressed that drug pharmacokinetic distribution in vivo need to be extrapolated from many experiments as the stability and distribution of the studied molecule can be influenced by many factors.

To our knowledge, the stability of nucleic acids in the presence of ocular tissues in vitro has not been studied previously. To specifically analyze the nuclease activity of ocular tissues and media and eliminate the in vivo clearance and distribution factors, we tested ODN stability after its in vitro incubation in the presence of various ocular tissues and fluids. We evaluated the degradation rate of a 53-mer ribozyme in the presence of isolated mice ocular tissues in vitro and after its application on the ocular surface in vivo. As shown in Figure 1, in vitro degradation of ribozymes was rapid in the presence of ocular tissues. In these experiments, ribozyme degradation was more rapid in the presence of posterior segment tissues (Figure 1B,C) than in the presence of anterior segment tissues (Figure 1D,E,F). Noteworthy is the observation that, in the presence of vitreous, no intact ribozyme was found after 10 min of incubation (Figure 1H). These results demonstrate that a high nuclease activity is taking place in the vitreous. Similar results were found with other types of nucleic acids injected into the vitreous of rabbits [9], or monkeys [10]. These findings explain the low stability of naked ODN after

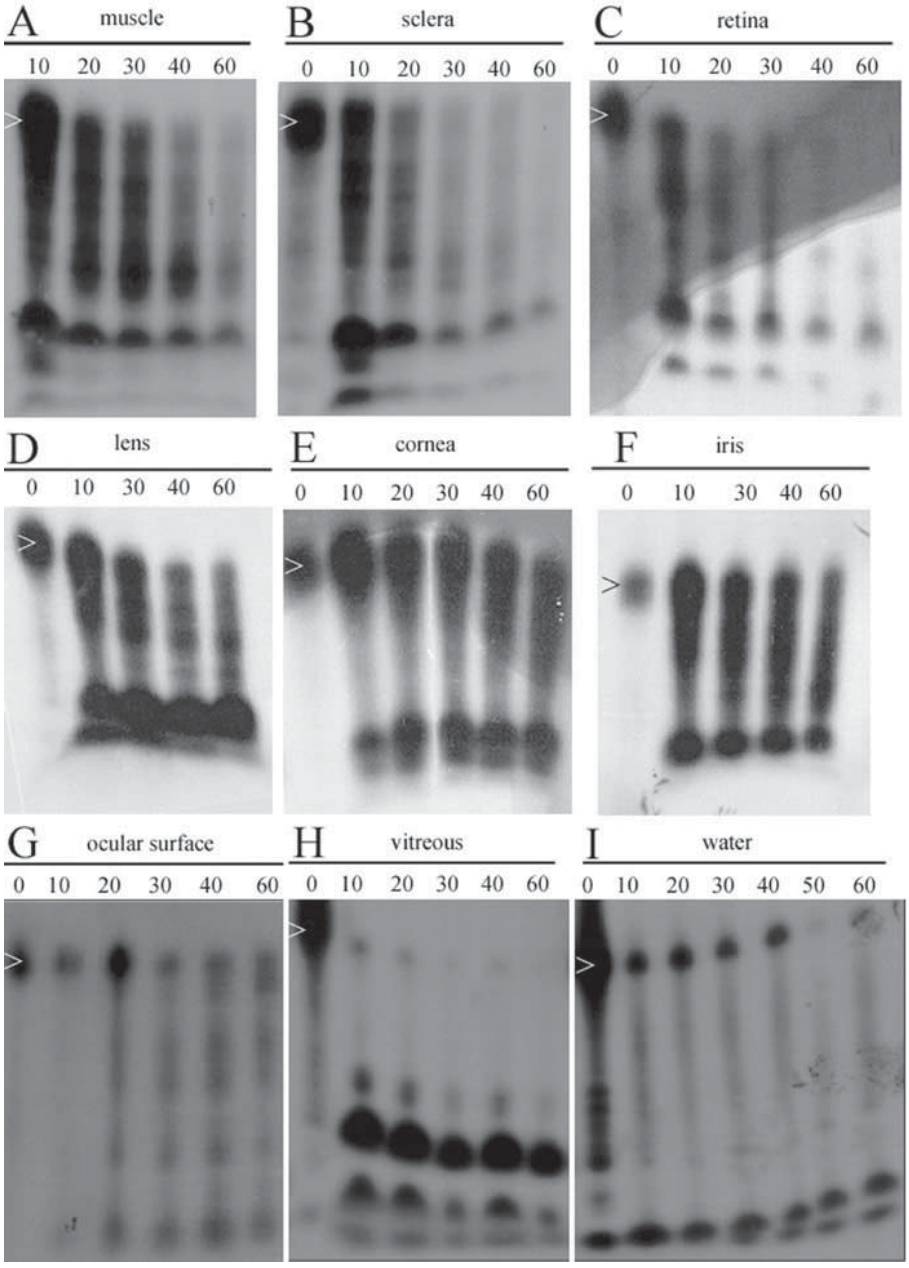

Figure 1. Degradation rate of a 53-mer ribozyme in mouse ocular tissues after application to the ocular surface. Ten week-old C57Bl6 mice were used for these experiments. After sacrifice, eyes were enucleated, tissues (cornea, iris, lens, ocular muscles, retina/choroid, and vitreous) were dissected and collected. Then 1.7-2 pmol of ^{32}P-labeled ribozymes were mixed with the tissues or in bidistilled water at 37 °C for different periods of time (10, 20, 30, 40, 50, 60 min, n=4 per timepoint). To evaluate the degradation rate of nucleic acids in vivo, 2 pmol (in 20 µl) of radiolabeled ribozymes were instilled on the eye surface of anesthetized mice (n=2 per timepoint). At various timepoints after instillation the eye surface was rinsed with 120 µl of bidistilled water and collected. The ribozymes were then extracted as described in reference [73] for the extraction of ODNs from ocular tissues, and their migration was observed on denaturating acrylamid gel. ^{32}P-labeled ribozymes were mixed with muscle (**A**), sclera (**B**), retina (**C**), lens (**D**), cornea (**E**), iris (**F**), ocular surface (**G**), vitreous (**H**), or water (**I**) at 37 °C for different periods of time: 10, 20, 30, 40, 50, 60 min, n=4 per timepoint. White arrows indicate full length ribozyme.

its injection into the vitreous. During incubation with sclera (Figure 1B) or exposure to the ocular surface tissues (Figure 1G), intact ribozyme molecules were still detected after 20 and 60 min, respectively. These results indicate that topical or transscleral administration of unprotected nucleic acids would be more appropriate than intravitreous delivery. Thus, if delivery by the vitreous route is to be chosen, methods of protection against nucleic acid degradation and retinal diffusion facilitation have to be used.

Topical instillation of nucleic acids: Instillation of an active compound is the first choice method of delivery for ocular therapy. However, due to the innate protective characteristics of the eye against the entry of foreign compounds (tear drainage and corneal epithelium barrier), the bioavailability of an instilled compound is generally low. To overcome this limiting factor, frequent instillations or the use of specific formulations which increase the corneal contact time have been developed. Despite these improvements, after instillation, drug levels within the posterior segment of the eye are, in most instances negligible.

Naked plasmids: The fate and biodistribution of an instilled plasmid has not been studied, but its indirect biological effect (as immunomodulation) shows that local as well as systemic expression of the encoding protein of interest occurs. The type and location of transfected cells remain unclear.

Several reports have demonstrated that viral infection is decreased by instillation of plasmids encoding for immunomodulator protein genes. In a model of immune-mediated herpetic stromal keratitis, a single topical administration of naked plasmid encoding interleukin 10 (IL-10) significantly reduced the extent of corneal lesions [11-14].

Topical instillation (or subconjunctival injection) of plasmids may also have "vaccine" potentials. Topical (or subconjunctival) administration of plasmid encoding for herpes simplex virus (HSV) glycoprotein D or for a chimeric construct of glycoprotein D- and IL-2-induced cellular and humoral responses against the virus [15,16]. However, this mode of administration is not efficient for transfection of intraocular cells.

Naked oligonucleotides: Following instillation to the rabbit eye, a 16-mers oligothymidylate DNA ODN remained localized in the conjunctiva, corneal epithelium, and sclera. Only negligible levels were found in the iris [17]. Similarly, a 21-mers DNA ODN topically applied in the rat eye remained localized on the corneal epithelial surface [18].

Plasmids in liposomes: Complex of nucleic acids with lipids facilitates their compaction, increases their lipophilicity and enhances their intracellular delivery. The topical instillation of liposome carrying *lacZ* plasmid in rat allows β-galactosidase expression in retinal ganglion cells (RGCs) [19]. Similarly, β-galactosidase encoding plasmid in liposome preparations TMAG (N-(α-trimethylammonioacetyl)-didodecyl-D-glutamate) or DC-cholesterol (3-β[N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol) induce a specific gene expression in corneal and, conjunctival epithelial cells as well as in RGCs, for up to 1 month [20]. The mode and mechanism of liposome penetration from the ocular surface to the retina remains enigmatic.

Oligonucleotides in liposomes: Interestingly, using liposomes to deliver ODNs yielded different results when these were instilled locally. Formulated liposomes suspension or dispersed within a poloxamer gel, resulted in a low transfection rate which was limited to the superficial layers of the conjunctiva and cornea epithelium [17]. Therefore, if intraocular tissues are targeted, liposomes are not appropriate potential carriers of oligonucleotides through topical delivery.

Plasmids in polyplex: Repeated instillations of plasmids encoding gene formulated in nonionic copolymeric micelles, resulted in transient specific gene expression in the iris root, sclera, conjunctiva and lateral rectus muscle of rabbit and nude mouse eyes. In these experiments, no gene expression was detected in the retina [21]. Thus, this type of topical formulation may be considered for the targeting of ocular surface tissues and possibly also the tissues within the anterior chamber angle including the trabecular meshwork, the iris root, and the ciliary body.

Intracameral and intracorneal injections: Intracameral injections are rarely used in clinic because of the rapid turnover of aqueous humor resulting in very short-time contact time between the active compound and the tissue and because adequate formulations result in efficient concentration of instilled drugs in the anterior segment tissues. However, for the direct delivery of nucleic acids to corneal cells or iris cells, intracameral injections have been evaluated. On the other hand, injection into the corneal stroma may enhance the transfection of corneal cells because it induce mechanical pressure and allows for a prolonged direct contact of the nucleic acids with the target cells.

Naked plasmids: Sustained expression of the encoding genes has been observed in the cornea with the help of direct intrastromal injection of plasmids.

Stromal hydration technique (injection under pressure into the cornea) was used to deliver plasmids encoding β-galactosidase and green fluorescent protein (GFP) as well as plasmids encoding the vascular endothelial growth factor (VEGF) and soluble VEGF receptor Flt-1 (s-Flt1). Following stromal injection, β-galactosidase expression was initially detected at 1 h after treatment persisting for up to 10 days later. Injection of VEGF plasmid induced corneal and anterior chamber neovascularization while injection of s-Flt1 plasmid prevented corneal neovascularization [22]. Injection of the plasmid to the cornea did not induce any inflammation or detectable pathology within the treated tissue.

In a mouse model of epithelial injury, a plasmid encoding for IL-1 receptor antagonist (IL-1RA) was injected into the corneal stroma. Expression of the IL-1RA protein in the cornea peaked between 12 and 24 h lasting for up to 2 weeks. Subsequently, inhibition of corneal neovascularization induced by a combination of alkali and scrape injury was observed [23].

Similarly, intrastromal corneal injections of plasmid encoding the VEGF-binding domain of the Flt-1 receptor coupled with an endoplasmic reticulum retention peptide inhibited the VEGF-induced leukocyte infiltration and corneal neovascularization [24].

Naked oligonucleotides: The injection of naked PS DNA oligonucleotides (3 μM) into the anterior chamber led to a low percentage of DNA molecules transfecting the anterior chamber tissues in rats and primates [25]. These results were probably due to the rapid turn over of aqueous humor and the short contact time of the oligonucleotides with ocular cells.

Confinement of injected ODNs to the treated tissue enhances the cellular uptake. Three subepithelial corneal injections of a DNA antisense against tumor necrosis factor alpha (TNFα) were performed in a mouse model of immune-mediated herpetic stromal keratitis. Fluorescein isothiocyanate (FITC) labeled ODNs were detected in the cornea for up to 10 days after the injection. These injected anti-TNFa ODNs decreased the clinical course of the herpes virus infection and its morbidity [26].

Plasmids in liposomes: Injection into the rat anterior chamber with liposomes carrying *lacZ* plasmid resulted in β-galactosidase expression in the basal layer of the corneal epithelium, the ciliary epithelium, the ciliary body stroma, the iris, and RGCs [19]. Due to their relatively large size, liposomes may have accumulated in the aqueous humor-draining pathways, delaying the outflow and increasing the contact time with ocular tissues. Whether increased pressure may have influence this process has not been evaluated but should be taken into account.

Engineering of protein-bound liposomes can enhance their efficacy and specificity: A plasmid encoding *lacZ* was encapsulated in hemagglutinating virus of Japan (HVJ) fusogenic liposome which contains the viral high-mobility group 1, a nonhistone nuclear protein able to mediate nuclearization. Following injection of this formulation into the anterior chambers of rats and rhesus monkeys, β-galactosidase blue staining was localized in the trabecular meshwork of both rat and primate eyes. Iris and ciliary body localization, however, was detected in rat eyes only [25].

Oligonucleotides in liposomes: Similarly, HSV fusigenic liposomes injected I the anterior chamber have been used to deliver PS oligonucleotides in anterior segment tissues of rat and monkey eyes. Enhanced intracellular delivery of PS oligonucleotides was achived in all anterior segment tissues compared to the injection of naked PS oligonucleotides. Whether this enhanced delivery results in longer life-time or improved biological efficacy remains to be evaluated [25].

RNA aptamer in sustained-release hydron polymer pellets: For the potential modulation of corneal neovascularization, RNA aptamer binding and inhibiting angiopoietin 2 were delivered in sustained-release pellets of hydron polymer (poly[2-hydroxylethylmethacrylate]). Rat cornea neovascularization induced by basic fibroblast growth factor (bFGF) was inhibited by pellets containing an RNA aptamer using the corneal micropockets system [27].

Subconjunctival delivery: Subconjunctival injection is minimally invasive and can be repeated. The subconjunctival space allows for the injection of large volumes and can be used as a reservoir. Thus it may replace the need for repeated instillations. After subconjucntival injections, the intraocular penetration depends on the physicochemical properties of the active compound used. Few informations are available regarding the pharmacokinetics and concentrations of drugs in ocular tissues after subconjunctival injections. In general, high levels of drugs may be detected in the anterior and the posterior segments of the eye. However, high systemic absorption has also been observed. Therefore, after subconjunctival injections, drug diffusion is not restricted to ocular tissues. In this respect, this mode of delivery is not a strict "local" route of administration.

Naked plasmids: Plasmids delivered by the subconjunctival route transfect cells in the periocular tissues but not intraocular cells. In the prospect of preventing fibrosis of blebs after filtering surgery, a plasmid encoding the chloramphenicol acetyltransferase (CAT) gene was injected subconjunctivally in rabbit eyes. Expression was detected in the bleb tissues (conjunctiva, tenon's capsule, and sclera) but not in cornea, iris, or ciliary body [28].

Due to the potential systemic absorption when using this mode of delivery, subconjunctival administration of plasmids can be considered for vaccination purposes, as previously mentioned [15].

Naked oligonucleotides or siRNAs: ODNs and siRNAs are smaller than plasmids and allow for potential targeting of corneal cells after subconjunctival injections [29].

Subconjunctival injection of VEGF-specific siRNA mix significantly inhibited corneal neovascularization or HSV-1 infection in respective animal models [30].

A single administration of an anti-tumor growth factor b (TGFb) PS DNA ODN in a rabbit model of glaucoma filtration surgery allowed for prolonged bleb survival. Similarly, reduction of conjunctival scarring was achieved in a mouse model [31]. In all experiments, ODNs were well tolerated and no side effects observed.

Plasmids in liposomes: Plasmids in liposomes enhance the transfection of corneal cells. After subconjunctival injection of a plasmid encoding the extracellular region of brain-specific angiogenesis inhibitor 1 (BAI1-ECR) gene fused to GFP, green fluorescence was observed in the rabbit corneal stroma for seven days. Furthermore, after two weekly injections, a significant inhibition of corneal neovascularization was also achieved [32].

Plasmids in collagen shield: The slow release of plasmids in the subconjunctival space, using collagen shield, increased markedly the reporter genes expression within tissues after filtering surgery [28].

siRNA mixed in lipid polymer: Subconjunctival injection of siRNAs targeting the type 2 receptor of TGFb (TGFb-R2), mixed to a lipid polymeric transfecting reagent (TransIT-TKO), reduced the subconjunctival inflammation and matrix fibrosis induced in a mouse model [33].

Whether siRNAs subconjunctival injection can be used when intraocular cells are targeted has not been carefully investigated so far.

Intravitreous injection: Direct injection of active compounds into the vitreous cavity is the simplest way to target intraocular tissues. However, molecules injected into the vitreous are cleared rapidly through diffusion anteriorly or

transretinally. Therefore, long-standing vitreous therapeutic levels can be achieved either by repeated injection, the use of high initial concentrations or slow release formulations. Shortly after vitreous injection, initial high peak of local drug levels can be toxic to adjacent ocular tissues. For example, high ODNs levels may induce severe inflammatory response, maculopathy, and permanent retinal damage [34,35]. Complications, such as vitreous hemorrhage, endophthalmitis, and/or retinal tear, although rare, may occur after intravitreal injection. These risks are obviously increased when repeated injections are given.

Naked plasmids: Following intravitreous injection of naked plasmids, no significant expression of the reporter gene was observed in the retina [36-38].

Naked oligonucleotide: The kinetics and biodistribution of PS DNA oligonucleotide, injected in the vitreous cavity depends on the animal model and the injected dose.

In the mouse eye, increasing the dose of fluorescent PS ODN from 3 μM to 30 μM raised internalization rate in RGCs from 13% to 59%. However, observed fluorescence is of short duration, decreasing in intensity within a day [39]. In a murine model of proliferative retinopathy (PVR), antisense DNA ODN directed at VEGF induced a 30% reduction of new blood vessel growth and 50% of VEGF levels [40].

In the rat eye, 2.5 mM PS DNA ODN directed at VEGF was detected in the RGCs and in all other retinal layers. A preferential accumulation into retinal pigmented epithelium (RPE) was observed [41]. The ODNs were also observed in endothelial cells of the retinal microvasculature [42]. In the krypton laser-induced choroidal neovascularization (CNV) model, ODNs concentrated in the laser photocoagulated site for at least eight weeks, with a preferential localization in macrophages, RPE and choroidal tissue [41]. An apparent reduction in the intensity of CNV was observed following treatment with anti-VEGF ODN [43]. Also, the survival of RGCs was increased when using anti-Bax ODNs eight days after axotomy [44]. In RCS-*rdy* rat, a PS antisense ODN directed against the alveoli cathepsin 5 significantly increased the number of phagosomes in RPE cells at 28 days [45].

In the rhesus monkey, PS ODNs designed to target both human and rat VEGF were injected into the vitreous [35]. Using PS ODN concentration of 7.5 mM resulted in penetration of the ODN in all retinal layers with a preferential accumulation in the RPE cells during the first week after injection. Three weeks after injection, the level of ODNs in all neural retinal layers had decreased while the concentration in the RPE cells increased. A transient vitreous haze was observed but no evident retinal damage was detected. At lower doses of 0.75 mM, DNA ODN localized mostly in RGCs and RPE. Intravitreal injections of VEGF DNA antisense ODN (3 μM) induced a significant reduction of iris neovascularization [35].

Extensive pharmacokinetic studies were performed prior to the clinical use of a PS anti- CMV DNA ODN. Clearance from the vitreous of rabbits and monkeys followed a first order kinetics, resulting from diffusion and redistribution from the surrounding ocular tissues [10,46]. Elimination was quicker in the monkey than in the rabbit, with half-lives calculated at about 20 h and 60 h, respectively. Changing the chemistry to a mixed PS DNA/RNA ODN backbone anti-CMV prolonged the vitreous half-life in the rabbit to 48 h. In the high doses injected group of animals, both the electroretinograms (ERG) and tissues anatomy were affected when examinations were carried out four weeks after the intravitreal injections [34].

The anti-CMV DNA ODN (ISIS 2922) was evaluated by the Fomivirsen study group in CMV-infected patients with AIDS. Different intravitreal injection regimens were scheduled, depending on the severity of the retinitis. In these evaluations, the ocular tolerance was acceptable despite the presence of anterior chamber inflammation and increased intraocular pressure. No systemic absorption of the drug was detected [47]. As for the treatment outcome, Fomivirsen reduced the severity of retinitis and prevented the occurrence of retinal detachments. Higher efficacy was observed when treatment was initiated early during the disease course [48]. As the lower dose regimen (165 μg/injection) demonstrated similar treatment outcomes, it is the preferable treatment dose in terms of convenience and safety [49].

Pegylated RNA aptamers: To limit oligonucleotides transretinal diffusion from increasing their vitreous half-life and stability, oligo-aptamers coupled to polyethylene glycol (PEG) were formulated.

The anti-VEGF pegylated aptamer (pegaptanib, EYE001®) was intravitreally injected in different animal models of ocular neovascularization. In the mouse retinopathy of prematurity (ROP) model, retinal neovascularization was inhibited by 80% in the treated eyes [50]. In monkeys, plasma and vitreous concentrations were linearly related to the injected dose of the anti-VEGF pegylated RNA aptamer. It is of interest that the injected aptamer had a vitreous half-life of approximately 94 h and was found intact in the plasma. Despite its relatively rapid clearance, active molecules of the aptamer were detected in the vitreous up to 28 days after injection. No toxicity to ocular tissue was observed in the treated eyes [51]. In two randomized double-masked studies in patients with CNV secondary to age-related macular degeneration (AMD; n=1208), 70% of patients treated with repeated injection of 0.3 mg of aptamer lost fewer than 15 letters of visual acuity compared to only 55% in the sham-injected group 54 weeks after initiation of the study [50]. Treatment of CNV associated with AMD with anti-VEGF pegylated RNA aptamer reduced the extent of CNV leakage. Only mild to moderate side effects were encountered in these studies [52]. Moreover, the same anti-VEGF pegylated aptamer induced a significant suppression of retinal leukostasis and blood-retinal barrier breakdown in diabetic rats [53].

Plasmids in liposomes: Liposomes have been used to increase the half-life time of intravitreally injected nucleic acids, reduce toxicity, and minimize intraocular side effects. After their injection, these liposome formulations induce the formation of vitreal bodies (small white sparking opacities) which localize in the lower quadrants. Vitreal condensations and transient clouding interfere with visual acuity [54]. Despite these drawbacks, plasmid encapsulation within liposomes allows for the transfection of cells in the anterior segment of the eye (cili-

ary epithelium, stroma of the ciliary body, and iris), inner retinal layers and RPE cells [19]. Yet, injection into the vitreous of naked plasmid does not induce any reporter gene expression.

Oligonucleotides in liposomes: Liposomes significantly increase the stability of ODNs in the vitreous. In the rabbit eye, 37% of the oligonucleotides within liposomes were still present in the vitreous 15 days after their administration [55].

Liposomes coated with the envelope of inactivated HVJ were used to encapsulate a PS DNA ODN. When injected intravitreally to mice eyes, ODNs were found in 50% of RGCs and in retinal vessels for up to 3 days. In photoreceptors, however, ODNs were only occasionally observed [39]. In a rat model of laser-induced CNV, administration of anti-VEGF ODN within the same liposome preparation showed the presence of ODNs in RGCs and in the CNV 3-14 days after the injection. In these experiments, however, the extent of CNV in treated eyes was not, affected [56].

To increase the transfection efficiency of liposomes, strategies such as pegylation have been developed. PEG coating induces a repulsive effect toward protein aggregation at the vesicle surface, facilitating its disruption. Using this technique, ODNs uptake was increased in retina/choroid but was associated with a moderate and transient intraocular inflammation [9].

Oligonucletides in nanoparticles: Small nanoparticles follow a transretinal migration and home in RPE cells [57]. Also, plasmids encoding for GFP or red nuclear fluorescent protein (RNFP) encapsulated in poly(lactic) acid (PLA) or poly(lactide-co-glycolide) acid (PLGA) nanoparticles induced gene expression within the RPE cell layer, the homing site of these intravitreally injected nanoparticles [58].

More recently, it was reported that encapsulation of an anti-CMV DNA oligonucletide in albumin nanoparticles improved the antiviral activity of this oligonucletide construct [59].

Oligonucletides in polyplex: Complexes of nucleic acids with polymers improve their stability and cellular internalization.

An intravitreally injected antifibronectin antisense PS DNA ODN complexed with polyoxyethylene-polyspermine (PEO-PSP) copolymers reduced fibronectin expression in retinal vessel cells of treated rat eyes. This effect was observed at the mRNA and protein level. The complexed ODN was detected in retinal capillary cells until day 6 after injection [42].

In RCS-rdy+ rat, the use of lipophilic amino acid dendrimers allowed the penetration of anti-VEGF ODN to the nuclei of RPE, outer nuclear layer (ONL), inner nuclear layer (INL) and ganglion cell layer (GCL) up to four months after the initial intravitreous injection. When injected alone, ODNs reached non-detectable levels two months after their administration. In a laser-induced CNV model, ODNs complexed with dendrimer significantly inhibited CNV development for four to six months, whereas ODNs alone did not show any significant effect [60].

It is interesting that dendrimers allowed the delivery of ODNs to photoreceptor cells while polyplex or lipoplex did not have a similar effect.

Oligonucletides in VP22 vectosomes: VP22 is a cationic peptide that form well characterized nanoparticles (vectosomes) when complexed with ODNs [61]. We have observed that after intravitreous injection in the rat eye, vectosomes followed a transretinal migration accumulating in the cytoplasm of RPE cells [62]. The VP22 vectosomes show a specific sensitivity to light. After their internalization by the RPE cells, the vectosomes remain stable within the cytoplasm. However, when the cells are illuminated, the vectosomes are destabilized releasing their ODN. The released ODN then moves from the cytoplasm to the cell nucleus expressing its genetic load. These interesting characteristics of VP22 vectosomes can therefore be used to control release of ODNs at chosen sites using white light (or laser) beam; Figure 2A,B show the distribution of vectosomes at 24 h after their intravitreous injection. As illustrated, vectosomes infiltrated all retinal layers and internalized with the cytoplasm of retinal cells (particularly RPE cells) remaining stable for at least 10 days. By illuminating the treated rat eyes with a white light, we could observe that the released fluorescent ODNs concentrated in the cell nuclei of RPE, INL, and GCL cells (Figure 2C,D). This demonstration highlights the interesting and most appealing possibility of using a light source or laser beam to carry out a localized and specific release of ODNs from their "storing place" within the cytoplasm endosomes allowing their migration and expression within the targeted cell nuclei. This strategy was implemented and found applicable in vitro and in vivo systems [62].

Subretinal delivery: To increase the local concentration of active compounds in the posterior retinal layers (photoreceptors and RPE cells) and prolong their contact time with the target cells, direct injection in the subretinal space can be performed. Injections can be carried out externally through the sclera or internally through the retina entering into the eye via the pars plana. These types of injections require the use of pressure and fluid or air to detach the retina. The disadvantage of this method is that the area of targeted cells is limited to the locally detached retina. Furthermore, multiple complications may occur: Of these, most notable are lesions of RPE cells, hemorrhages, retinal tears, sub- or pre-retinal fibrosis, and uncontrolled retinal detachments. Subretinal delivery of nucleic acids has been used to target RPE and photoreceptors in preclinical studies as a proof of concept. However, due to the expected high rate of complications, particularly if reinjections are needed and to the potential difficulty to detach a pathological retina where sub retinal fibrosis takes place, subretinal injections may remain an investigative tool and not a practical method for application to the human eye.

Naked plasmids: Subretinal injection of plasmid encoding *lacZ* under a CMV promoter did not result in any significant specific gene expression [38].

Naked oligonucleotides: In rat eyes, the distribution of PS DNA ODNs injected to the subretinal space remains limited to the retina around the detached area. In this area, it has been observed in RPE cells and in all retinal layers. The ODN concentration within the intraocular tissues was dose and site

dependent. Although ODN doses injected to the subretinal space were lower (1.29 and 12.9 μg) than those delivered into the vitreous (64.5 and 129 μg), similar or even better uptake dynamics were observed after subretinal injections [41]. These findings indicate that the clearance of PS ODNs from the subretinal space is much slower than from the vitreous. Injection of ODNs at the site of laser photocoagulation in CNV model in rat eyes, caused its localization in the RPE, macrophages, and in a few cells within the choroid. In this model, ODNs were detected and identified within the ocular tissues for at least 56 days after injection [63]. Injecting labeled PS DNA ODN for human and rat VEGF in the subretinal space of rhesus monkeys led to a strong labeling of RPE cells and all neural layers during the first week after the injection. After three weeks, the ODN signal went down dramatically in the neuroretina but remained constant in the RPE. A dose-related cell infiltration was observed along with a transient fluid accumulation and slight distortion of the neuroretina. In these experiments the injected ODNs partially inhibited the CNV disease in treated animals [35].

Complexed plasmids: An Epstein-Barr virus (EBV)-based plasmid encoding human bFGF was complexed in K8/JTS-1 oligopeptides and subretinally delivered in Royal College of Surgeon (RCS) rats [64]. Rodent cells are not permissive for EBV in term of plasmid replication. However, the EBV vector facilitated not only the long-term maintenance of the plasmid but also allowed for a high level of gene expression after transfection. Plasmids enter the cells, presumably via receptor-mediated endocytosis. Expression of bFGF was detected in choroidal and RPE cells. Interestingly the photoreceptor cell degeneration in this animal model was delayed for 60 days post-injection [64].

After subretinal injection of plasmid encoding *lacZ* in medium containing 40% of Lipofectamine 2000, blue staining was detected in RPE cells. However, in treated animals, significant damage to photoreceptors was observed [38].

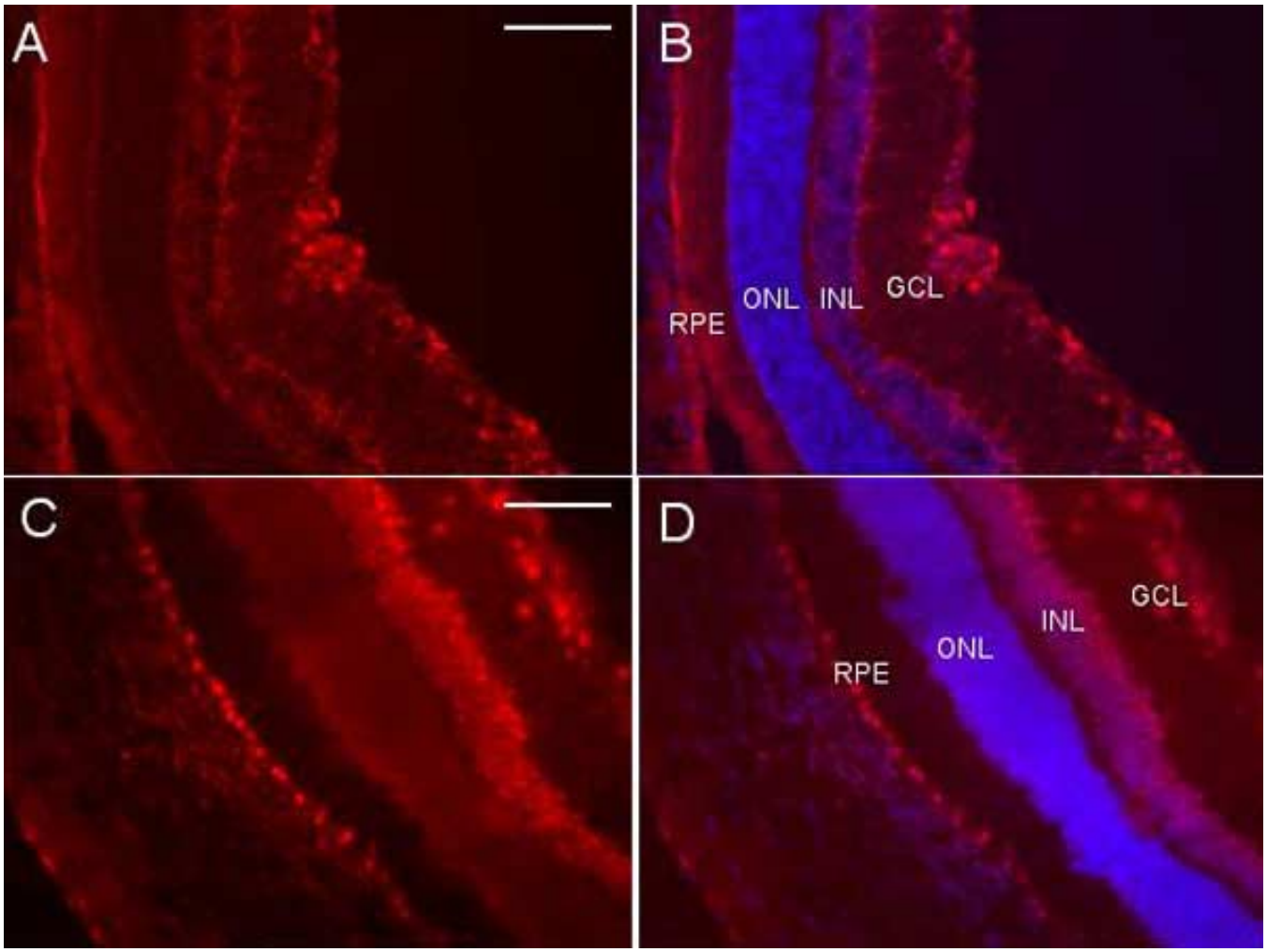

Figure 2. Retinal distribution of Hex-labeled oligonucletides. In vivo light-controlled distribution of Hex-labeled oligonucletides in the retina after intravitreous injection of vectosomes in the rat eye. Fluorescence microphotographs of retinal sections 24 h after intravitreous injections of vectosomes without illumination (**A**) and (**B**): with Dapi nuclei co-staining, or with illumination (**C**) and (**D**): with Dapi nuclei co-staining. Retinal pigment epithelium (RPE), outer nuclear layer (ONL), inner nuclear layer (INL), and ganglion cell layer (GCL). Scale bars represents 100 μm.

Subretinal injection of the plasmid in medium containing 10% NeuroPorter induced transfection of RPE cells for at least 14 days, without any observed damage to retinal tissues [38]. Thus, in practical terms, the use of 10% NeuroPorter should be more applicable for potential therapeutic use.

Intravenous delivery

Plasmids in liposomes: To allow for the crossing of the blood-ocular barriers, pegylated immunoliposomes tagged with the rat 8D3 monoclonal antibody targeted to transferrin receptor-rich structures were used. Cellular β-galactosidase expression was differentially regulated by gene promoters. Using the glial fibrillary acidic protein (GFAP) promoter, expression of the reporter gene was observed in the inner retina, ciliary body, iris, sebaceous glands of the tarsal plate and corneal epithelium [65].

Similarly, pegylated immunoliposomes tagged with a human anti-insulin receptor antibody were intravenously injected in monkeys. The pattern of organ expression was closely related to the type of promoter used. β-galactosidase was expressed throughout the entire retina (including the photoreceptor cells) with either a SV40 (simian virus) or an opsin promoter. The SV40 promoter also enabled gene expression in other organs such as brain, liver and spleen. The opsin promoter helped restrict the transgene expression to the eye. Plasmid-based gene expression remained within the expected therapeutic range for two-three weeks after a single intravenous administration [66].

siRNA in polyplex: When systemic administration of a polymer delivery vehicle (TargeTran) VEGF specific siRNA mix was used, corneal neovascularization induced by CpG ODNs or HSV-1 infection was significantly inhibited [30].

Axonal delivery

Naked plasmids: Localization study of plasmids encoding the luciferase gene under the SV40 promoter or encoding *lacZ* under either the CMV or RSV (respiratory syncytial virus) promoters was performed in rat eyes after delivery by retrograde transport from intact axon terminals of RGCs. Using this technique, RGCs could be specifically targeted for at least 10 days after the injection [67].

Naked siRNA: siRNAs against c-Jun and Apaf-1 were injected into the optic nerve stump to inhibit axotomy-induced apoptosis. Retinal flat-mounts of treated eyes demonstrated neuronal localization of the injected siRNAs and a significant of RGCs survival [68].

Plasmids in polyplex: Polyplex formed with poly(L-lysine) and the same plasmids used for the experiments previously detailed in increased the duration of expression within the intraocular tissues [67].

Delivery by iontophoresis: Application of low density iontophoretic electric current to the eye is safe and may enhance the penetration of nucleic acids without the need for injection. Iontophoresis acts on the movement of charged molecules through cells and tissues. Iontophoresis has also an effect on the organization of the tissue itself, allowing for postiontophoretic drug penetration and electroosmosis [69].

Naked plasmids: Full-thickness human scleral fragments in an agarose gel were used to test if the transfer of nucleic acids across the sclera could be enhanced by an electric field. It was found that a 3 kb plasmid-encoding GFP could cross the sclera [70].

Transcorneal iontophoresis was performed in rabbit eye after instillation of a plasmid-encoding GFP. Green fluorescence was detected in the cornea, the anterior chamber angle and the ciliary body [71]. In our hands, however, direct transcorneal iontophoresis of plasmids in rabbit and rat eyes did not enhance the transfection of reporter gene to corneal or intraocular tissues [unpublished data].

Naked oligonucleotides: With the use of full-thickness human scleral fragments in an agarose gel, the previously described method revealed that an electric field enhanced the transfer of a single-stranded 51-mer DNA ODN and a double hairpin 68-mers RNA/DNA oligonucleotide (ODN) through the sclera [70,72].

Transcorneal iontophoresis was performed in rabbit eyes after instillation of PS ARN ODN. These ODNs were detected in the anterior chamber within 5 min after the application of the iontophoresis current. ODNs were observed in the vitreous 10 min after treatment and the posterior retina after 20 min [71].

The potential of iontophoresis to enhance the intraocular penetration of topically applied ODNs was also evaluated. One hour after transcorneoscleral iontophoresis of antinitric oxide synthase-2 (NOS2) DNA ODN in normal rat eyes, ODNs were detected in all cornea layers, conjunctiva, sclera, iris/ciliary body and internal retinal layers. Five hours later, ODNs were observed in the external retinal layers, the RPE, and episcleral vessel walls [73]. Furthermore, antisense ODN directed against NOS2 downregulated NOS2 expression in iris/ciliary body of rat eyes with endotoxin-induced uveitis (EIU). In these treated rats, nitrite production was also significantly reduced. No inflammatory cell infiltrate nor any signs of tissue damage were observed after iontophoresis [73].

In order to understand the route of penetration of ODNs using low current density iontophoresis, labeled ODNs were used. After transcorneoscleral iontophoresis of a fluorescent PS anti-VEGF receptor 2 (VEGF-R2/Flk/KDR) DNA ODN in rat eyes, ODNs were observed in all layers of the corneal epithelium and endothelium, and also in the iris. However, the kinetics of penetration showed that while intraocular delivery was achieved at the limbus and through the anterior sclera immediately after the application of current. The penetration of ODNs in the corneal layers was delayed. In the cornea, ODNs diffused slowly from the periphery and the corneal superficial layers where ODNs had accumulated. In corneas with neovascularization, ODNs were detected in vascular endothelial cells of the stroma and in infiltrating leucocytes. ODNs extracted from treated tissues 90 min after iontophoresis were recovered intact [18].

In mice eyes, we have used low current iontophoresis to enhance the intraocular delivery of fluorescent ribozymes [74]. C56Bl6 mice were treated with a 100 $\mu A/mm^2$ current applied with a cup covering the cornea and first millimeters of

sclera. The fluorescent (CY5) ribozymes (1.6 pmoles/μl) were applied the eye surface through the cup. The negative electrode was connected at the bottom of the cup and the positive electrode was a needle inserted subcutaneously in the base of the neck. The current was applied for 10 min and the mice were sacrificed 1 h later. As illustrated in Figure 3, no fluorescence was observed in eyes receiving saline iontophoresis (Figure 3A) or application of fluorescent ribozymes without current (Figure 3B). On the other hand, application of iontophoresis and fluorescent ribozymes, resulted in strong fluorescence of the cornea, iris, iris vessels and ciliary body (Figure 3C). Strong fluorescence was also detected in the posterior segment of these treated eyes with a preferential localization to the outer retina (Figure 3D). These electrical iontophoretic parameters did not result in any structural damages when applied to adult mice eyes (Figure 3E). Extraction of ribozymes from the tissues and migration on denaturing acrylamid gel demonstrated that, intact ODNs were still present in the anterior segment tissues of treated eyes at one hour (data not shown).

Delivery by electrotransfer: Electroporation uses short electric pulses to enhance the penetration of high molecular weight compounds into cells. Success of electrotransfer depends on the delivery of a sufficient amount of plasmid to the target tissue. Efficient tissue delivery is associated with the use of appropriate pulse intensity and its repetition. Electroporation applies a high voltage (typically >100 V) pulse for a short (μ-ms) duration to allow for infiltration of the ocular tissue cells in a reversible manner.

Naked plasmids: Reporter plasmid was efficiently transfected into retinal explants ex vivo by high-voltage pulses [75].

Both intracameral and intracorneal injections can be performed efficiently to transfect corneal cells. After injection in the anterior chamber of a plasmid encoding for reporter gene and application of low voltage electrotransfer, a specific expression in corneal endothelial cells was observed for 21 days [76]. Intracameral injection of a plasmid encoding plasminogen activator in the rat eye coupled to electroporation reduced fibrin formation and was safe [77].

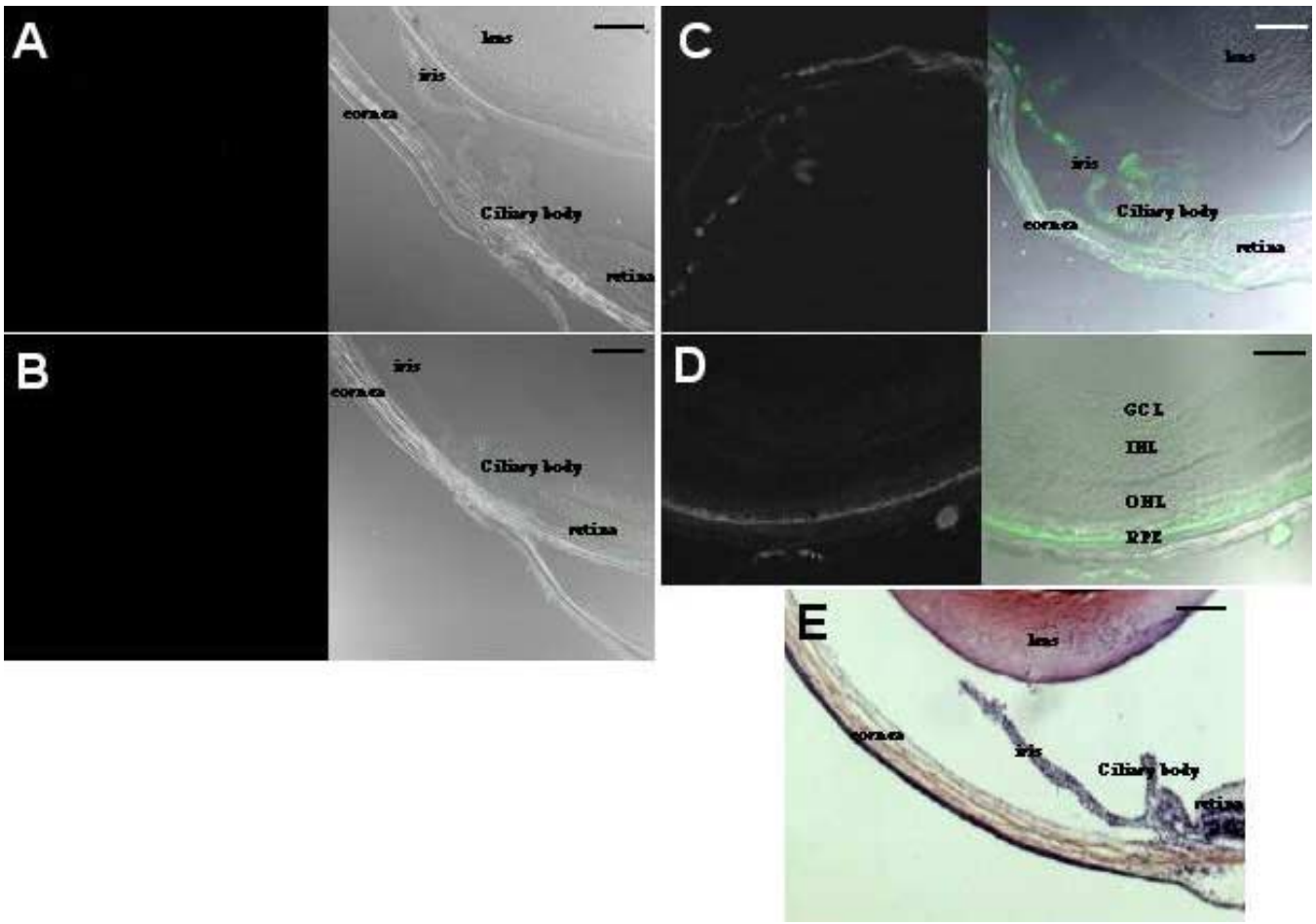

Figure 3. Adult mice eye sections one hour after transcorneosceral iontophoresis of CY5-labeled ribozymes. Confocal microscopy of anterior segment in phase contrast and fluorescence one hour after saline iontophoresis (**A**). Confocal microscopy of anterior segment in fluorescence and phase contrast one hour after application of fluorescent ribozymes without current (**B**). Confocal microscopy of anterior segment in fluorescence and phase contrast one hour after application of fluorescent ribozymes with current (**C**). Confocal microscopy of posterior segment in fluorescence and phase contrast one hour after application of fluorescent ribozymes with current (**D**). Hematoxylin-eosin stained sections one hour after application of fluorescent ribozymes with current (**E**). Scale bars represents 100 μm.

When the plasmid was injected in the stroma of the rat cornea, followed by electric pulses, expression of reporter genes was observed within keratocytes in a localized area for 15 days [78]. Similarly, intracorneal or subconjunctival injection of plasmids encoding luciferase or GFP in the mouse eye, followed by electric pulses to the corneas, induced a thousand fold more gene expression as compared with injection of plasmid alone over the entire surface of the cornea in both epithelial and stromal layers. Gene expression was detected, in these cases, as early as 6 h post-electroporation. Gene expression remained high for three days, and decreased by the seventh day after treatment. When an optimal field strength of 200 V/cm was used, no trauma, corneal edema, or inflammation were observed. However, at higher field strengths, corneal damage was detected [79].

These electrotransfer techniques have been used to induce the expression of specific genes in different models of filtering surgery or corneal neovascularization. Injection into rabbit conjunctiva of a plasmid encoding matrix metalloproteinase-3 (MMP-3) induced the expression of MMP-3 for 30 days after treatment. Applying this treatment after filtering surgery reduced the postoperative subconjunctival fibrosis [80].

Gene transfer of a plasmid expressing the human plasminogen kringle 5 by subconjunctival injection and electrotransfer inhibited the corneal neovascularization induced in a rat model [81].

To target the retina, plasmids were injected into the vitreous cavity and electric pulses were applied with one spoon electrode covering the cornea and the other spoon electrode on the sclera in the back of the eye, resulting in an axial electric field [36]. Using this technique, expression of the transgene was limited to encoding brain-derived neurotrophic factor (BDNF) [37] or glial cell line-derived neurotrophic factor (GDNF), expression of gene was observed in RGCs for 4 weeks. The extent of expression correlated well with biological effects [82]. Other reports confirmed that electrotransfer (3.4 V/mm) induced transduction of intravitreally injected *lacZ* reporter plasmid without any observed retinal damage [38]. After intravitreous injection, electrotransfer has little influence on the transretinal permeation of injected plasmids. Therefore, if cells within the ONL are targeted, this delivery system should not be considered.

To circumvent the low retinal transfection efficacy obtained with intravitreous injection, subretinal injection of reporter plasmid in neonatal rodent pups' eyes, coupled with several pulses of high voltage electroporation, was performed. In the treated eyes, gene expression was detected in retinal cells for at least 50 days with little damage to the intraocular tissues [75]. Reporter plasmids carrying retinal cell type-specific promoters were engineered to target retina cells. Moreover, when using DNA-based RNA interference vectors directed against two transcription factors crucial for photoreceptor development, photoreceptor phenotypes were similar to those of the corresponding knockout mice [75].

A recent report demonstrated that electrotransfer (3.4 V/mm) induced the safe transduction of subretinally injected *lacZ* reporter plasmid in the mice RPE cells for at least 14 days [38].

In rat eyes, the subretinal injection of reporter plasmid followed by electrotransfer led to expression in RPE cells for three to four weeks. Interestingly, the co-injection of a plasmid encoding the bacteriophage phiC31 integrase conferred genomic integration. In these experiments, a long-term stable gene expression was observed for at least four to five months after treatment [83].

In ovo electroporation can be used to manipulate gene expression in the eye during embryonic development. GFP plasmid was injected into the lumen of the lens vesicle in chicken embryos at stage 15 and electroporation was performed with one electrode above the eye and the other underneath the head of the embryo. A strong GFP signal was detected in lens cells 4 h after electroporation, lasting for at least five days. Fluorescence was also detected in corneal and retinal cells [84].

A new concept of ocular eletcrotransfer was recently developed in our laboratory. It is designed to specifically transfect the ciliary muscle, used as a reservoir for long lasting expression secreted proteins into the ocular media [85]. Muscle cells are good candidates for electrotransfer allowing for a long lasting expression. This technique has been used to produce TNFa soluble receptor (sTNFaR) in the rat eye. High levels plasmid expression was observed in the ocular media of treated eyes. The level of expression correlated well with the decrease of TNFa levels for at least one month. No sTNFaR protein was detected in the serum, demonstrating the potential for achieving a localized therapeutic effect within the eye.

Ballistic delivery of naked plasmids: High velocity gold microparticles coated with DNA can be used as gun projectiles. This technique is called "gene gun."

Gene gun of a plasmid encoding GFP was efficient in the delivery of genes to corneal epithelium. In rabbit eyes, the corneal epithelium was labeled in 100% without any detectable corneal damage [86].

Gene gun system delivery was also efficient for the delivery of plasmid molecules in rat eyes [87].

DISCUSSION

Nonviral methods of nucleic acid delivery offer a wide range of possibilities for the targeting of ocular cells. For one specific delivery system, efficacy depends on the nature and length of the nucleic acid, the type of targeted cells or tissue, the experimental animals age and species, and the mode of detection and quantification. When using a nonviral method of nucleic acid delivery, the route of administration has to be chosen as a function of the targeted cells or tissue. Conclusions regarding the potential clinical use have to be derived from data obtained from multiple experiments. Nonetheless, some nonviral systems have shown efficiency in various experimental animal models while a few strategies have already reached the stage of clinical application.

Specific routes of administration are more adapted to target the anterior segment while others may be better for the posterior segment. Unfortunately, the topical route is not suit-

able for the delivery of nucleic acids to the anterior segment of the eye due to the low transfection rate. However, intracorneal administration can be considered for the transfection of corneal cells with a high efficiency. Subconjunctival injection of plasmids, siRNAs, or other types of nucleic acids is the route of choice if tissue remodeling or scar formation of periocular tissues is to be influenced, as in glaucoma surgery. Systemic diffusion of the injected molecules occurs after subconjunctival injection. Therefore, large volumes cannot be administered when using this route of delivery. Liposomes, dendrimers, and iontophoresis or elecrotransfer of nucleic acids can efficiently enhance the transfection rate for anterior segment tissues and cells.

To target posterior segment tissues, intravitreous injection, transscleral delivery technique, or subretinal injections can be considered depending on the target cells. Receptor-mediated gene delivery strategy is another way to improve the targeting specificity. For example, liposomes tagged with human antiinsulin receptor can efficiently transfect retinal cells after their intravenous injection [66].

The delivery efficiency of antisense ODNs or siRNAs can be enhanced by their combination with polymers, cationic lipids, peptides or iontophoresis. For example, while intravitreous injection of ODNs is not optimal for targeting photoreceptor cells, the combination of ODNs with dendrimers allows for a higher transfection rate of photoreceptors. Interestingly, topical instillation of plasmids within liposomes can lead of RGSs transfection in animal models. However, the mechanism of liposomes migration to the inner retina is not clear. Furthermore, whether this phenomenon will also be observed in humans remain to be explored.

To limit the growth of CNV, an anti-VEGF pegylated aptamer (pegaptanib, EYE001®) can be repeatedly injected into the vitreous of patients [50]. The fate of the nonbiodegradable PEG in the eye and its potential accumulation in RPE cells is a pertinent question that unfortunately remains unanswered at the present. In the future, other routes of administration as well as sustained delivery systems will probably improve the risks:benefits ratio of this promising strategy.

Once in the target cell, nucleic acids must freely interact with the cell machinery to retain their activity. For this purpose, some proteins (Tat from HIV and VP22 from HSV-1) that are internalized by cells and can localize in the cytoplasm or nucleus may be used. Vectosomes, particles formed between the VP22 cationic peptide of HSV-1 and ribozymes, DNA, hybrids DNA/RNA or proteins, are nontoxic an easily gain access into the cell cytoplasm. Due to their light sensitive property, site-specific and time-controlled release of the vectosome complexed ODN tissues can be achieved in the eye [61]. The VP22 gene also enhance adenovirus-mediated transgene delivery to neuron retinal cells via intercellular trafficking [88].

An additional approach for enhancement of the therapeutic potential is through chemical or structural modification of the nucleic acid molecule to be used. The replacement of a single nonbridging oxygen by a sulfur (PS ODN) causes an enhanced resistance to endonucleases and a marked increase of in vivo stability [89]. Addition of locked nucleic acids (LNAs), which contain a methylene bridge connecting the 2'-oxygen of ribose with the 4'-carbon are under investigation. Preliminary results showed these changes enhanced ODN stability and reduced toxicity [90,91]. Other types of nucleic acid molecules as the siRNA are being introduced and have demonstrated efficiency for the inhibition of specific protein expression [92].

Somatic therapies using growth factors and anti-apoptosis genes enhance cell survival and slow down degenerative processes but are short of inducing a cure. Genoplasty, on the other hand, may be a strategy of choice due to its ability to induce site-specific point mutation correction in vitro and in vivo. Using RNA-DNA hybrid ODNs, or more recently single-stranded DNA ODNs, this technique has yielded promising results [91].

In conclusion, nonviral systems have been developed for the delivery of nucleic acids to eye tissues and cells. Ongoing studies with these nonviral strategies show that some are more adapted for specific purposes than others. Thus, different strategies have to be used and modulated according to the location and type of affected tissues targeted for therapy. Following these adaptations, the type of genetic construct with most promising beneficial outcome has to be selected. Optimization of these techniques and overcoming their present limitations and potential pitfalls may boost clinical application in the field of ocular gene therapy. It is hoped that, assimilation of the available knowledge described in this review with expected improvements of the present technologies will propel the therapeutic potential for eye diseases into a new era within the foreseeable future.

ACKNOWLEDGEMENTS

Charlotte Andrieu-Soler is funded by EyeGatePharma and the French Ministry of Research and Education. We thank the Fondation de l'Avenir and ANR emergence for their financial support of this project.

REFERENCES

1. Bennett J, Tanabe T, Sun D, Zeng Y, Kjeldbye H, Gouras P, Maguire AM. Photoreceptor cell rescue in retinal degeneration (rd) mice by in vivo gene therapy. Nat Med 1996; 2:649-54.
2. Acland GM, Aguirre GD, Ray J, Zhang Q, Aleman TS, Cideciyan AV, Pearce-Kelling SE, Anand V, Zeng Y, Maguire AM, Jacobson SG, Hauswirth WW, Bennett J. Gene therapy restores vision in a canine model of childhood blindness. Nat Genet 2001; 28:92-5.
3. Mori K, Ando A, Gehlbach P, Nesbitt D, Takahashi K, Goldsteen D, Penn M, Chen CT, Mori K, Melia M, Phipps S, Moffat D, Brazzell K, Liau G, Dixon KH, Campochiaro PA. Inhibition of choroidal neovascularization by intravenous injection of adenoviral vectors expressing secretable endostatin. Am J Pathol 2001; 159:313-20.
4. Bainbridge JW, Mistry A, De Alwis M, Paleolog E, Baker A, Thrasher AJ, Ali RR. Inhibition of retinal neovascularisation by gene transfer of soluble VEGF receptor sFlt-1. Gene Ther 2002; 9:320-6.
5. Takahashi K, Saishin Y, Saishin Y, Silva RL, Oshima Y, Oshima S, Melia M, Paszkiet B, Zerby D, Kadan MJ, Liau G, Kaleko M,

Connelly S, Luo T, Campochiaro PA. Intraocular expression of endostatin reduces VEGF-induced retinal vascular permeability, neovascularization, and retinal detachment. FASEB J 2003; 17:896-8.
6. Rasmussen H, Chu KW, Campochiaro P, Gehlbach PL, Haller JA, Handa JT, Nguyen QD, Sung JU. Clinical protocol. An open-label, phase I, single administration, dose-escalation study of ADGVPEDF.11D (ADPEDF) in neovascular age-related macular degeneration (AMD). Hum Gene Ther 2001; 12:2029-32.
7. Provost N, Le Meur G, Weber M, Mendes-Madeira A, Podevin G, Cherel Y, Colle MA, Deschamps JY, Moullier P, Rolling F. Biodistribution of rAAV vectors following intraocular administration: evidence for the presence and persistence of vector DNA in the optic nerve and in the brain. Mol Ther 2005; 11:275-83.
8. Chen X, Dudgeon N, Shen L, Wang JH. Chemical modification of gene silencing oligonucleotides for drug discovery and development. Drug Discov Today 2005; 10:587-93.
9. Bochot A, Fattal E, Boutet V, Deverre JR, Jeanny JC, Chacun H, Couvreur P. Intravitreal delivery of oligonucleotides by sterically stabilized liposomes. Invest Ophthalmol Vis Sci 2002; 43:253-9.
10. Leeds JM, Henry SP, Bistner S, Scherrill S, Williams K, Levin AA. Pharmacokinetics of an antisense oligonucleotide injected intravitreally in monkeys. Drug Metab Dispos 1998; 26:670-5.
11. Daheshia M, Kuklin N, Kanangat S, Manickan E, Rouse BT. Suppression of ongoing ocular inflammatory disease by topical administration of plasmid DNA encoding IL-10. J Immunol 1997; 159:1945-52.
12. Noisakran SJ, Carr DJ. Therapeutic efficacy of DNA encoding IFN-alpha1 against corneal HSV-1 infection. Curr Eye Res 2000; 20:405-12.
13. Noisakran S, Carr DJ. Topical application of the cornea post-infection with plasmid DNA encoding interferon-alpha1 but not recombinant interferon-alphaA reduces herpes simplex virus type 1-induced mortality in mice. J Neuroimmunol 2001; 121:49-58.
14. Noisakran S, Campbell IL, Carr DJ. Ectopic expression of DNA encoding IFN-alpha 1 in the cornea protects mice from herpes simplex virus type 1-induced encephalitis. J Immunol 1999; 162:4184-90.
15. Inoue T, Inoue Y, Nakamura T, Yoshida A, Takahashi K, Inoue Y, Shimomura Y, Tano Y, Fujisawa Y, Aono A, Hayashi K. Preventive effect of local plasmid DNA vaccine encoding gD or gD-IL-2 on herpetic keratitis. Invest Ophthalmol Vis Sci 2000; 41:4209-15.
16. Inoue T, Inoue Y, Hayashi K, Shimomura Y, Fujisawa Y, Aono A, Tano Y. Effect of herpes simplex virus-1 gD or gD-IL-2 DNA vaccine on herpetic keratitis. Cornea 2002; 21:S79-85.
17. Bochot A, Mashhour B, Puisieux F, Couvreur P, Fattal E. Comparison of the ocular distribution of a model oligonucleotide after topical instillation in rabbits of conventional and new dosage forms. J Drug Target 1998; 6:309-13.
18. Berdugo M, Valamanesh F, Andrieu C, Klein C, Benezra D, Courtois Y, Behar-Cohen F. Delivery of antisense oligonucleotide to the cornea by iontophoresis. Antisense Nucleic Acid Drug Dev 2003; 13:107-14.
19. Masuda I, Matsuo T, Yasuda T, Matsuo N. Gene transfer with liposomes to the intraocular tissues by different routes of administration. Invest Ophthalmol Vis Sci 1996; 37:1914-20.
20. Matsuo T, Masuda I, Yasuda T, Matsuo N. Gene transfer to the retina of rat by liposome eye drops. Biochem Biophys Res Commun 1996; 219:947-50.
21. Liaw J, Chang SF, Hsiao FC. In vivo gene delivery into ocular tissues by eye drops of poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide; PEO-PPO-PEO) polymeric micelles. Gene Ther 2001; 8:999-1004.
22. Stechschulte SU, Joussen AM, von Recum HA, Poulaki V, Moromizato Y, Yuan J, D'Amato RJ, Kuo C, Adamis AP. Rapid ocular angiogenic control via naked DNA delivery to cornea. Invest Ophthalmol Vis Sci 2001; 42:1975-9.
23. Moore JE, McMullen TC, Campbell IL, Rohan R, Kaji Y, Afshari NA, Usui T, Archer DB, Adamis AP. The inflammatory milieu associated with conjunctivalized cornea and its alteration with IL-1 RA gene therapy. Invest Ophthalmol Vis Sci 2002; 43:2905-15.
24. Singh N, Amin S, Richter E, Rashid S, Scoglietti V, Jani PD, Wang J, Kaur R, Ambati J, Dong Z, Ambati BK. Flt-1 intraceptors inhibit hypoxia-induced VEGF expression in vitro and corneal neovascularization in vivo. Invest Ophthalmol Vis Sci 2005; 46:1647-52.
25. Hangai M, Tanihara H, Honda Y, Kaneda Y. Introduction of DNA into the rat and primate trabecular meshwork by fusogenic liposomes. Invest Ophthalmol Vis Sci 1998; 39:509-16.
26. Wasmuth S, Bauer D, Yang Y, Steuhl KP, Heiligenhaus A. Topical treatment with antisense oligonucleotides targeting tumor necrosis factor-alpha in herpetic stromal keratitis. Invest Ophthalmol Vis Sci 2003; 44:5228-34.
27. White RR, Shan S, Rusconi CP, Shetty G, Dewhirst MW, Kontos CD, Sullenger BA. Inhibition of rat corneal angiogenesis by a nuclease-resistant RNA aptamer specific for angiopoietin-2. Proc Natl Acad Sci U S A 2003; 100:5028-33.
28. Angella GJ, Sherwood MB, Balasubramanian L, Doyle JW, Smith MF, van Setten G, Goldstein M, Schultz GS. Enhanced short-term plasmid transfection of filtration surgery tissues. Invest Ophthalmol Vis Sci 2000; 41:4158-62.
29. Andrieu-Soler C, Berdugo M, Doat M, Courtois Y, BenEzra D, Behar-Cohen F. Downregulation of IRS-1 expression causes inhibition of corneal angiogenesis. Invest Ophthalmol Vis Sci 2005; 46:4072-8. Erratum in: Invest Ophthalmol Vis Sci. 2006 May;47(5):1784.
30. Kim B, Tang Q, Biswas PS, Xu J, Schiffelers RM, Xie FY, Ansari AM, Scaria PV, Woodle MC, Lu P, Rouse BT. Inhibition of ocular angiogenesis by siRNA targeting vascular endothelial growth factor pathway genes: therapeutic strategy for herpetic stromal keratitis. Am J Pathol 2004; 165:2177-85.
31. Cordeiro MF, Mead A, Ali RR, Alexander RA, Murray S, Chen C, York-Defalco C, Dean NM, Schultz GS, Khaw PT. Novel antisense oligonucleotides targeting TGF-beta inhibit in vivo scarring and improve surgical outcome. Gene Ther 2003; 10:59-71.
32. Yoon KC, Ahn KY, Lee JH, Chun BJ, Park SW, Seo MS, Park YG, Kim KK. Lipid-mediated delivery of brain-specific angiogenesis inhibitor 1 gene reduces corneal neovascularization in an in vivo rabbit model. Gene Ther 2005; 12:617-24.
33. Nakamura H, Siddiqui SS, Shen X, Malik AB, Pulido JS, Kumar NM, Yue BY. RNA interference targeting transforming growth factor-beta type II receptor suppresses ocular inflammation and fibrosis. Mol Vis 2004; 10:703-11.
34. Dvorchik BH, Marquis JK. Disposition and toxicity of a mixed backbone antisense oligonucleotide, targeted against human cytomegalovirus, after intravitreal injection of escalating single doses in the rabbit. Drug Metab Dispos 2000; 28:1255-61.
35. Shen WY, Garrett KL, Wang CG, Zhang K, Ma ZZ, Constable IJ, Rakoczy PE. Preclinical evaluation of a phosphorothioate oligonucleotide in the retina of rhesus monkey. Lab Invest 2002; 82:167-82.

36. Dezawa M, Takano M, Negishi H, Mo X, Oshitari T, Sawada H. Gene transfer into retinal ganglion cells by in vivo electroporation: a new approach. Micron 2002; 33:1-6.
37. Mo X, Yokoyama A, Oshitari T, Negishi H, Dezawa M, Mizota A, Adachi-Usami E. Rescue of axotomized retinal ganglion cells by BDNF gene electroporation in adult rats. Invest Ophthalmol Vis Sci 2002; 43:2401-5.
38. Kachi S, Oshima Y, Esumi N, Kachi M, Rogers B, Zack DJ, Campochiaro PA. Nonviral ocular gene transfer. Gene Ther 2005; 12:843-51.
39. Hangai M, Tanihara H, Honda Y, Kaneda Y. In vivo delivery of phosphorothioate oligonucleotides into murine retina. Arch Ophthalmol 1998; 116:342-8. Erratum in: Arch Ophthalmol 1998; 116:976.
40. Robinson GS, Pierce EA, Rook SL, Foley E, Webb R, Smith LE. Oligodeoxynucleotides inhibit retinal neovascularization in a murine model of proliferative retinopathy. Proc Natl Acad Sci U S A 1996; 93:4851-6.
41. Shen WY, Garrett KL, da Cruz L, Constable IJ, Rakoczy PE. Dynamics of phosphorothioate oligonucleotides in normal and laser photocoagulated retina. Br J Ophthalmol 1999; 83:852-61.
42. Roy S, Zhang K, Roth T, Vinogradov S, Kao RS, Kabanov A. Reduction of fibronectin expression by intravitreal administration of antisense oligonucleotides. Nat Biotechnol 1999; 17:476-9.
43. Garrett KL, Shen WY, Rakoczy PE. In vivo use of oligonucleotides to inhibit choroidal neovascularisation in the eye. J Gene Med 2001; 3:373-83.
44. Isenmann S, Engel S, Gillardon F, Bahr M. Bax antisense oligonucleotides reduce axotomy-induced retinal ganglion cell death in vivo by reduction of Bax protein expression. Cell Death Differ 1999; 6:673-82.
45. Rakoczy PE, Lai MC, Watson M, Seydel U, Constable I. Targeted delivery of an antisense oligonucleotide in the retina: uptake, distribution, stability, and effect. Antisense Nucleic Acid Drug Dev 1996; 6:207-13.
46. Leeds JM, Henry SP, Truong L, Zutshi A, Levin AA, Kornbrust D. Pharmacokinetics of a potential human cytomegalovirus therapeutic, a phosphorothioate oligonucleotide, after intravitreal injection in the rabbit. Drug Metab Dispos 1997; 25:921-6.
47. Vitravene Study Group. Safety of intravitreous fomivirsen for treatment of cytomegalovirus retinitis in patients with AIDS. Am J Ophthalmol 2002; 133:484-98.
48. Vitravene Study Group. A randomized controlled clinical trial of intravitreous fomivirsen for treatment of newly diagnosed peripheral cytomegalovirus retinitis in patients with AIDS. Am J Ophthalmol 2002; 133:467-74.
49. Vitravene Study Group. Randomized dose-comparison studies of intravitreous fomivirsen for treatment of cytomegalovirus retinitis that has reactivated or is persistently active despite other therapies in patients with AIDS. Am J Ophthalmol 2002; 133:475-83.
50. Eyetech Study Group. Preclinical and phase 1A clinical evaluation of an anti-VEGF pegylated aptamer (EYE001) for the treatment of exudative age-related macular degeneration. Retina 2002; 22:143-52.
51. Drolet DW, Nelson J, Tucker CE, Zack PM, Nixon K, Bolin R, Judkins MB, Farmer JA, Wolf JL, Gill SC, Bendele RA. Pharmacokinetics and safety of an anti-vascular endothelial growth factor aptamer (NX1838) following injection into the vitreous humor of rhesus monkeys. Pharm Res 2000; 17:1503-10.
52. Siddiqui MA, Keating GM. Pegaptanib: in exudative age-related macular degeneration. Drugs 2005; 65:1571-7;discussion1578-9.
53. Ishida S, Usui T, Yamashiro K, Kaji Y, Ahmed E, Carrasquillo KG, Amano S, Hida T, Oguchi Y, Adamis AP. VEGF164 is proinflammatory in the diabetic retina. Invest Ophthalmol Vis Sci 2003; 44:2155-62.
54. Bejjani RA, Jeanny JC, Bochot A, Behar-Cohen F. [The use of liposomes as intravitreal drug delivery system]. J Fr Ophtalmol 2003; 26:981-5.
55. Fattal E, De Rosa G, Bochot A. Gel and solid matrix systems for the controlled delivery of drug carrier-associated nucleic acids. Int J Pharm 2004; 277:25-30.
56. Ogata N, Otsuji T, Matsushima M, Kimoto T, Yamanaka R, Takahashi K, Wada M, Uyama M, Kaneda Y. Phosphorothioate oligonucleotides induction into experimental choroidal neovascularization by HVJ-liposome system. Curr Eye Res 1999; 18:261-9.
57. Bourges JL, Gautier SE, Delie F, Bejjani RA, Jeanny JC, Gurny R, BenEzra D, Behar-Cohen FF. Ocular drug delivery targeting the retina and retinal pigment epithelium using polylactide nanoparticles. Invest Ophthalmol Vis Sci 2003; 44:3562-9.
58. Bejjani RA, BenEzra D, Cohen H, Rieger J, Andrieu C, Jeanny JC, Gollomb G, Behar-Cohen FF. Nanoparticles for gene delivery to retinal pigment epithelial cells. Mol Vis 2005; 11:124-32.
59. Irache JM, Merodio M, Arnedo A, Camapanero MA, Mirshahi M, Espuelas S. Albumin nanoparticles for the intravitreal delivery of anticytomegaloviral drugs. Mini Rev Med Chem 2005; 5:293-305.
60. Marano RJ, Toth I, Wimmer N, Brankov M, Rakoczy PE. Dendrimer delivery of an anti-VEGF oligonucleotide into the eye: a long-term study into inhibition of laser-induced CNV, distribution, uptake and toxicity. Gene Ther 2005; 12:1544-50.
61. Normand N, van Leeuwen H, O'Hare P. Particle formation by a conserved domain of the herpes simplex virus protein VP22 facilitating protein and nucleic acid delivery. J Biol Chem 2001; 276:15042-50.
62. Normand N, Valamanesh F, Savoldelli M, Mascarelli F, BenEzra D, Courtois Y, Behar-Cohen F. VP22 light controlled delivery of oligonucleotides to ocular cells in vitro and in vivo. Mol Vis 2005; 11:184-91.
63. Shen WY, Rakoczy PE. Uptake dynamics and retinal tolerance of phosphorothioate oligonucleotide and its direct delivery into the site of choroidal neovascularization through subretinal administration in the rat. Antisense Nucleic Acid Drug Dev 2001; 11:257-64.
64. Neuner-Jehle M, Berghe LV, Bonnel S, Uteza Y, Benmeziane F, Rouillot JS, Marchant D, Kobetz A, Dufier JL, Menasche M, Abitbol M. Ocular cell transfection with the human basic fibroblast growth factor gene delays photoreceptor cell degeneration in RCS rats. Hum Gene Ther 2000; 11:1875-90.
65. Zhu C, Zhang Y, Pardridge WM. Widespread expression of an exogenous gene in the eye after intravenous administration. Invest Ophthalmol Vis Sci 2002; 43:3075-80.
66. Zhang Y, Schlachetzki F, Li JY, Boado RJ, Pardridge WM. Organ-specific gene expression in the rhesus monkey eye following intravenous non-viral gene transfer. Mol Vis 2003; 9:465-72.
67. Garcia-Valenzuela E, Rayanade R, Perales JC, Davidson CA, Hanson RW, Sharma SC. Axon-mediated gene transfer of retinal ganglion cells in vivo. J Neurobiol 1997; 32:111-22.
68. Lingor P, Koeberle P, Kugler S, Bahr M. Down-regulation of apoptosis mediators by RNAi inhibits axotomy-induced retinal ganglion cell death in vivo. Brain 2005; 128:550-8.

69. Curdy C, Kalia YN, Guy RH. Post-iontophoresis recovery of human skin impedance in vivo. Eur J Pharm Biopharm 2002; 53:15-21.
70. Davies JB, Ciavatta VT, Boatright JH, Nickerson JM. Delivery of several forms of DNA, DNA-RNA hybrids, and dyes across human sclera by electrical fields. Mol Vis 2003; 9:569-78.
71. Asahara T, Shinomiya K, Naito T, Shiota H. Induction of gene into the rabbit eye by iontophoresis: preliminary report. Jpn J Ophthalmol 2001; 45:31-9.
72. Shuler RK Jr, Dioguardi PK, Henjy C, Nickerson JM, Cruysberg LP, Edelhauser HF. Scleral permeability of a small, single-stranded oligonucleotide. J Ocul Pharmacol Ther 2004; 20:159-68.
73. Voigt M, de Kozak Y, Halhal M, Courtois Y, Behar-Cohen F. Down-regulation of NOSII gene expression by iontophoresis of anti-sense oligonucleotide in endotoxin-induced uveitis. Biochem Biophys Res Commun 2002; 295:336-41.
74. Millington-Ward S, O'Neill B, Tuohy G, Al-Jandal N, Kiang AS, Kenna PF, Palfi A, Hayden P, Mansergh F, Kennan A, Humphries P, Farrar GJ. Strategems in vitro for gene therapies directed to dominant mutations. Hum Mol Genet 1997; 6:1415-26.
75. Matsuda T, Cepko CL. Electroporation and RNA interference in the rodent retina in vivo and in vitro. Proc Natl Acad Sci U S A 2004; 101:16-22.
76. Oshima Y, Sakamoto T, Yamanaka I, Nishi T, Ishibashi T, Inomata H. Targeted gene transfer to corneal endothelium in vivo by electric pulse. Gene Ther 1998; 5:1347-54.
77. Sakamoto T, Oshima Y, Nakagawa K, Ishibashi T, Inomata H, Sueishi K. Target gene transfer of tissue plasminogen activator to cornea by electric pulse inhibits intracameral fibrin formation and corneal cloudiness. Hum Gene Ther 1999; 10:2551-7.
78. Oshima Y, Sakamoto T, Hisatomi T, Tsutsumi C, Sassa Y, Ishibashi T, Inomata H. Targeted gene transfer to corneal stroma in vivo by electric pulses. Exp Eye Res 2002; 74:191-8.
79. Blair-Parks K, Weston BC, Dean DA. High-level gene transfer to the cornea using electroporation. J Gene Med 2002; 4:92-100.
80. Mamiya K, Ohguro H, Ohguro I, Metoki T, Ishikawa F, Yamazaki H, Takano Y, Ito T, Nakazawa M. Effects of matrix metalloproteinase-3 gene transfer by electroporation in glaucoma filter surgery. Exp Eye Res 2004; 79:405-10.
81. Yu WZ, Li XX, She HC, He PY, Dong JQ, Rui M, Ma DL. Gene transfer of kringle 5 of plasminogen by electroporation inhibits corneal neovascularization. Ophthalmic Res 2003; 35:239-46.
82. Ishikawa H, Takano M, Matsumoto N, Sawada H, Ide C, Mimura O, Dezawa M. Effect of GDNF gene transfer into axotomized retinal ganglion cells using in vivo electroporation with a contact lens-type electrode. Gene Ther 2005; 12:289-98.
83. Chalberg TW, Genise HL, Vollrath D, Calos MP. phiC31 integrase confers genomic integration and long-term transgene expression in rat retina. Invest Ophthalmol Vis Sci 2005; 46:2140-6.
84. Chen YX, Krull CE, Reneker LW. Targeted gene expression in the chicken eye by in ovo electroporation. Mol Vis 2004; 10:874-83.
85. Bloquel C, Bejjani R, Bigey P, Bedioui F, Doat M, BenEzra D, Scherman D, Behar-Cohen F. Plasmid electrotransfer of eye ciliary muscle: principles and therapeutic efficacy using hTNF-alpha soluble receptor in uveitis. FASEB J 2006; 20:389-91.
86. Tanelian DL, Barry MA, Johnston SA, Le T, Smith G. Controlled gene gun delivery and expression of DNA within the cornea. Biotechniques 1997; 23:484-8.
87. Zagon IS, Sassani JW, Verderame MF, McLaughlin PJ. Particle-mediated gene transfer of opioid growth factor receptor cDNA regulates cell proliferation of the corneal epithelium. Cornea 2005; 24:614-9.
88. Kretz A, Wybranietz WA, Hermening S, Lauer UM, Isenmann S. HSV-1 VP22 augments adenoviral gene transfer to CNS neurons in the retina and striatum in vivo. Mol Ther 2003; 7:659-69.
89. Sands H, Gorey-Feret LJ, Cocuzza AJ, Hobbs FW, Chidester D, Trainor GL. Biodistribution and metabolism of internally 3H-labeled oligonucleotides. I. Comparison of a phosphodiester and a phosphorothioate. Mol Pharmacol 1994; 45:932-43.
90. Jepsen JS, Sorensen MD, Wengel J. Locked nucleic acid: a potent nucleic acid analog in therapeutics and biotechnology. Oligonucleotides 2004; 14:130-46.
91. Andrieu-Soler C, Casas M, Faussat AM, Gandolphe C, Doat M, Tempe D, Giovannangeli C, Behar-Cohen F, Concordet JP. Stable transmission of targeted gene modification using single-stranded oligonucleotides with flanking LNAs. Nucleic Acids Res 2005; 33:3733-42.
92. Campbell TN, Choy FY. RNA interference: past, present and future. Curr Issues Mol Biol 2005; 7:1-6.

The print version of this article was created on 30 Oct 2006. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.