Vitreous Is a Barrier in Nonviral Gene Transfer by Cationic Lipids and Polymers

Leena Pitkänen,[1,2] Marika Ruponen,[1] Jenni Nieminen,[1] and Arto Urtti[1,3]

Received May 13, 2002; accepted January 2, 2003

Purpose. To investigate the role of vitreous in nonviral gene delivery into retinal pigment epithelial (RPE) cells.

Methods. Human RPE cell line D407 was cultured in six-well plates. Bovine vitreous, hyaluronan, or DMEM was added on the cells. Complexes of DNA and cationic carriers (polyethyleneimine, poly-L-lysine, DOTAP liposomes) were pipetted onto the vitreous, hyaluronan, or DMEM. Cellular uptake of DNA was studied with ethidium monoazide DNA and gene expression with GFP–plasmid complexes. FITC-dextrans and FITC-polylysines were used to probe the effects of the size and cationic charge on permeation in the vitreous in a similar experimental setup. Fluorescent cells were analyzed by flow cytometry.

Results. Vitreous decreased the cellular uptake of DNA complexes 2–30 times, and GFP expression was also impaired. In hyaluronan solutions the cellular uptake of the complexes was also decreased significantly in most cases. In vitreous, cellular uptake of all FITC-dextrans decreased slightly, and uptake of poly-L-lysines was decreased substantially, whereas in hyaluronan solutions the effects were mild or nonexistent.

Conclusions. Polymeric and liposomal gene delivery is substantially limited by the vitreous. This is probably because of the size and charge of the retinal gene delivery after intravitreal injections.

KEY WORDS: gene transfer; intravitreal drug delivery; vitreoretinal pharmacology; liposome; polymer.

INTRODUCTION

Transfer of therapeutic genes to the retina may open new possibilities in the treatment of severe eye diseases. Potential targets of gene therapy include age-related macular degeneration, proliferative vitreoretinopathy, retinal and choroidal neovascularization, and retinitis pigmentosa. For example, gene therapies based on neurotropins and growth factors are possible treatments against retinal degenerations (1–3), and angiostatic factors have potential in the treatment of neovascularization (4,5).

Safe and efficient gene transfer is a prerequisite for gene therapy. Genes have been successfully transferred to the neural retina and retinal pigment epithelial (RPE) cells with various viral vectors *in vitro* and *in vivo* (6–10). However, safety concerns may limit the use of some viral vectors, such as adenovirus, in retinal gene therapy. Alternative nonviral systems are based on cationic lipids or cationic polymers. They are considered to be safe and biocompatible, but they transfer genes *in vivo* at lower efficacy than many viral vectors. Although there are some published data on nonviral gene transfer *in vivo* in animal eyes (11,12) and *in vitro* in retinal pigment epithelial cells (13,14), the specific ocular factors and mechanisms of nonviral gene transfer in posterior segment have not been studied.

Liposomal and polymeric vectors form complexes with DNA by electrostatic forces. Positive surface charge of the complexes facilitates their attachment on the cell surface and, subsequently, DNA delivery into the cells. First, the complexes must be able to reach the target cells. Because of pharmacokinetic limitations, intravenous administration of the complexes is not a practical choice in retinal gene delivery. Likewise, topical ocular administration of the complexes seems impossible because even small-molecular-weight drugs show very limited permeation from the ocular surface to the retina (15–17). Subretinal administration locates the gene transfer complexes in the vicinity of retina, but this is technically demanding and might not be a clinically feasible route of gene delivery.

Intravitreal injection would be safer and easier than subretinal injections, but the vitreous might interact with the nonviral gene delivery systems. The vitreous contains water (98%) and colloids (0.1%), and the rest of the solid material is ions and low-molecular-weight solutes. The two major structural components are collagen (40–120 μg/ml) and hyaluronic acid (100–400 μg/ml). A number of noncollagenous proteins have also been isolated (18). Hyaluronan is the most common negatively charged glycosaminoglycan in human vitreous, but it also contains chondroitin sulfate and possibly heparan sulfate (19). These glycosaminoglycans are known to interact with polymeric and liposomal DNA complexes (20). Vitreous is a complicated three-dimensional network of collagens, GAGs, and noncollagenous structural proteins. The structure of the vitreous functions as a molecular sieve that may particularly restrict the diffusion of large molecules (17,19). In general, only sparse information is available about the influence of extracellular barriers on the transport of macromolecular drugs and drug delivery systems (21).

We investigated the effects of bovine vitreous and hyaluronan on gene transfer with liposomal (DOTAP) and polymeric poly-L-lysine (PLL) and polyethyleneimine (PEI) DNA complexes to the cultured D407 RPE-cell line. We also used FITC-dextrans and FITC-poly-L-lysines to investigate the effects of the size and charge of macromolecules on their permeation in the vitreous.

MATERIALS AND METHODS

Hyaluronan and Vitreous

Hyaluronic acid sodium (H 1876; molecular weight 3.0–5.8 × 10^6) from Sigma (St. Louis, MO) was dissolved in Dulbecco's Modified Eagles Medium (DMEM, high glucose,

[1] Department of Pharmaceutics, University of Kuopio, Kuopio, Finland.

[2] Department of Ophthalmology, Kuopio University Hospital, Kuopio, Finland.

[3] To whom correspondence should be addressed. (e-mail: Arto.Urtti@uku.fi)

ABBREVIATIONS: DMEM, Dulbecco's Modified Eagles Medium; DOTAP, 1,2-dioleyl-3-trimethylammonium propane; PEI, polyethyleneimine; PLL, poly-L-lysine; GFP, green fluorescent protein; FITC, fluorescein isothiocyanate; RPE, retinal pigment epithelium; GAG, glycosaminoglycan; kDa, kilodalton; mM, millimolar; CMV, cytomegalovirus; PBS, phosphate-buffered saline; rpm, rounds per minute; EMA, ethidium monoazide.

0724-8741/03/0400-0576/0 © 2003 Plenum Publishing Corporation

41966, GibcoBRL Life Technologies, Paisley, Scotland) at concentrations of 0.3 mg/ml and 1.0 mg/ml.

The bovine eyes were obtained from a local slaughterhouse. Fresh bovine eyes were cleaned of extraocular material and opened circumferentially about 8 mm behind the limbus; the anterior tissues were removed, and the vitreous was separated gently from the neural retina. Vitreous was pushed with a syringe (opening diameter 1.5 mm) through a nylon mesh (holes 1 mm × 1 mm) to facilitate handling and administration into the cell culture plates.

DNA Complexes with Liposomes and Polymers

PEI with a mean molecular weight of 25,000 was obtained from Aldrich (St. Louis, MO) and was used as 10 mM aqueous stock solution (22). PLLs with mean molecular weights of 20,000 and 200,000 were from Sigma, and they were diluted with water (3 mg/ml). PLL of mean molecular weight 200,000 was used for complexation. 1,2-Dioleyl-3-trimethylammonium-propane (DOTAP) was purchased from Avanti Polar Lipids (Pelham, AL). Cationic liposomes composed of DOTAP were prepared by evaporating a chloroform solution of lipids, resuspending the lipid in water at a concentration of 3.2 mM, and sonication under argon until a translucent lipid solution was obtained.

The green fluorescent protein GFP S65T mutant was excised from pTR5-DC/GFP plasmid (a gift from Dr. D. D. Mosser, Montreal, Canada) (23) as a BamHI fragment that was inserted into the BamHI-site of a CMV-driven pCR3-plasmid (InVitrogen, Carlsbad, CA) to yield the plasmid (pGFP) that was used in the complexes. The pGFP was labeled with ethidium monoazide (EMA, Molecular Probes, Eugene, OR), which forms covalent bonds with DNA bases during photoactivation. The labeling procedure has been described previously (24). Briefly, EMA in water (5 μg/ml) was added to GFP-expressing plasmid in water (200 μg/ml). After incubation (10 min) at room temperature, the solution was exposed to UV light (wavelength 312 nm) for 2 min. Gel filtration on NAP-10 columns (Pharmacia) was used to purify labeled DNA from free EMA. To remove intercalated but not covalently bound EMA, cesium chloride was added to a concentration of 1.1 g/ml, and the plasmid was extracted with CsCl-saturated isopropanol. CsCl was removed by dialysis against Tris-EDTA buffer, and the labeled EMA–DNA plasmid was recovered by ethanol precipitation.

The reporter gene plasmid that encodes β-galactosidase under the control of cytomegalovirus promoter was a gift from Dr. F. C. Szoka, Jr. (University of California San Francisco, CA) (25).

Plasmid DNA (pGFP) and the cationic polymers or cationic liposomes were both diluted first in 5% glucose solution. Solutions of DNA and carrier were mixed before transfection to yield complexes at charge ratios of 2:1 or 4:1 (positive charges of carrier over negative charges of DNA; DNA 20 μg/ml). DNA–carrier complexes were prepared at room temperature, and before transfection the solutions were allowed to remain for at least 20 min.

The sizes of the complexes were determined with a NICOMP 380 submicron particle sizer that is based on light scattering (NICOMP Particle Sizing Systems Inc., Santa Barbara, CA). For the measurements PEI, PLL, and DOTAP were complexed with plasmid DNA (pGFP) as described above. The complexes were made in 5% glucose, and the concentration of DNA was 20 μg/ml. Size distributions were assessed on the basis of NICOMP volume-weighted analysis.

The FITC-Dextrans

The FITC-dextran with mean molecular weight of 4400 was from Sigma, and the FITC- dextrans with mean molecular weights 70,000, 500,000 and 2,000,000 were from Molecular Probes. Different concentrations of FITC-dextrans were tested in order to avoid saturation of their cellular uptake in flow cytometer measurements. FITC-dextrans were dissolved in DMEM at 1.5 mg/ml.

The molecular size of the FITC-dextrans was measured with a NICOMP 380 submicron particle sizer at 3 mg/ml, and the mean diameter of the molecules was assessed on the basis of NICOMP number-weighted analysis.

FITC-Labeled Poly-L-Lysines

FITC-labeled poly-L-lysine (PLL) of mean molecular weight 20,000 and unlabeled PLLs of mean molecular weights 20,000 and 200,000 were purchased from Sigma. PLL of MW 200,000 was labeled with FITC. First, PLL was diluted in 0.05 M sodium bicarbonate (5 mg/150 μl), and FITC-label was diluted in the same buffer (1 mg/ml). The solutions were combined and stirred for 2 h at +5°C. Free label was separated by gel filtration (NAP 10 Column, Sephadex® G-25 1.3 × 2.6 cm, Pharmacia Biotech, Uppsala Sweden). Labeled PLL was precipitated with ethanol, centrifuged, and dissolved in sterile water. Dialysis was performed overnight in PBS at +4°C in darkness. Precipitation was repeated as described above, and yield was determined. The FITC-labeled PLLs were used at a concentration of 6.25 μg/ml, and 744 μg/ml of unlabeled PLL was added. PLL solutions were diluted in DMEM before testing.

Cell Culture and Transfection

The D407 cell line (human retinal pigment epithelial cells) (26) was a gift from the laboratory of Dr. Richard Hunt (University of South Carolina, Medical School, Columbia, SC). The D407 RPE cells were cultured in DMEM supplemented with 1% penicillin–streptomycin (Gibco BRL, Grand Island, NY), 5% fetal bovine serum (Gibco), and 2 mM L-glutamine (Gibco) at +37°C in 7% CO_2.

One day before a cellular uptake or transfection experiment the cells were divided into six-well plates (Costar, Radnor, PA), 350,000 cells per well. DMEM culture medium was aspirated, the cells were washed with PBS, and 1 ml (about a 1-mm-thick layer) of vitreous, hyaluronan solution (0.3 mg/ml or 1.0 mg/ml) or DMEM was added on the cells. Then, 150 μl of DNA-complexes, FITC-dextran, or FITC-PLL was pipetted carefully onto the surface of the wells. For cellular uptake experiments EMA-labeled plasmid was used, and GFP-coding plasmid was complexed in transfection experiments. Three different treatments were used at +37°C: incubation of 5 or 48 h without stirring and 5 h with stirring (horizontal rotational movement with diameter 2.5 cm, at 50 rpm). For the measurements of uptake the cells were washed twice with PBS, detached from the bottom of the well with trypsin-EDTA (Gibco) (5 min at +37°C), and fixed with 1% paraformaldehyde. In GFP-transfections the cells were washed

twice with PBS after incubation, and the culture medium was added for 24 h. Thereafter, the cells were treated as described above.

Flow Cytometry Analysis

Cellular uptake and transgene expression were measured with a fluorescence-activated cell sorter (FACS-scan flow cytometry, Becton Dickinson, San Jose, CA) with an argon ion laser (488 nm) as the excitation source. Fluorescence of GFP was collected at 525 nm (FL1) and fluorescence of EMA was collected at 670 nm (FL3). For each sample, 10,000 events were collected. The cells cultured under normal culture conditions (control cells) were visualized on a FSC (Forward Angle Light Scatter) vs. SSC (90 degrees light scatter) display. Those cells that were living before fixation were selected for analysis by gating the major cell population. Only these cells were analyzed.

Cellular Uptake of EMA–DNA

EMA–DNA was used as a marker for intracellular delivery of DNA as described previously (24). The number of positive events was analyzed from the FL3 histogram. The gate of positive events for each carrier was adjusted according to the negative control (i.e., transfections were carried out with unlabeled β-galactosidase DNA–carrier complexes). The percentage of positive cells was calculated as the number of positive events in FL3 divided by the total number of events in the gate of living cells.

GFP Expression

The number of GFP-positive cells was analyzed from the FL1 vs. FL3 dot plot. The positive events were separated from the autofluorescence by setting a gate, and the percentage of positive cells among living cells was calculated.

Confocal Microscopy

An additional experiment was made to clarify the role of diffusion in the vitreal barrier. Divided cover glasses (Lab-Tek II Chambered coverglass, Nalge Nunc International, Naperville, IL) were covered by FITC-poly-L-lysine. First, they were incubated for 1 h with 400 μl of 10 μg/ml FITC-PLL of molecular weight 20,000 in water at + 37°C. Then the excess of FITC-PLL was pipetted away, and the glass was allowed to dry. About 1 mm thickness of vitreous (190 μl) was pipetted onto the wells, and 28 μl of DOTAP, PLL, and PEI complexed with rhodamine-labeled DNA (Rhodamine/β-Gal, pGeneGrip, Gene Therapy Systems, Inc., San Diego, CA) at charge ratios ±2 and ±4 were added on the vitreous. After 2 h of incubation at +37°C, the sample was examined with confocal microscopy on an UltraVIEW confocal imaging system (Perkin-Elmer Life Sciences, Boston, MA) with an Eclipse TE 300 microscope (Nikon, Melville, NY) using a 100 × oil immersion objective (Plan Fluor, Nikon). The bottom of the glass was detected by the FITC-PLL dots that were imaged by using the 488-nm excitation line of krypton/argon laser, and green fluorescence was detected at 515–545 nm. Rhodamine-labeled DNA was detected at 590–610 nm after excitation at 568 nm. Exposure times were between 0.4 and 0.7 s, and confocal images were collected with a cooled digital charge-coupled device camera (Perkin Elmer Life Sciences). Ten serial images with fluorescein and rhodamine fluorescence at about 25 μm Z intervals were recorded and then colocalized. Thus, the topmost images were about 250 nm upward from the bottom of the glass. Images were processed and analyzed by using the confocal assistant software program (UltraVIEW). To get a semiquantitative conception of the diffusion of the complexes, the rhodamine fluorescent dots were counted from the two lowest and two topmost images of each sample. For controls, the same amount of each complex solution (28 μl) was mechanically mixed evenly into 95 μl of vitreous and, thereafter, handled and detected as described above.

Determination of Nucleases in Vitreous and Hyaluronan

Plasmid that encodes β-galactosidase under the control of cytomegalovirus promoter (10 μg/2.3 μl) and DMEM, hyaluronan (1 mg/ml in DMEM), or vitreous that had been handled as described before were mixed (at 200 μl/sample). The solutions were incubated at +37°C. At time points 0, 2.5, 5, 24, and 48 h, the reactions were stopped by 50 μl of 5× bromophenol blue (30% glycerol, 0.25% SDS, 50 mM Tris-HCl, 50 mM NaCl, 20 mM EDTA, 0.1% bromophenol blue) and stored at −20°C until gel electrophoresis. Aliquots of 18 μl of the samples were pipetted into the wells of gel (1% agarose in 1% TAE). The gel electrophoresis was performed in 1% TAE buffer with ethidium bromide by EPS 600 (Pharmacia Biotech) (75 V, 1 h). For detection of effect of nucleases on complexed DNA, β-galactosidase-coding plasmid was complexed with PEI, PLL, and DOTAP at charge ratios ±2 and ±4 as described before, and 125 μl of the complex solution was mixed with 75 μl of DMEM, 1mg/ml hyaluronan, or vitreous. Samples were taken at time points 0 min, 24 h, and 48 h, and gel electrophoresis was performed as described above.

Viscosity Measurements

The viscosities of 5% glucose (1.1418 mPa), DMEM (1.0380 mPa), and hyaluronan (0.3 mg/ml 1.211 mPa and 1.0 mg/ml 1.7646 mPa) were measured by capillary viscometer. The viscosity of vitreous [7–24 mPa, depending on the shear rate (6–100 rpm) at +21°C] was measured by rotation viscometer (Brookfield, Middleboro, MA).

Statistical Analysis of FACS Data

The Mann–Whitney *U*-test was used for statistical analysis. The results of experiments with FITC-dextrans of molecular weights 500,000 and 2,000,000 were also analyzed with a *t* test.

RESULTS

Cellular Uptake of DNA Complexes

According to the FACS analysis, 35–42% of D407 cells took up PEI/DNA complexes, and the cellular uptakes of DOTAP/DNA and PLL complexes were about 20% and 6–15% over 5 h in DMEM (Fig. 1). In the presence of vitreous, the cellular uptake of all carrier/DNA complexes decreased to less than 2% ($p < 0.005$), and hyaluronan de-

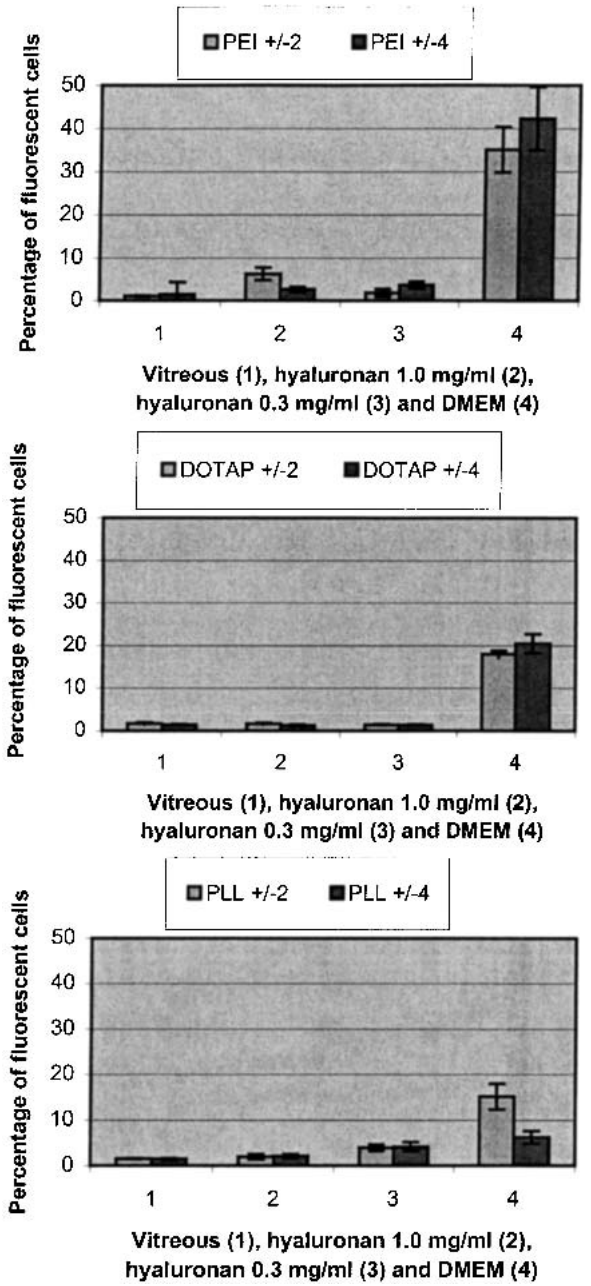

Fig. 1. Uptake of carrier–DNA complexes by D407 cells after 5 h of incubation. All three carriers (PEI, DOTAP, and PLL) were examined at charge ratios ±2 and ±4 in vitreous (1), hyaluronan 1.0 mg/ml (2), hyaluronan 0.3 mg/ml (3), and DMEM (4). Means ± S.E.M. (n = 6–10) are shown.

creased the uptake of DNA significantly ($p < 0.05$) (Fig. 1). The only exception was PLL ±4 in 0.3 mg/ml hyaluronan. Decreased ($p < 0.01$) cellular uptake of the complexes was also seen when stirring was used during the 5 h of incubation (Fig. 2). Decreased uptake of labeled DNA in the complexes was not dependent on incubation time. Vitreous significantly decreased ($p < 0.005$) the cellular uptake of all complexes during incubations of 48 h.

The overall range of relative changes in cellular uptake, compared to cellular uptake levels in DMEM, were the following: 4- to 30-fold decrease in the vitreous over 5 h, 4- to 14-fold decrease over 48 h, and 2- to 30-fold decrease over 5 h in the presence of stirred vitreous.

Transfection Efficacy

GFP expression was below 1% of the cells after incubation of 5 h with the complexes in DMEM (Fig. 3). Notably, with PLL ±4 and PEI ±2 complexes, the transfection efficacy was also practically zero (< 0.06%) in the DMEM experiment (Fig. 3). Vitreous and hyaluronan decreased the expression levels of GFP to practically zero ($p < 0.01$) (Fig. 3). Blocked transfection was not dependent on incubation period. During 48 h of incubation, the fraction of GFP-expressing cells decreased 30–85 times from 0.2–0.4% in DMEM to less than 0.02% in the vitreous (data not shown; $p < 0.005$).

When stirring was used during incubation, PEI ±2 and PEI ±4 complexes caused GFP expression in 4.5 ± 1.6% (SEM) and 9.7 ± 1.3% (SEM) of the cells, respectively. The other complexes mediated transfection levels of less than 1%. Again, the vitreous decreased the GFP-transfection by about 2 orders of magnitude to less than 0.01% in all cases ($p < 0.01$) (data not shown).

Cellular Uptake of FITC-Dextrans

FITC-dextrans were used as probe molecules to evaluate the effects of molecular size on permeation in vitreous. The cellular uptake of FITC-dextrans of molecular weights 4400 and 70,000 was decreased by vitreous from almost 100% to 65% ($p < 0.005$). At higher molecular weights (500,000 and 2,000,000), the vitreous decreased the cellular uptake from 60–70% to about 35% ($p < 0.005$) (Fig. 4). Hyaluronan 0.3 and 1.0 mg/ml caused only modest and mostly not significant changes in the cellular uptake of FITC-dextrans.

Cellular Uptake of FITC-PLL

The cellular uptake of positively charged FITC-labeled PLL 20,000 and 200,000 were 99% and 76% in DMEM, respectively. Vitreous decreased the cellular uptake of FITC-PLL significantly ($p < 0.005$) (Fig. 5). The cellular uptake of FITC-PLL probes was affected by the vitreous more than the uptake of any FITC-dextran ($p < 0.005$) (Fig. 5).

The cellular uptake of FITC-labeled PLL 20,000 was significantly decreased by hyaluronan ($p < 0.001$), although to lesser extent than by the vitreous (Fig .5). In the case of PLL 200,000 hyaluronan solutions did not cause significant decrease in the cellular uptake.

Sizes of the Complexes and Macromolecules

The mean diameters of the complexes were: 180 nm (PEI ±2), 110 nm (PEI ±4), 200 nm (DOTAP ±2), 90 nm (DOTAP ±4), 110 nm (PLL ±2), and 170 nm (PLL ±4). The sizes of PEI complexes varied especially greatly, and in number-weighted analysis there were mostly very small complexes (<10 nm). The mean diameter of the largest FITC-dextran (MW 2,000,000) was 30 nm, and that of other FITC-dextrans was below 10 nm.

Confocal Microscopy

In confocal microscopy rhodamine fluorescent particles were seen in the bottom of the vitreous in all samples, and

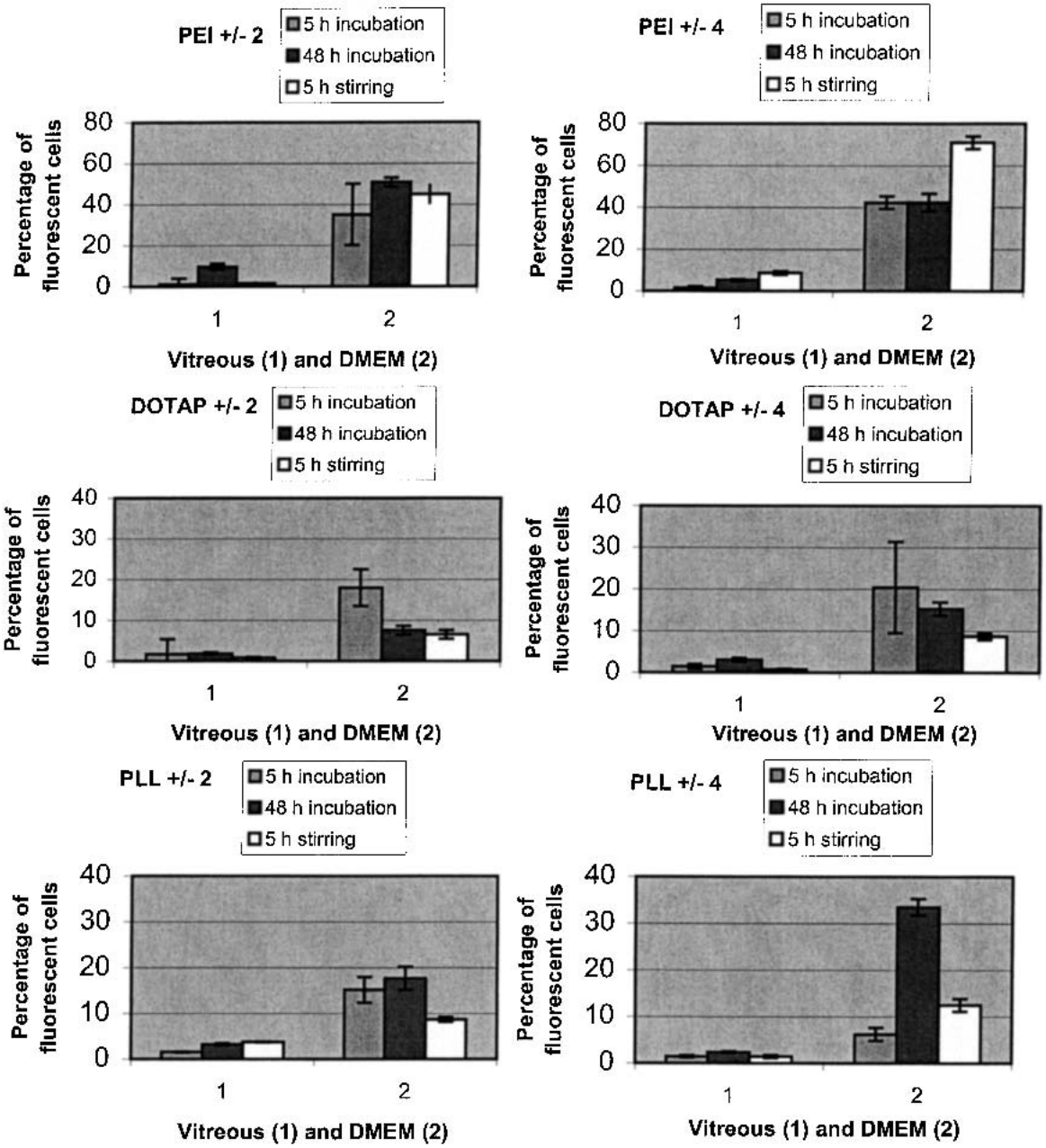

Fig. 2. Uptake of PEI, DOTAP, and PLL complexes by D407 cells at charge ratios ±2 and ±4 after incubation of 5 h (with and without stirring) and 48 h in vitreous and DMEM. Means ± S.E.M. (n = 5–10) are shown. Note the different scale of PEIs compared to other vehicles.

there were not remarkably more particles in the upper part of vitreous (about 250 μm upward from the bottom of the well) in any case. Compared to the mixed control samples there was no less rhodamine fluorescence in the bottom of unmixed vitreous samples.

Determination of Nucleases

No nuclease activity was seen in DMEM or hyaluronan solutions. In the vitreous there were possibly partial changes from the supercoiled DNA to circular plasmid after 2.5 and 5 h of incubation. After 24 and 48 h of incubation in the vitreous, the plasmid had degraded.

When DNA was complexed with DOTAP at a charge ratio ±2 or ±4, DNA was separated from complexes by the electric current and/or SDS (0.05%) present in samples. The separated DNA was detectable in electrophoresis, but the released DNA appeared to be intact even after 48 h of incubation. In the case of PLL and PEI complexes, no release of degradation of DNA was seen.

DISCUSSION

Therapeutic gene transfer into RPE and other retinal cells is a promising concept for treating many severe retinal diseases. For example, gene transfer into the RPE cells and subsequent secretion of neurotropic factors, growth factors, or inhibitors of neovascularization to the surrounding cells

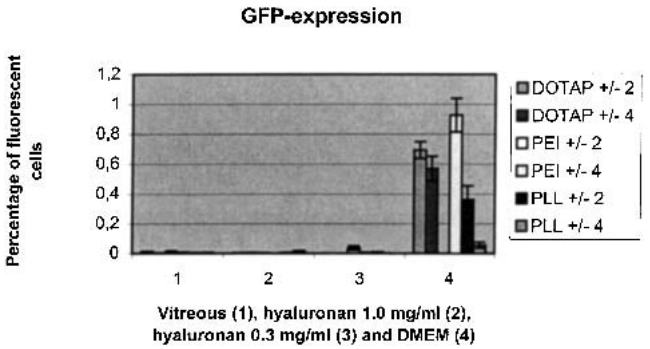

Fig. 3. GFP-expression in D407 cells mediated by DOTAP, PEI, and PLL complexes at charge ratios ±2 and ±4 after 5 h incubation with vitreous (1), hyaluronan 1.0 mg/ml (2), 0.3 mg/ml (3), and DMEM (4). Means ± S.E.M. are shown ($n = 5$).

has great therapeutic implications. Transfection of only a fraction of the RPE cells may be adequate, if the gene product is secreted and diffuses to the surrounding tissue.

Nonviral vectors are easier to produce than viral vectors, and they might be developed as potentially safer alternatives for the delivery of genes into the RPE cells. Although primary (13,14) and secondary RPE cells (D407) (27,28) have been transfected successfully with various liposomal and polymeric vectors, the efficacy of intravitreal *in vivo* transfections of the rat retinal cells is very low (unpublished data, 2000). However, nonviral systems can be modified in various ways. For novel strategies in nonviral gene delivery, it is important to know what the limiting factors are in gene transfer by the existing nonviral gene delivery systems.

Cationic liposomal or polymeric vectors and plasmid DNA form complexes by electrostatic forces. The diameters of the complexes are usually 20–300 nm, and their shape and morphology vary depending on the carrier, charge ratio, and medium (27–30). The complexes with positively charged surface are taken up by endocytosis into the target cells (30). After endocytosis the complexes or DNA must release from the endosomal vesicles and pass the plasmid DNA into nucleus of the cell. It is possible, e.g., to add ligands to the complexes, modify their DNA binding properties, or change the surface of the complexes with coating molecules. These coating molecules can be used to mask partly or completely

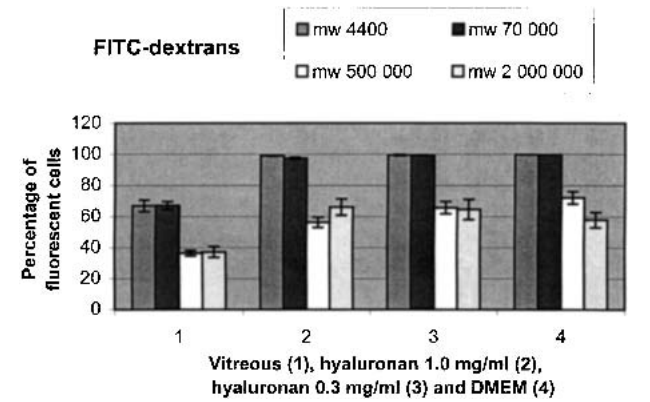

Fig. 4. Cellular uptake of FITC-dextrans of molecular weights 4400, 70,000, 500,000, and 2,000,000 in vitreous (1), hyaluronan 1.0 mg/ml (2), hyaluronan 0.3 mg/ml (3), and DMEM-medium (4). Means ± S.E.M. are illustrated ($n = 5$–27).

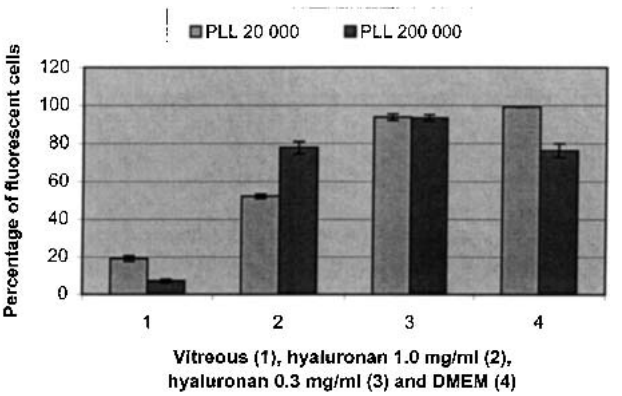

Fig. 5. Cellular uptake of PLL of molecular weights 20,000 and 200,000. Amounts of FITC-labeled and nonlabeled PLL are 6.25 μg/ml and 744 μg/ml, respectively. FITC-PLL was applied on (1) vitreous, (2) 1.0 mg/ml hyaluronan, (3) 0.3 mg/ml hyaluronan, and (4) DMEM. Means ± S.E.M ($n = 9$–12) are shown.

the positive surface charges. Overall, the properties of the complexes can be modified in many ways.

As a polyanionic gel, vitreous may affect the gene transfer in various ways. It might bind the positively charged complexes, reorganize their structure, release DNA from the complex too early, and even for inert complexes, the vitreous may act as a diffusional barrier that slows down the distribution of the complexes after the injection. We found that a layer of 1 mm of vitreous almost completely blocks the cellular uptake and transfection of the complexes at charge ratios of ±2 and ±4 (Figs. 1–2). Hyaluronan solutions also decreased the cellular uptake of complexes (Fig. 1), suggesting that the impaired cellular uptake of the complexes is partly caused by hyaluronan in vitreous. However, there may be additional factors (e.g., other glycosaminoglycans) in the vitreous contributing to the barrier effect.

Interactions between the GAGs and carrier–DNA complexes are dependent on the structures of GAG and on the gene delivery system (20). Polymeric and liposomal complexes of DNA bear cationic surface groups that may bind negatively charged GAGs. At a threefold excess of negative charges, chondroitin sulfates and heparan sulfate totally inhibited transfection of RAA smooth muscle cells *in vitro* with DOTAP, PEI, and PLL complexes (20). Apparently the cationic complexes were coated by these sulfated GAGs. In a similar experiment, hyaluronan partly or completely inhibited transfections mediated by PEI but not those mediated by DOTAP or PLL (20). When the complexes were coated with hyaluronan or heparan sulfate, the cellular uptake of DOTAP and PLL complexes did not decrease, but in the case of PEI, heparan sulfate blocked and hyaluronan decreased the cellular uptake (24). In the previous experiments (20,24) the complexes were coated with GAGs in solution without a gel layer, and, thus, no diffusional barrier was present between the cells and the complexes. In this study, hyaluronan and particularly the vitreous decreased the cellular uptake of all tested complexes. When complexes were made with rhodamine-labeled DNA and pipetted on a layer of vitreous, there were also rhodamine fluorescent particles in the bottom of the vitreal layer after 2 h of incubation, suggesting that the mechanism of the decrease in cellular uptake is not a simple diffusional barrier for intact complexes. The DNA may have been re-

leased, or the structure of the complexes may have changed in a way that the complexes are not taken into cells. Various lipoplexes and polyplexes are known to interact differently with GAGs, and, for example, PEI complexes may release DNA in the presence of sulfated GAGs (20).

In vivo the vitreous is not a completely stagnant gel. Intravitreal flow causes some convection, which may affect drug transport in the posterior segment (31). Ocular movements also cause vitreal movement (32). Therefore, experiments with strirring were conducted. Again, vitreous blocked the cellular uptake of the complexes almost completely (Fig. 2). A network structure of vitreous restricts the passage of big molecules, and the density of vitreous is highest in the marginal parts. The basal layer of the vitreous allows passage of molecules of mean molecular size of 15–20 nm and smaller (33). In order to elucidate the importance of complex size in the limited permeation in the vitreous, we investigated FITC-dextrans in similar conditions. In the presence of vitreous the cellular uptake levels of the FITC-dextrans with mean molecular weights 500,000 and 2,000,000 were smaller than those of FITC-dextrans 4400 and 70,000. The size of FITC-dextran 2,000,000 (diameter 30 nm) is in the same range as number-weighted size of some complexes (DOTAP ±4; 50 nm) and larger than PEI–DNA complexes at ±2 (<10 nm). Still, vitreous decreased the permeation of the DNA complexes much more than it affected the permeation of FITC-dextran 2,000,000. In addition to the size of the molecule or complex, other factors must also be involved.

Because the FITC-dextran data suggest that the size of the complexes alone does not explain the decreased cellular uptake and transfection, further experiments with cationic FITC-PLL polymers were performed to evaluate the effect of charge. Our results show that the positive charge further restricts the polymer permeation in the vitreous or hyaluronan. The cellular uptake of FITC-PLL 20,000 decreased in the presence of vitreous or hyaluronan more than the uptake of the FITC-dextrans, even with MW 500,000 and 2,000,000. Therefore, it appears that the interplay of the size and positive surface charge immobilizes the DNA complexes in the vitreous.

In our experiments we used a 1-mm-thick layer of the vitreous, and still the cellular uptake of the complexes was blocked. Importantly, the effect was also clear in the experiments with longer incubations of 48 h. Vitrectomy might partly eliminate the problems of poor vitreal permeation of the gene delivery complexes, but the surgical removal of the posterior cortical gel is technically difficult. Even a thin layer of vitreous may be adequate to disturb the cellular uptake of the complexes. Furthermore, the interphotoreceptor matrix of the neural retina also contains glycosaminoglycans (e.g., hyaluronan, chondroitin sulfates) (34) that may impair the permeation and cellular uptake of carrier–DNA complexes. The permeation of the gene delivery complexes through the retina and their possible interactions in the intercellular matrix are not known. This aspect needs more investigation.

The handling of biogel samples may alter the network structure of the gels and may also influence macromolecule transport (21). Vitreous has a tendency to liquefy when collagen is removed or destroyed (33). We can assume that our handling procedure partly collapsed the collagen network of the vitreous. In *in vivo* conditions the complexes may actually stick more easily to the network of the healthy vitreous than to the partially liquefied vitreous in our experiments. Therefore, the sieve factor *in vivo* may be more effective than *in vitro.* On the other hand, the human vitreous has a tendency to liquefy with age, and the conditions for permeation also vary *in vivo* (33).

FACS cannot identify whether the fluorescent probe is inside a cell or attached to the cell surface. It is unlikely that the neutral or negatively charged dextrans would attach firmly to the cell surface, but in the case of positively charged molecules this might happen. However, trypsinization of the RPE cells during the detaching procedure cuts the proteoglycans on the cell surface and may separate the attached molecules from the cell surface. The same experimental procedure was used in all cases, and, therefore, we can conclude that hyaluronan and vitreous clearly are barriers to the uptake of DNA complexes when compared to DMEM.

The vitreous in these experiments was collected from cows. The structural components in human and bovine vitreous are the same (hyaluronan, other GAGs, collagen). The exact organization of human and bovine vitreous may differ, but it is highly probable that the human vitreous is also a significant block for the permeation of cationic DNA complexes. This is supported by the observation that hyaluronan limited the gene transfer at similar concentration (0.3 mg/ml) and mean molecular weight ($3.0–5.8 \times 10^6$) that exist in human vitreous.

In conclusion, vitreous limits the retinal gene transfer. Neural retina and internal limiting membrane may constitute additional barriers, but nevertheless, the vitreal barrier must be overcome. This should be taken into account in the development of intravitreal gene delivery systems. The vector should be small enough to permeate through the vitreous and retina. Shielding of the charges in the complexes should reduce interactions with negatively charged glycosaminoglycans in the posterior segment. Retinal gene transfer has a unique environment, and the factors described limit the ocular use of the current nonviral DNA delivery systems that were originally designed for other medical targets.

ACKNOWLEDGMENTS

We thank Ilpo Jääskeläinen for professional help in particle sizing with light scattering and Seppo Rönkkö for his help in confocal imaging. We are also grateful to the project technician Kaarina Pitkänen for her skilful assistance and advice during these experiments. We would also like to acknowledge Mika Reinisalo for cloning the green fluorescent protein expressing plasmid and Pekka Suhonen for his help in statistical analysis. This work was financed by Academy of Finland and EVO funding from the Kuopio University Hospital.

REFERENCES

1. M. Cayouette, D. Behn, M. Sendtner, P. Lachapelle, and C. Gravel. Intraocular gene transfer of ciliary neurotrophic factor prevents death and increases responsiveness of rod photoreceptors in the retinal degeneration slow mouse. *J. Neurosci.* **18**:9282–9293 (1998).
2. M. Akimoto, S. Miyatake, J. Kogishi, M. Hangai, K. Okazaki, J. C. Takahashi, M. Saiki, M. Iwaki, and Y. Honda. Adenovirally expressed basic fibroblast growth factor rescues photoreceptor cells in RCS rats. *Invest. Ophthalmol. Vis. Sci.* **40**:273–279 (1999).
3. J. Bennett, Y. Zeng, R. Bajwa, L. Klatt, Y. Li, and A. M. Magu-

ire. Adenovirus-mediated delivery of rhodopsin-promoted bcl-2 results in a delay in photoreceptor cell death in the rd/rd mouse. *Gene Ther.* **5**:1156–1164 (1998).
4. M. Honda, T. Sakamoto, T. Ishibashi, H. Inomata, and H. Ueno. Experimental subretinal neovascularization is inhibited by adenovirus-mediated soluble VEGF/flt-1 receptor gene transfection: a role of VEGF and possible treatment for SR in age-related macular degeneration. *Gene Ther.* **7**:978–985 (2000).
5. W. V. Hauswirth and L. Beaufrere. Ocular gene therapy: Quo vadis? *Invest. Ophthalmol. Vis. Sci.* **40**:2821–2826 (2000).
6. H. Miyoshi, M. Takahashi, F. Gage, and I. Verma. Stable and efficient gene transfer into the retina using an HIV-based lentiviral vector. *Proc. Natl. Acad. Sci. USA* **94**:10319–10323 (1997).
7. T. Li, M. Adamian, D. Roof, E. Berson, T. Dryja, B. Roessler, and B. Davidson. *In vivo* transfer of a reporter gene to the retina mediated by an adenoviral vector. *Invest. Ophtalmol. Vis. Sci.* **35**:2543–2549 (1994).
8. B. Spencer, S. Agarwala, M. Miskulin, M. Smith, and C. R. Brandt. Herpes simplex virus-mediated gene delivery to the rodent visual system. *Invest. Ophtalmol. Vis. Sci.* **41**:1392–1401 (2000).
9. J. Ray, J. H. Wolfe, G. D. Aguirre, and M. E. Haskins. Retroviral cDNA transfer to the RPE: stable expression and modification of metabolism. *Invest. Ophtalmol. Vis. Sci.* **39**:1658–1666 (1998).
10. Y. K. Lai, P. Rakoczy, I. Constable, and F. Rolling. Adeno associated virus-mediated gene transfer into human retinal pigment epithelium cells. *Aust. N. Z. Ophtalmol.* **26**:77–79 (1998).
11. I. Masuda, T. Matsuo, T. Yasuda, and N. Matsuo. Gene transfer with liposomes to the intraocular tissues by different routes of administration. *Invest. Ophthalmol. Vis. Sci.* **37**:1914–1920 (1996).
12. T. Matsuo, I. Masuda, T. Yasuda, and N. Matsuo. Gene transfer to the retina of rat by liposome eye drops. *Biochem. Biophys. Res. Commun.* **219**:947–950 (1996).
13. A. Urtti, J. Polansky, G. M. Lui, and F. Szoka. Gene delivery and expression in human retinal pigment epithelial cells: effects of synthetic carriers, serum, extracellular matrix and viral promoters. *J. Drug Target.* **7**:413–421 (2000).
14. K. Abul-Hassan, R. Walmsley, and M. Boulton. Optimization of non-viral gene transfer to human primary retinal pigment epithelial cells. *Curr. Eye Res.* **20**:361–366 (2000).
15. A. M. Tonjum. Permeability of horseradish peroxidase in the rabbit corneal epithelium. *Acta Ophtalmol.* **52**:650–658. (1974).
16. A. M. Tonjum. Movement of horseradish peroxidase in the cornea, sclera and the anterior uvea. *Acta Ophtalmol.* **55**:771–780 (1977).
17. D. Maurice and S. Mishima. Ocular pharmacokinetics. In: L. M. Sears (ed.), *Pharmacology of the Eye*, Springer-Verlag, Berlin, 1984, pp. 19–102.
18. E. R. Berman. *Biochemistry of the Eye*. Plenum Press, New York, 1991, p. 292.
19. P. N. Bishop. Structural molecules and supramolecular organisation of the vitreous gel. *Prog. Retin. Eye Res.* **19**:323–344 (2000).
20. M. Ruponen, S. Ylä-Herttuala, and A. Urtti. Interactions of polymeric and liposomal gene delivery systems with extracellular glycosaminoglycans: physicochemical and transfection studies. *Biochim. Biophys. Acta* **1415**:331–341 (1999).
21. N. N. Sanders, S. C. Smedt, and J. Demeester. The physical properties of biogels and their permeability for macromolecular drugs and colloidal drug carriers. *J. Pharm. Sci.* **89**:835–849 (2000).
22. O. Boussif, F. Lezoualch, M. D. Zanta, D. Mergny, D. Scherman, B. Demeneix, and J. P. Behr. A versatile vector for gene and oligonucleotide transfer into cells in culture and *in vivo*: polyethyleneimine. *Proc. Natl. Acad. Sci. USA* **92**:7297–7301 (1995).
23. D. D. Mosser, A. W. Caron, P. Bourget, P. Jolicoeur, and B. Massie. Use of a discistronic expression cassette encoding the green fluorescent protein for the screening and selection of cells expressing inducible gene products. *BioTech* **22**:150–161 (1997).
24. M. Ruponen, S. Rönkkö, P. Honkakoski, J. Pelkonen, and A. Urtti. Extracellular glycosaminoglycans modify cellular trafficking of lipoplexes and polyplexes. *J. Biol. Chem.* **276**:33875–33880 (2001).
25. G. R. McGregor and C. T. Caskey. Construction of plasmids that express *E. coli* beta-galactosidase in mammalian cells. *Nucleic Acids Res.* **17**:2365 (1989).
26. A. A. Davis, P. S. Bernstain, D. Bok, J. Turner, M. Nachtigal, and R. C. Hunt. A human retinal pigment epithelial cell line that retains epithelial characteristics after prolonged culture. *Invest. Ophtalmol. Vis. Sci.* **36**:955–964 (1995).
27. Z. Hyvönen, A. Plotniece, I. Reine, B. Checkavichus, G. Duburs, and A. Urtti. Novel cationic amphiphilic 1,4-dihydropyridine derivatives for DNA delivery. *Biochim. Biophys. Acta* **1509**:451–466 (2000).
28. I. Jääskeläinen, S. Peltola, P. Honkakoski, J. Mönkkönen, and A. Urtti. A lipid carrier with a membrane active component and a small complex size are required for efficient cellular delivery of anti-sense phosphorothioate oligonucleotides. *Eur. J. Pharm. Sci.* **10**:187–193 (2000).
29. I. Jääskeläinen, B. Sternberg, J. Mönkkönen, and A. Urtti. Physicochemical and morphological properties of complexes made of cationic liposomes and oligonucleotides. *Int. J. Pharm.* **167**:191–203 (1998).
30. R. I. Mahato, Y. Takakura, and M. Hashida. Nonviral vectors for *in vivo* gene delivery: physicochemical and pharmacokinetic considerations. *Crit. Rev. Ther. Drug Carrier Syst.* **14**:133–172 (1997).
31. J. Xu, J. J. Heys, V. H. Barocas, and T. W. Randolph. Permeability and diffusion in vitreous humor: Implications for drug delivery. *Pharm. Res.* **17**:664–669 (2000).
32. I. C. Michaelsson. *Textbook of the Fundus of the Eye*, 3rd ed.. Churchill Livingstone, Edinburgh, 1980, p. 84.
33. E. A. Balazs and J. L. Denlinger. The vitreous. In H. Davidson (ed.), *The Eye.* Academic Press, New York 1984, pp. 533–589.
34. G. S. Hageman and L. V. Johnson. Structure, composition and function of the retinal interphotoreceptor matrix. *Prog. Ret. Res.* **10**:207–249 (1991).