Intravitreal Delivery of Oligonucleotides by Sterically Stabilized Liposomes

Amélie Bochot,1 Elias Fattal,1 Valérie Boutet,2 Jean Robert Deverre,2 Jean Claude Jeanny,3 Hélène Chacun,1 and Patrick Couvreur1

PURPOSE. The efficacy of sterically stabilized liposomes for delivering a model phosphodiester oligonucleotide intravitreally was investigated in the rabbit.

METHODS. Ocular distribution and clearance from the vitreous humor of a model 16-mer oligothymidylate (pdT16) were evaluated in the rabbit by radioactivity measurements after intravitreal injection of either a solution or liposomes containing the [33P]pdT16 oligonucleotide. The integrity of pdT16 was investigated using a competitive hybridization assay.

RESULTS. The residual concentration of the [33P]pdT16 oligonucleotide within the ocular tissues was significantly increased after intravitreal administration of the liposomal suspension compared with a simple solution. Administration of liposome-encapsulated pdT16 oligonucleotide resulted in sustained release into the vitreous and the retina-choroid compared with release from the solution and in a reduced distribution to nontarget tissues (sclera, lens). In addition, liposomes protected the phosphodiester oligonucleotide against degradation. This was not observed after administration of the free oligonucleotide.

CONCLUSIONS. The intravitreal injection of a phosphodiester oligonucleotide encapsulated within liposomes is a new way of delivering intact oligonucleotide to the eye in a controlled manner. This offers interesting prospects for the treatment of retinal diseases. (Invest Ophthalmol Vis Sci. 2002;43:253–259)

Antisense oligonucleotides have great potential in the field of antiviral therapy, particularly for intraocular infections that mainly affect the posterior segment of the eye. These antisense molecules, with an oligodeoxyribonucleotide structure, are complementary to specific sequences of the viral mRNA or DNA and thus may inhibit viral multiplication.1 This strategy has recently led to the first antisense oligonucleotide used therapeutically. Vitrovane (Isis Pharmaceuticals, Carlsbad, CA) is a 21-mer phosphorothioate oligonucleotide delivered intravitreally to the eye for the local treatment of cytomegalovirus (CMV) retinitis in patients with acquired immunodeficiency syndrome (AIDS). Oligonucleotides have also been suggested for the treatment of herpes simplex virus (HSV) infections2 or as a tool for understanding the mechanism of retinal development (neural retina cell proliferation, differentiation, and survival).3,4

However, although the use of phosphorothioate oligonucleotides has led to a great improvement in stability and cell penetration, it has resulted in a variety of nonantisense activities. In addition, phosphorothioate oligonucleotides have been described as capable of binding to a large number of proteins in a sequence-independent manner, thus causing serious side effects.5,6 However, intravitreal delivery of drugs with a short half-life (t1/2) requires repeated administrations that can induce damage (risks of endophthalmitis, damage to lens, retinal detachment, and poor tolerance by terminal-stage patients).7

To improve efficacy and comfort of intravitreal delivery, liposomes are an interesting system.8 These lipid vesicles enclosing an aqueous core are small and biodegradable and can encapsulate a variety of drugs. In addition, they can provide a convenient way of obtaining slow drug release from a relatively inert depot without changing the intrinsic properties of the molecule administered.9 Intravitreally administered liposomal systems are able to both significantly increase drug t1/2 and to minimize intraocular side effects. In general, drugs encapsulated within liposomes are less toxic than their free counterparts.10,11

In this study, liposomes were investigated for the first time for the intravitreal delivery of phosphodiester oligonucleotides. This approach is intended to deliver oligonucleotides in a sustained manner from the vitreous humor by using sterically stabilized liposomes, to decrease the distribution in other nonrelevant tissues of the eye to maintain oligonucleotide integrity for a prolonged period, and to avoid the unwanted aptamer effects associated with the use of phosphorothioate oligomers.

MATERIALS AND METHODS

Materials

Soybean phospholipid (PC) was provided by Lipoïd (KG, Ludwigshafen, Germany). 1,2-Distearoyl-sn-glycero-3-phosphatidylethanolamine-N-[poly(ethylene glycol)-2000] (PEG-DSPE) was purchased from Avanti Polar Lipids (Birmingham, AL). Cholesterol (CHOL) was obtained from Sigma Chemical Co. (St. Louis, MO), and the model oligonucleotide 5′-phosphorylated oligothymidylate (pdT16) was from Eurogentec (Seraing, Belgium).

Oligonucleotide Radiolabeling

The 5′-radiolabeled model oligonucleotide was obtained by the following procedure. Ten microliters of the following components was added to 50 μL of a dT16 oligonucleotide solution (10 μM): T4 polynucleotide kinase (T4pnk; Roche Molecular Biochemicals, Mannheim, Germany), buffer for T4 polynucleotide kinase (Roche Molecular Biochemicals), and [33P] adenosine triphosphate (ATP; Isotopchim, Ganagobie, France). Twenty microliters of distilled water was added to this mixture, and it was incubated for 1.5 hours at 37°C. The T4pnk was inactivated by heating for 15 minutes at 65°C. The preparation was

From the 1Laboratoire de Physico-Chimie, Pharmacotechnie, Bio-Pharmacie, Unité Mixte de Recherche Centre National de la Recherche Scientifique (CNRS) 8612, Faculté de Pharmacie, Château-Malabry, France; 2Service de Pharmacologie et d’Immunologie, Département de la Recherche Médicale/Direction des Sciences du Vivant, Commissariat à l’Energie Atomique (CEA)-Saclay, Gif-sur-Yvette, France; and 3Développement, Vieillissement et Pathologie de la Réteine, Institut National Claude Bernard, affilié CNRS, Paris, France. Submitted for publication January 3, 2001; revised July 16, 2001; accepted August 10, 2001.

Commercial relationships policy: N.

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Corresponding author: Elias Fattal, UMR CNRS 8612, Université Paris-Sud, Faculté de Pharmacie, 5 rue J. B. Clément, 92296 Château-Malabry Cedex, France; elias.fattal@cep.u-psud.fr.

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diluted with distilled water. Pure radionuclide-labeled oligonucleotides were finally recovered after chromatography using a spin column (Bio-Spin; Bio-Rad, Richmond, CA) centrifuged at 2400 rpm for 1 minute.

**Preparation of Liposome-Encapsulated pdT16**

The liposomes used to encapsulate the oligothymidy late were composed of PC, CHOL, and PEG-DSPE (molar ratio 64:36:6). Total lipids (195 mg/mL) were dissolved in chloroform in a round-bottomed flask, using a rotary evaporator, and dried in a vacuum. The resultant lipid film was then hydrated using 3 mL HEPES buffer (145 mM NaCl containing 10 mM HEPES [pH 7.4]). The suspension was calibrated using an extrusion device (Lipex, Vancouver, British Columbia, Canada) by repeated extrusion cycles through polycarbonate membranes (pore sizes, successively 0.4 and 0.2 μm). This suspension was then used to generate freeze-thaw vesicles containing pdT16. For each batch, the liposomal suspension was mixed with an aqueous solution containing 5% radiolabeled oligothymidy late and unlabeled oligothymidy late and then shaken with a vortex mixer for 1 or 2 minutes. The final volume of the mixture was 1.1 mL and the final pdT16 concentration was 250 μM. The preparation was frozen in liquid nitrogen for 5 minutes and then thawed at +32°C or +35°C for 2 to 3 minutes. This procedure was repeated 10 times. Free pdT16 was separated from liposome-encapsulated pdT16 by ultracentrifugation at 150,000 rpm for 1 hour at 4°C. The supernatant was removed, and the pellet was resuspended in HEPES buffer to obtain a final volume of 1.1 mL. This washing process was repeated three times. The encapsulation efficiency of pdT16 within the liposomes was determined by measuring the total radioactivity of pdT16 in nonwashed liposomes and in both supernatants and liposomes after separation. The percentage of oligonucleotide encapsulated within the liposomes was calculated according to the following equation: (amount of pdT16 entrapped in liposomes after separation)/(total amount of pdT16 before separation) × 100.

**Size Determination of the Liposomes**

Quasi-elastic light scattering with a nanosizer (Coulter N4 Plus; Coultronics, Margency, France) was used to evaluate liposome diameter before and after encapsulation of pdT16. The liposomal suspension (40 μL) was diluted to 6 mL HEPES buffer. Light scattering was recorded at an angle of 90°. Measurements were made in triplicate.

**In Vitro Release of pdT16 from Liposomes in HEPES Buffer**

In vitro release studies were performed in a water bath maintained at +37°C using a membrane-free release model. Liposomes (0.5 mL; 100.5 pmol of pdT16) were introduced into four vials, and 6 mL HEPES buffer at +37°C was used as the release medium. After 0.5, 2, 4, and 24 hours, the entire contents of the vial were removed and centrifuged as described before, and the amount of free [33P]pdT16 was determined by radioactivity measurement.

**Pharmacokinetics Studies**

Thirty New Zealand White albino rabbits weighing 4 to 4.5 kg were used in these experiments. The animals were maintained and used in accordance with the ARVO Statement for the Treatment of Animals in Ophthalmic and Vision Research. One eye of each animal received a single intravitreal injection of [33P]pdT16 in HEPES buffer solution (solution) and served as a control and the other eye received liposome-encapsulated [33P]pdT16 (liposomes) and served as the experimental eye. Liposomes were diluted before use in HEPES/NaCl buffer. None of these treatments affected the vision. In each case, the intravitreal dose containing 1.05 nmol of pdT16 in a volume of 0.1 mL (50.4 μg/0.1 mL) was delivered 5 mm posterior to the limbus, using a syringe with a 28-gauge needle. The needle was inserted into the center of the vitreous humor. The preparations, stored at +4°C, were injected slowly. Rabbits were then killed at 1, 6, and 24 hours and 7 and 14 days for the solution, and at 24 hours and 4, 7, 14, and 30 days for liposomes by injection of an overdose of sodium pentobarbital. The eyes were immediately enucleated and the globe dissected. The aqueous and vitreous humors were aspirated with a syringe. The cornea and the iris were excised and the lens removed. The sclera was divided into four segments from which the attached choroid-retina was scraped. Samples of conjunctiva and blood were collected. The amount of [33P]pdT16 in ocular tissues was expressed as a percentage of the total injected dose. The amount of [33P]pdT16 in the whole eye was calculated by adding the quantities of radioactivity detected in all ocular tissues and fluids. The t1/2 of [33P]pdT16 in the vitreous humor was calculated from the curves.

**Assay**

Tissues were assayed by measurements of the radioactivity of [33P]pdT16. Samples of conjunctiva, cornea, iris, lens, sclera, choroid-retina, aqueous humor, vitreous humor, and blood were collected. The intact pdT16 in the vitreous humor was evaluated by heterogeneous competitive hybridization, as described previously, except that the hybridization temperature was 25°C instead of 40°C, taking into account the pdT16 fusion temperature.2 Oligonucleotides used in the assay (Table 1) were synthesized by Eurogentec. Data were analyzed by enzyme and radioimmunoassay software (Immunofit EIA/RIA; Beckman Coulter) and by applying the four parameters’ logistic transformation. The method was validated in untreated rabbit vitreous humor with four quality-control samples. Acceptable intra- and interassay agreement was obtained in the range of calibration 50 to 500 pM. The limit of quantification was 50 pM. The percentage of cross-reactivity obtained with the pdT16 oligomer deleted at the 5′-end (N1 to N-3) were lower than 40% (Table 1). No interference with the liposome formulation was observed.

**Analysis of Intact Oligonucleotide in the Vitreous by Heterogeneous Competitive Hybridization Assay**

In this experiment, liposomes were prepared as described previously, using only nonradiolabeled pdT16. Six New Zealand White albino rabbits were given 0.1 mL of a single intravitreal injection of pdT16 in solution in the left eye and liposome-encapsulated pdT16 in the right eye. After 1 and 14 days, rabbits were killed, and the vitreous humor collected. The intact pdT16 in the vitreous humor was evaluated by competitive hybridization, as described previously, except that the hybridization temperature was 25°C instead of 40°C, taking into account the pdT16 fusion temperature.2 Oligonucleotides used in the assay (Table 1) were synthesized by Eurogentec. Data were analyzed by enzyme and radioimmunoassay software (Immunofit EIA/RIA; Beckman Coulter) and by applying the four parameters’ logistic transformation. The method was validated in untreated rabbit vitreous humor with four quality-control samples. Acceptable intra- and interassay agreement was obtained in the range of calibration 50 to 500 pM. The limit of quantification was 50 pM. The percentage of cross-reactivity obtained with the pdT16 oligomer deleted at the 5′ end (N1 to N-3) were lower than 40% (Table 1). No interference with the liposome formulation was observed.

**Histologic Studies**

Six New Zealand rabbits received blank liposomes, pdT16 nonradiolabeled in solution or liposome-encapsulated nonradiolabeled pdT16. One animal was used as a control and did not receive any injection. After 1 or 14 days, animals were killed by administration of an overdose of sodium pentobarbital, and the eyes were promptly enucleated and placed in Bouin liquid. After fixation and a wash in tap water, the anterior chamber was removed, dehydrated in a graded ethanol series, and embedded in wax oriented to obtain sagittal sections. Serial sections (7 μm thick) were collected every 350 μm on gelatin-coated slides. The sections made with a microtome (Jung RM/2055; Leica, Rueil Malmaison, France) were stained using hemalun-eosin-orange G and examined for retinal abnormalities at magnification ×10 with a light microscope (Leitz, Wetzlar, Germany).
RESULTS

The entrapment efficacy of pdT16 in liposomes prepared by the freeze-thaw method was 17.7% corresponding to a concentration of pdT16 of 44.3 ± 11.8 μM. The initial diameter of PC/CHOL/PEG-DSPE liposomes before encapsulation (189 nm) decreased slightly after freeze thawing and oligonucleotide encapsulation (150 nm). The release kinetics of pdT16 from liposomes in HEPES buffer were also investigated. After a burst release corresponding to approximately 10% of the total amount of encapsulated oligonucleotide, a steady state was obtained (Table 2).

Table 1 shows the sequence of the oligonucleotides used and the percentage of cross-reactivity measured in rabbit vitreous humor. pdT16 was the tracer oligonucleotide, and the capture oligonucleotide was fixed to microwells and complementary to pdT16. Deletion of pdT16 resulted in N-1, N-2, and N-3, with cross-reactivities of 38%, 5%, and <5%, respectively.

Table 2 presents the release kinetics of [33P]pdT16 from PC/CHOL/PEG-DSPE liposomes. The release was measured at different time points, and the data are expressed as the mean ± SD. The experiments were performed in HEPES buffer at +37°C (n = 3).

Formulations (solution and liposomes), the radioactivity was mainly distributed in the posterior segment of the eye. As well as the vitreous humor, the ocular sites that contained more than 1% of the injected dose of radiolabeled pdT16 were the retina-choroid (Fig. 2B), the lens (Fig. 2C), and the sclera (Fig. 2D). A small fraction of [33P]pdT16 (approximately 0.5%) was also detected in the iris. For all tissues, a peak concentration was observed at the earlier time points when [33P]pdT16 was administered intravitreally as a simple solution. In contrast, [33P]pdT16 liposomes induced a more long-lasting retention of the radioactivity in the tissue. This was particularly remarkable for the retina-choroid, in which the solution provided a peak of radioactivity at day 1 (16% at 6 and 24 hours), followed by a rapid decrease (1.8% at day 14). In contrast, the concentration of radioactivity remained almost constant in this tissue (around 3.5% of the injected dose) when pdT16 liposomes were used.

Because the pharmacokinetic studies were performed using a 33P-radiolabeled oligonucleotide, which did not allow the distinction between intact and degraded pdT16, we performed...
additional experiments using an original competitive hybridization assay. This methodology, which was applied for the first time to the vitreous humor, provided a clear evaluation of the oligonucleotide’s integrity. The results (Table 3) show that pdT16 in solution was immediately degraded in the vitreous, whereas a significant part of the pdT16 encapsulated within liposomes was efficiently protected from degradation. More than one third of the injected dose of oligonucleotides was found intact 2 weeks after administration.

One day after injection, light microscopy showed only a moderate and localized inflammation in the vitreous (presence of polymorphonuclear leukocytes and macrophages) for the three injected formulations (pdT16 in solution, pdT16 encapsulated with liposomes and blank liposomes) without any retinal abnormalities (data not shown). Fourteen days after injection, histologic examinations showed edema in the inner retinal layer cells for the pdT16 in solution (Fig. 3B), pdT16-loaded liposomes (Fig. 3C), and empty liposomes (data not shown) compared with the reference (no injection, Fig. 3A). There was no difference in the intensity of the effect provoked by the three formulations injected. The edema affected only a part of the surface of the retina. The structures of the outer retina and the choroid appeared completely normal. At day 14, the number of inflammatory cells in the vitreous was decreased compared with that at day 1, and these cells were more

**TABLE 3. Analysis of Intact Oligonucleotide into the Vitreous from the Solution or Liposomes**

<table>
<thead>
<tr>
<th>Formulation</th>
<th>1 Day</th>
<th>14 Days</th>
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<tbody>
<tr>
<td>Solution</td>
<td>0.76 ± 0.02</td>
<td>0.47 ± 0.1</td>
</tr>
<tr>
<td>Liposomes</td>
<td>52.2 ± 7.5</td>
<td>37.25 ± 9</td>
</tr>
</tbody>
</table>

Data are expressed as mean percentage of the injected dose into the vitreous ± SD, determined by heterogeneous competitive hybridization assay.
The purpose of this study was to investigate the intravitreal delivery of a model phosphodiester oligonucleotide encapsulated within sterically stabilized (pegylated) liposomes to obtain a slow release of this oligomer into the eye. Therefore, the clearance and the ocular distribution of \(^{33}\)P-pdT16 were studied after intravitreal administration of this formulation and compared with the distribution obtained after the administration of the same oligonucleotide in solution.

Results of the \(^{33}\)P-pdT16 ocular distribution studies gave evidence of the elimination pathway of \(^{33}\)P-pdT16 after clearance from the vitreous humor. Indeed, drugs are usually eliminated by two principal pathways: the anterior route, through passage into the aqueous humor, and the posterior route, involving transport (active or passive) across the retina. The distribution in the ocular tissues of \(^{33}\)P-radiolabeled pdT16 injected as a solution or as liposomes showed that the major part of the radioactivity was found within the posterior segment of the eye (vitreous, retina-choroid, and sclera). Only a small amount was found within the anterior segment (lens, iris). This distribution clearly indicates that \(^{33}\)P-pdT16 removal occurred mainly by the posterior pathway. However, the rapid decline of \(^{33}\)P in the retinal structure when pdT16 was administered in a solution was probably a consequence of the extensive passage of pdT16 or its degradation products across the retinal epithelial membrane and its transfer to the blood through the choroid.

Taking into account the nonphagocytic character of neural retinal cells and the ability of retinal pigment epithelial (RPE) cells to take up large molecules, including oligonucleotides, these results strongly suggest that RPE cells are a target for antisense oligonucleotides, as proposed by Rakoczy et al.\(^{13,14}\)

**DISCUSSION**

The purpose of this study was to investigate the intravitreal delivery of a model phosphodiester oligonucleotide encapsulated within sterically stabilized (pegylated) liposomes to obtain a slow release of this oligomer into the eye. Therefore, the clearance and the ocular distribution of \(^{33}\)P-pdT16 were studied after intravitreal administration of this formulation and compared with the distribution obtained after the administration of the same oligonucleotide in solution.

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**Figure 3.** (A) Photomicrograph of normal rabbit retina (reference). (B) Photomicrograph of rabbit retina 14 days after intravitreal injection of pdT16 in solution. Fourteen days after injection, histologic examinations showed edema in the inner retinal layer cells for the pdT16 in solution compared with the reference (no injection, A). The edema affected only a part of the surface of the retina. The structures of the outer retina and the choroid appeared completely normal. At day 14, the number of inflammatory cells in the vitreous was decreased compared with day 1, and these cells were more particularly localized in contact with the inner limiting membrane. (C) Photomicrograph of rabbit retina 14 days after intravitreal injection of pdT16-loaded liposomes. Fourteen days after injection, histologic examinations showed edema in the inner retinal layer cells for pdT16-loaded liposomes compared with the reference (no injection, A). The edema affected only a part of the surface of the retina. The structures of the outer retina and the choroid appeared completely normal. At day 14, the number of inflammatory cells in the vitreous was decreased compared with day 1 and these cells were more particularly localized in contact with the inner limiting membrane. There was no difference in the intensity of the effect provoked by the liposomal formulations and the solution (B). V, vitreous; GCL, ganglion cell layer; IPL, inner plexiform layer; OPL, outer plexiform layer; INL, inner nuclear layer; ONL, outer nuclear layer; SP, segments of photoreceptors; RPE, retinal pigmented epithelium; C, choroid; S, sclera; IC, inflammatory cells. Bars, 10 \(\mu\)m.
More important was the observation that the stability of the phosphodiester, the clearance of \(^{33}P\)-labeled pdT16 from the vitreous humor, and its ocular distribution were all strongly influenced by the nature of the formulation (simple solution or liposomes). When the oligonucleotide was administered in a liposomal suspension, it was retained in the vitreous humor to a much higher extent than when it was administered in a solution and its stability was dramatically improved. To our knowledge, the intraocular stability of phosphodiester oligonucleotides has never been investigated. Using a heterogeneous competitive hybridization assay, it was possible to show clearly that the free oligonucleotide was, in fact, very poorly stable in the vitreous, because only 0.7% of the injected dose was intact 24 hours after administration, whereas the amount of radioactivity recovered was 26% ± 6% of the injected dose. When liposomes were administered, the stability of the oligonucleotide was considerably improved. Two weeks after intravitreal administration, the amount of radiolabeled oligonucleotide recovered in the vitreous was quite similar to the amount of intact oligonucleotide (57% ± 9% of the injected dose).

Because of methodologic difficulties, it was impossible to distinguish between free \(^{33}P\)-labeled pdT16 (released from liposomes) and \(^{33}P\)-labeled pdT16 still encapsulated within liposomes in the vitreous humor. Only the total amount of \[^{33}P\]pdT16 could be determined within this compartment. As a consequence, the data presented in this article do not indicate whether \(^{33}P\) measured in the tissues corresponded to the presence of an oligonucleotide–liposome complex or to the oligonucleotide already released from the liposomes. However, several hypotheses can be proposed concerning the fate of oligonucleotides and of encapsulated pdT16 after intravitreal administration: (1) Intact vesicles containing pdT16 are cleared from the vitreous humor; (2) the vesicles are degraded within the vitreous humor and their content cleared as free molecules; or (3) pdT16 is released from intact vesicles by passive diffusion through the phospholipid bilayers and cleared as free molecules.

It is unlikely that intact vesicles containing pdT16 could be cleared from the vitreous humor, either by diffusion through the anterior route or through phagocytosis by retinal cells. Indeed, it has been shown that large unilamellar vesicles (LUV) do not cross the inner limiting membrane. Moreover, the small amount of pdT16 measured in the iris and the lens after administration of liposomes also suggests that the diffusion of intact liposomes through the anterior route does not occur.

Therefore, it may be assumed that only nonencapsulated pdT16 or its degradation products were able to diffuse through the ocular tissues. Thus, it may be hypothesized that the radioactivity associated with pdT16 may be released after disruption of the liposomes within the vitreous humor. The destruction of liposomes could, indeed, result from interactions of the lipid vesicles with proteins that are present in the vitreous humor. Although the main cellular component of the vitreous humor, the hyalocytes, are poorly characterized, they have been classified in at least one report as macrophages and thus could play a role in the uptake of liposomes. In the current study, however, \[^{33}P\]pdT16 encapsulated within liposomes was cleared very slowly compared with \[^{33}P\]pdT16 in solution, indicating that the rate of degradation of the vesicles was very slow and probably not the main factor involved in the release of \[^{33}P\]pdT16. In this work, we used sterically stabilized liposomes containing polyethyleneglycol (PEG) chains coupled to DSPE. This composition may protect liposomes from degradation or accelerated leakage, because the PEG constitutes a hydrophilic layer surrounding the vesicles. This coating layer has been shown in several studies to induce a repulsive effect toward proteins that can adsorb onto the vesicle surface and induce a disruption of the liposomes, leading to a total release of their content. In addition, the presence of PEG on the liposome’s surface would result in a low cellular uptake of these vesicles. Sterically stabilized liposomes display a longer \(t_{1/2}\) than conventional ones and allow a controlled release of the encapsulated molecules.17–20

The presence of cholesterol (CHOL) has also been shown to reduce liposome fluidity and permeability. This effect is due to the suppression by cholesterol at a molar ratio of 50% of the phase transition that generally induces passage from a solid state to a fluid state, in which the liposomes become very leaky. Thus, the interactions between the surface of the vesicles and the proteins would be limited because of the steric repulsion (PEG) and to the increase in bilayer rigidity (CHOL).

Thus, it can be concluded that the release of pdT16 from liposomes probably occurred before the vesicles were disrupted. This is consistent with the in vitro release profile of \[^{33}P\]pdT16 from liposomes, which occurred in the HEPES-NaCl buffer without liposome disruption. Accordingly, the liposomes would act as a slow-release delivery system for the oligonucleotides into the vitreous, which explains both the reduction in \[^{33}P\]pdT16 clearance and its improved stability in the vitreous.

Another great advantage of using liposome-encapsulated oligonucleotide is to induce a sustained drug delivery to the retina. This opens up interesting perspectives for the treatment of retinitis but also for reducing overall eye toxicity. Indeed, an independent recent study has shown that dose-related ocular toxicity was observed after intravitreal injections of oligonucleotides in solution.22 This was probably due to an extensive ocular tissue distribution of the oligomer combined with the use of phosphodiester analogues in which nonbridging oxygen of the internucleotide linkage is replaced by sulfur. Although phosphorothioates are more nuclease resistant than phosphodiester, these analogues bind promiscuously to proteins, leading to aptameric effects that may also account for the toxicity. In this study, as calculated in Table 4, the controlled release of \[^{33}P\]pdT16 from liposomes clearly decreased the amount of oligonucleotides in the nontargeted tissues (sclera, lens). In addition, the encapsulation within liposomes of the native oligonucleotides as phosphodiester analogues may avoid unwanted aptameric effects of phosphorothioates while keeping the sequence intact for a prolonged period, which, as demonstrated in this study, was not possible for the phosphodiester free in solution.

The histologic studies did not allow us to draw any conclusion about the influence of the preparation injected (pdT16 solution, empty liposomes, and pdT16-loaded liposomes) on the moderate inflammation observed in the vitreous humor and the localized edema of the inner retinal architecture. The intravitreal injection itself could induce a traumatic effect responsible for the inflammatory reaction observed. It is clear from our data and from the literature that liposomes do not display toxicity by different administration routes. For this reason, several formulations, including sterically stabilized liposomes, are now on the market. The major clinical changes observed after intravitreal injection of liposomes described in

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**Table 4. Ratio of \[^{33}P\]pdT16 in the Vitreous to Total \[^{33}P\]pdT16 in the Whole Eye**

<table>
<thead>
<tr>
<th>Time (d)</th>
<th>Solution</th>
<th>Liposomes</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>0.49</td>
<td>0.94</td>
</tr>
<tr>
<td>7</td>
<td>0.23</td>
<td>0.88</td>
</tr>
<tr>
<td>14</td>
<td>0.33</td>
<td>0.78</td>
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</table>

Ratios are expressed as a function of time after intravitreal injection of \[^{33}P\]pdT16.
the literature consisted of vitreal bodies—that is, small white sparkling opacities mainly located in the lower part of the eye. The liposome formulations spread diffusely within the vitreous cavity and cause cloudiness, interfering with the patient’s visual acuity and the ability of the ophthalmologist to examine the fundus until complete resorption of the formulation has occurred 14 to 21 days after administration. In addition, more than 12 studies have shown that liposomal systems are able to minimize intraocular side effects after intravitreal injection, because drugs encapsulated within liposomes were less toxic than the free form.

The present data are the first to demonstrate the use of liposomes as a delivery system for the phosphodiester oligonucleotide in the vitreous. By controlling the release of the oligonucleotide, the liposome formulation was able to protect this molecule from the degradation and to prolong its residence time in the vitreous. The liposomes also had a pronounced effect on the distribution of pdT16 in the posterior segment of the eye by reducing the amount of oligonucleotide in nontarget sites. Such a preferential pdT16 uptake by the retinal cells opens up interesting perspectives for using antisense phosphodiester oligonucleotides in the treatment of retinitis.

Acknowledgments

The authors thank Yvonne de Zok for discussion of histology studies.

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