Liposomes Dispersed Within a Thermosensitive Gel: A New Dosage Form for Ocular Delivery of Oligonucleotides

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Purpose. The main goal of this study was to develop an ocular controlled release formulation of a model oligonucleotide (pdT16), contained within liposomes dispersed within a thermosensitive gel composed by poloxamer 407.

Methods. The influence of the poloxamer concentration 2% or 27% on the stability of the liposomes (PC: CHOL and PC: CHOL: PEG-DSPE) was investigated. The in vitro release profiles of pdT16 from various poloxamer formulations (free pdT16 dispersed within 20% and 27% poloxamer gels, pdT16 encapsulated within liposomes dispersed within 20% and 27% poloxamer gels) were realized using a membrane-free release model.

Results. The dispersion of liposomes within a dilute 2% poloxamer solution resulted in a great leakage of pdT16 from liposomes. However, the destabilization effect of poloxamer was reduced when higher concentration (27%) was used. Poloxamer dissolution was found to control the release process of pdT16, whereas the dispersion of liposomes within 27% poloxamer gel was shown to slow down the diffusion of pdT16 out from the gel.

Conclusions. The dispersion of liposomes within a 27% poloxamer gel presented an interesting system to control the release of a model oligonucleotide compare to a simple gel.

KEY WORDS: drug delivery system; gel dissolution; liposomes; oligonucleotide; poloxamer 407 gels.

INTRODUCTION

Antisense oligonucleotides with base sequences complementary to specific genetic targets offer the possibility of selectively modulating the expression of gene (1). It has been shown that antisense oligonucleotides possess significant inhibitory activity against a number of DNA viruses responsible for ocular diseases: herpes simplex virus (HSV_1 and HSV_2) (2) in the anterior segment of the eye and human cytomegalovirus (CMV) in the posterior segment of the eye (3). Unfortunately, the use of these molecules is limited by their poor biological stability in biological fluids (4). One strategy to improve the protection of oligonucleotides from degradation involves their encapsulation within liposomes (5) which, in addition, presents further advantages as ocular delivery systems: they can provide increased efficacy (6), reduced toxicity (7) as well as prolonged activity (8).

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The instillation of conventional ocular forms results in extremely low bioavailability (9) due to the limited corneal absorption of drugs as well as the short residence time. Concerning the treatment of posterior segment disorders, the efficacy of conventional formulations for drug administration is also limited while direct injection into the vitreous humor is very delicate. Furthermore, repeated injections are necessitated by the short intraocular half-life of drugs when administered intravitreally (10).

One way to optimize topical or intravitreal delivery involves prolonging the retention of a liposomal suspension at the site of administration. We have attempted to achieve this by dispersing liposomes within a medium which would be able to form a gel in situ after administration. Poloxamer 407, which is a copolymer of polyoxyethylene, and polyoxypropylene offers the unique property of reversible thermal gelation (11). This would allow instillation or injection of a fluid solution which would form a semi solid gel at physiological temperature in the eye.

Thus, the aim of this study was to develop a system based on liposomes containing oligonucleotides and dispersed within poloxamer gels. This new formulation should be a novel ocular dosage form able to prolong the residence time, to control the release; and to protect against the degradation of the encapsulated antisense oligonucleotides when administrated into the eye. The influence of the poloxamer concentration and of the nature of the liposomes on oligonucleotide release was investigated.

MATERIALS AND METHODS

Materials

Poloxamer 407 (Lutrol® F127) was a gift from BASF. Soybean Phospholipid (PC) was provided by Lipoid (KG, Ludwishafen, Germany). 1, 2-distearoyl-sn-glycero-3 Phosphatidyl-ethanolamine-N-[Poly(ethyleneglycol)-2000] (PEG-DSPE) was purchased from Avanti Polar Lipids (Birmingham, AL, USA). Cholesterol (CHOL) was obtained from Sigma Chemical Co (St Louis, MO, USA) and the model oligonucleotide, 5′-phosphorylated oligothymidylate (pdT16) from Pharmacia Biotech (St Quentin-en-Yvelines, France).

Methods

Oligonucleotide Radiolabeling

The 5′-radiolabelled oligonucleotide was obtained by the following procedure. To 40 µl of dT16 solution (10 µM), 8 µl of the following components were added: T4 polynucleotide kinase (T4pnk), buffer for T4 polynucleotide kinase (Boehringer Mannheim, Germany) and [^{33}P] ATP (Isotopchim, France). To this mixture, 12 µl of distilled water were added and the whole was incubated for 1h30 at 37°C. The T4npk was inactivated by heating. The preparation was diluted with distilled water. Pure radiolabelled oligonucleotide was finally recovered after chromatography.

Liposome Preparations

The liposomes used to encapsulate oligothymidylate were composed of PC: CHOL (molar ratio 70:30) or PC:CHOL:

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PEG-DSPE (molar ratio 64:30:06). Practically, 195 μmoles of total lipids were dissolved in chloroform in a round-bottomed flask, using a rotary evaporator and dried under vacuum. The resulting lipid film was hydrated using 3 ml of HEPES buffer (145 mM NaCl containing 10 mM HEPES, pH 7.4). The suspension was extruded (Lipex, Vancouver, Canada) through polycarbonate membranes. The final diameter of the vesicles was 200 nm ± 40 nm. The liposomal suspension was mixed with a 5′-end radiolabelled oligothymidylate and unlabelled oligothymidylate solution. The final volume and the final pdT16 concentration of the mixture were 1.3 ml and 6 μM respectively. The preparation was frozen in liquid nitrogen for 5 minutes and then, thawed at 32°C–33°C for 3 minutes. This procedure was repeated 10 times. Free pdT16 was separated from liposome-encapsulated pdT16 by 3 repeated ultracentrifugation at 150,000 g for 1 hour at 4°C. The supernatant was removed and the pellet was resuspended in HEPES buffer to obtain a final volume of 1.3 ml. The entrapment efficiency of pdT16 in liposomes was determined by measuring the total pdT16 radioactivity in non washed liposomes and in both supernatants and liposomes after separation.

Stability of Liposomes Stored in HEPES Buffer at +4°C

Liposome-encapsulated pdT16 (PC:CHOL, PC:CHOL: PEG-DSPE) were stored at +4°C and after 1, 7, and 30 days, 1 ml of the suspensions was drawn off and ultracentrifuged to separate the free oligonucleotides which had leaked out and intact liposomes containing pdT16.

Characterization of the Liposomes-Gel System

Preparation of Poloxamer Gels

Poloxamer 407 gels were prepared by the cold process described by Schmolka (11).

Preparation of Gels Containing Free or Liposomal pdT16

A 5′-labelled oligothymidylate and unlabelled pdT16 solution was added to poloxamer to produce a final oligonucleotide concentration of 0.201 μM and a final poloxamer concentration of 20% or 27% (w/v). Liposomes were dispersed within poloxamer 407 gels under stirring at +4°C.

Electron Microscopy

Freeze-fracture electron microscopy was used to examine the suspension of the two liposomal formulations after their dispersion within a 27% poloxamer gel. A drop of the suspension containing 30% glycerol as a cryprotector was deposited on a thin copper planchett and rapidly frozen in liquid propane. Fracturing and shadowing using Pt-C, were performed in a Balzers BAF freeze-etch unit. The replicas were examined with a Philips 410 electron microscope.

Stability of Liposomes Dispersed Within 27% Poloxamer Gels Stored at +4°C

Release studies of pdT16 from the two liposomal formulations dispersed within a 27% poloxamer gel were performed at +4°C. After 1, 7 and 30 days, 1 ml of liposomes dispersed in the gel were drawn off and the released oligonucleotide was separated from encapsulated compound as described in the liposome preparation section.

IN VITRO RELEASE STUDIES

In vitro release studies were carried out in a water-bath maintained at +37°C using a membrane-free release model. 0.5g aliquots of various preparations (pdT16 dispersed within 20% and 27% poloxamer gels or liposome-encapsulated pdT16 dispersed within 20% and 27% poloxamer gels), corresponding to 100.5 pmol of pdT16, were introduced into vials and the formulations gelled when the experimental temperature was reached. 6 ml of HEPES buffer (+37°C) was used as the release medium. After 0.5, 2, 4, 6, 8 and 24 hours, the entire release medium was removed and pdT16 released by the formulation was obtained by measuring ^{33P}pdT16 radioactivity in fraction of this medium using scintillation counting. The percentage of pdT16 released was expressed as the ratio between pdT16 in the release medium and the total amount of pdT16 initially present in the preparations.

In the case of liposomes dispersed within gels, the proportions of free pdT16 and pdT16 still encapsulated into liposomes released from the gel, were determined after ultracentrifugation of release medium aliquots (150000g +4°C, 1 hour). Thereafter, the supernatant containing only free pdT16 were submitted to radioactivity counting. The difference between total pdT16 released into the medium and free pdT16 allowed us to calculate the amount of pdT16 released from the gel but still encapsulated into liposomes.

In Vitro Dissolution Profiles of 20% and 27% Poloxamer Gels

0.5g of 20% and 27% poloxamer gels was introduced into vials and 6 ml of buffer were layered over the surface of the gel formulations. The experiments were performed at +37°C. After 0.5, 2, 4, 6, 8, and 24 hours, the release medium was sampled and the amount of dissolved poloxamer was assayed by the colorimetric method described by Baleux (12). The dissolution gel profiles (20% and 27%) were expressed as the percentage of poloxamer gel dissolved as a function of time.

In Vitro Modelization of pdT16 Release from Gel and from Liposomes Dispersed Within Gel and Modelization of Poloxamer Dissolution

The amount of pdT16 released or the amount of poloxamer dissolved in the release medium were regressed against the experimental time using a first order kinetic following the equation:

$$\ln(M_t/M_o) = -kt$$

Mt/Mo represents the fraction of poloxamer dissolved or pdT16 released up to time t; k is the pdT16 release or dissolution rate (h^{-1}).

The half-life of pdT16 released or poloxamer dissolved is calculated by the equation:

$$T_{1/2} = \ln 2/k$$

In Vitro Release of pdT16 from Liposomes Dispersed Within a Dilute Poloxamer Solution (2%)

pdT16 release from the two formulations of liposomes dispersed within a dilute poloxamer solution was investigated. 0.5 ml of liposomes (100.5 pmol of pdT16), was introduced into each vial and 6 ml of 2% poloxamer solution at +37°C were used as release medium. As a control, 6 ml of HEPES buffer were used. After 0.5, 2, 4, and 24 hours, the entire contents of the vial were removed, centrifuged as described before and the amount of free pdT16 was determined.

RESULTS

Liposome Preparation and Characterization

Entrapment efficacy of pdT16 into liposomes reached 15% for the two formulations studied. For both liposome preparations, the vesicles appeared dispersed within the poloxamer gel. The poloxamer gel fracture surface is slightly rough, showing very small particles. The liposome fracture surface revealed the presence of oligolamellar vesicles (Fig. 1).

The presence or the absence of PEG-DSPE in the bilayer composition significantly influenced pdT16 leakage from liposomes. The presence of PEG-DSPE prevented liposome aggregation in HEPES buffer and leakage of pdT16 in both HEPES buffer and 27% poloxamer gel. Indeed, with PEG-DSPE, no vesicle aggregation was observed after one month in HEPES buffer as compared with the PC: CHOL formulation which became aggregated at 30 days. This aggregation was not observed when liposomes were dispersed within the poloxamer gel. In this case, pdT16 release from PC:CHOL:PEG-DSPE liposomes was slower than from PC:CHOL (10% compare to 20% at 1 and 7 days), but similar pdT16 release was measured after 1 month for both formulations (27%), (data not shown).

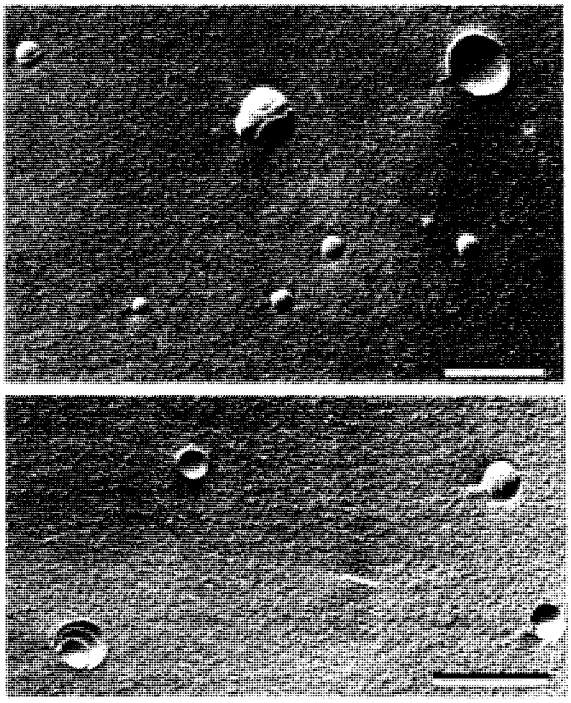

Fig. 1. Freeze-fracture of a liposomal dispersion (PC: CHOL) within the poloxamer gel. Bar = 300 nm.

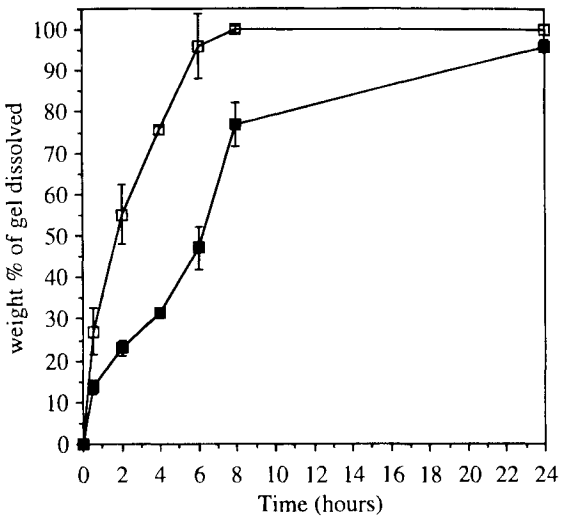

Fig. 2. Dissolution (weight % dissolved) of 20% (—□—) and 27% (—■—) poloxamer 407 gels as a function of time (hours). (n = 3).

In Vitro Release Studies

Effect of Poloxamer Concentration on the Dissolution of 27% and 20% Poloxamer Gels

Dissolution studies of poloxamer 407 gels showed that the 27% poloxamer gel exhibited slower dissolution than the 20% poloxamer gel (Fig. 2): complete dissolution occurred after 6 hours for the 20% poloxamer gel and between 8 hours and 24 hours for the 27% poloxamer gel. Poloxamer dissolution followed first order kinetic ($k_{27\%}$ = 0.135 h^{-1}, $T1/2_{27\%}$ = 5.13 hours and $k_{20\%}$ = 0.478 h^{-1}, $T1/2_{20\%}$ = 1.45 hours).

Effect of Poloxamer Concentration on Free pdT16 Release from Gels Without Liposomes

The release of pdT16 from poloxamer gels was shown to be influenced by the poloxamer 407 concentration (Fig. 3a and 3b). A marked reduction of pdT16 release was observed when the poloxamer 407 concentration was increased from 20% to 27%: T1/2 of pdT16 release was 1.28 hour (k = 0,539 h^{-1}) and 2.45 hours (k = 0,283 h^{-1}) respectively.

Effect of Liposomes on pdT16 Release from Poloxamer Gels

The dispersion of liposomes within the 27% poloxamer 407 gel led to a slower release of the pdT16 compared with the simple gel formulation without liposomes (Fig. 3a). However, pdT16 release was independent of the nature of the liposomes used (PC: CHOL data not shown or PC:CHOL:PEG-DSPE). T1/2 was 4,81 hours (k = 0,144 h^{-1}) for 27% poloxamer gel formulations containing liposomes, whereas it was only 2 hours (k = 0,346 h^{-1}) for 27% poloxamer gel formulations without liposomes. Thus, the introduction of liposomal formulations within poloxamer 407 at 27% resulted in a reservoir effect for pdT16. In contrast, pdT16 release from the 20% poloxamer gel and that from liposomes dispersed within the 20% poloxamer

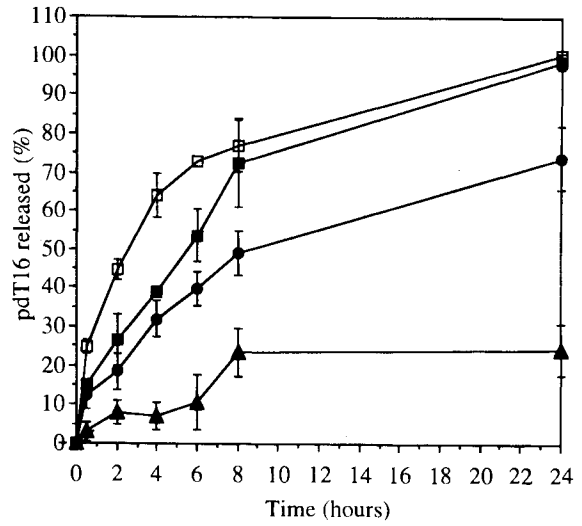

Fig. 3a. pdT16 release kinetics from 27% poloxamer gel without liposomes (—□—) and from PC:CHOL:PEG-DSPE liposomes dispersed within 27% poloxamer gels: total pdT16 (—■—), free pdT16 (—●—), pdT16 still encapsulated within liposomes (—▲—). (n = 3).

gel (Fig 3b) were found to be very similar (T1/2 = 1.28 hour and 2 hours respectively).

Investigation on the Form of Released pdT16: Free or Still Encapsulated Within Liposomes

The form of pdT16 released from 27% poloxamer gel containing liposomes (free pdT16 or pdT16 still encapsulated in liposomes) was quantified. The results (Fig. 3a) indicated that both free pdT16 and liposomally encapsulated pdT16 were present in the receptor medium and that free pdT16 represented the main part of pdT16. At 8 and 24 hours, about 25% of the pdT16 released was still encapsulated in PC: CHOL: PEG-DSPE liposomes and PC:CHOL liposomes (data not shown).

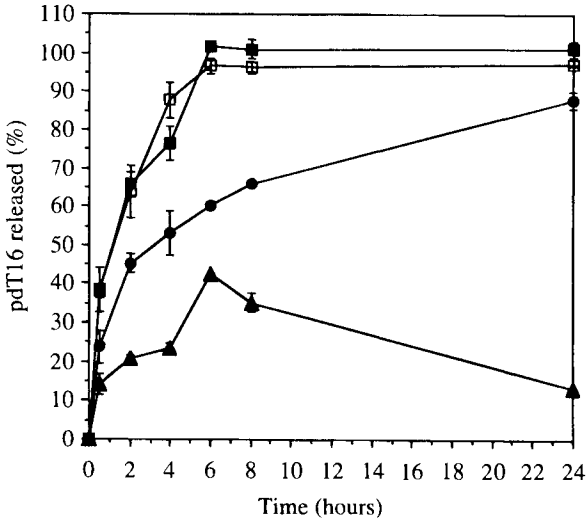

Fig. 3b. pdT16 release kinetics from 20% poloxamer gel without liposomes (—□—) and from PC:CHOL:PEG-DSPE liposomes dispersed within 20% poloxamer gels: total pdT16 (—■—), free pdT16 (—●—), pdT16 still encapsulated within liposomes (—▲—). (n = 3).

Concerning PC:CHOL:PEG-DSPE liposomes dispersed within the 20% poloxamer gel, the curve profile for pdT16 released still encapsulated within liposomes was quite different from that observed at 27%: during the first 6 hours of incubation, the amount of pdT16 released as the liposomal form increased, whereas after this time point, it decreased sharply to 13% (Fig. 3b).

Release of pdT16 From Liposomes Dispersed in HEPES Buffer or Within a 2% Poloxamer 407 Solution

To mimic in vivo situation after erosion and dissolution of the poloxamer gel, we studied the release of pdT16 from the two formulations of liposomes incubated in a dilute poloxamer 407 solution (2%), (Table 1). The leakage of pdT16 from liposomes increased dramatically in the presence of a 2% poloxamer 407 solution as compared with the HEPES buffer solution. Interestingly, the leakage of pdT16 from PC:CHOL liposomes after 0.5, 2 and 4 hours was greater than from PC:CHOL: PEG-DSPE vesicles. However, after 24 hours, the percentage of pdT16 released was similar for both formulations.

DISCUSSION

In an attempt to develop a controlled delivery system for ocular administration of antisense oligonucleotides, we have designed a new formulation based on the dispersion of liposomes containing a model oligonucleotide within poloxamer 407 gels.

An electron microscopy study revealed that the liposomes dispersed within gel corresponded to oligolamellar vesicles. This morphology, as well as the polarity of pdT16 and its high molecular weight (around 5 kDa) would help to explain the slow diffusion of the oligonucleotide molecules across the liposomal phospholipid bilayers during storage. Electron microscopy studies also demonstrated that liposomes were, surprisingly, intact within a 27% poloxamer gel. This is in accordance with the observed slow release of pdT16 from the 27% poloxamer gel containing liposomes stored at +4°C. In contrast, the dispersion of liposomes within a dilute 2% poloxamer solution (to mimic in vivo situation after gel dissolution in tears or in the vitreous humor) resulted in a considerable leakage of pdT16 from liposomes. Thus, it appeared that poloxamer 407 exhibited a quite different behavior depending on its concentration in the formulations. Poloxamer 407 is a non ionic copolymer of polyoxyethylene and polyoxypropylene exhibiting surfactant properties (11). Several authors (13–14) have reported that the preincubation of diluted poloxamer solutions with liposomes resulted in destabilization of the vesicles. At low concentration, the copolymer chains possess a greater fluidity and the insertion of the diblock polymer into the phospholipid bilayer could lead to the formation of pores or regions of membrane fluidity through which the oligonucleotide could leak. With respect to the influence of the liposomal composition, our studies revealed that PC:CHOL:PEG-DSPE liposomes were less sensitive to poloxamer than PC:CHOL vesicles: the incorporation of PEG-DSPE in the phospholipid bilayer reduced the leakage of pdT16 during the first 4 hours of the experiments and PEG-DSPE provided a steric barrier described by Torchillin et al. (15). This effect was limited to the first hours of incubation and the results obtained after 24 hours clearly showed that PEG-DSPE did not permit a permanent repulsion of the poloxamer chains.

Table 1. Release Kinetics of pdT16 from PC:CHOL and PC:CHOL:PEG-DSPE Liposomes Incubated in HEPES Buffer or 2% Poloxamer 407 Solution at +37°C

Formulation composition[a]	pdT16 leakage (%)			
	Time (hours)			
	0.5	2	4	24
PC:CHOL (HEPES buffer)	13.5 ± 2.1	17.2 ± 1.9	19.2 ± 3.2	27.6 ± 2.9
PC:CHOL (2% poloxamer solution)	50.8 ± 3.4	59.2 ± 1.8	67.1 ± 2.2	68.9 ± 3.6
PC:CHOL:PEG-DSPE (HEPES buffer)	14.1 ± 2.1	12.1 ± 2.5	10.0 ± 2.3	22.9 ± 3.0
PC:CHOL:PEG-DSPE (2% poloxamer solution)	34.2 ± 3.2	39.4 ± 1.4	47.0 ± 2.9	65.3 ± 2.0

[a] Molar ratio is 70:30 for PC:CHOL and 64:30:06 for PC:CHOL:PEG-DSPE. The experiments were realized at +37°C (n = 3).

The destabilization effect of poloxamer was dramatically reduced when higher polymer concentrations were used (20% or 27%). At 27%, the poloxamer preparation became highly viscous and gelified and the mobility of the copolymer chains supposedly could be greatly decreased. Interactions between the polyoxypropylene chains occurred preferentially, whereas interactions between the polyoxypropylene chains and the phospholipid bilayer decreased. In addition, at high concentration (27%), poloxamer appeared to have an interesting inhibitory effect on liposome aggregation.

The potential of the poloxamer gel containing dispersed liposomes to control the release of oligonucleotide was investigated in vitro. A study of the influence of poloxamer 407 concentration (20% or 27%) on pdT16 release kinetics from poloxamer gels showed that pdT16 release was more rapid with the lower (20%) poloxamer concentration. This was probably related to the reduction of the number and size of the micelles within the gel structure leading to a decrease in gel rigidity and increasing gel dissolution. Comparison of the pdT16 release from the different formulations and the dissolution of poloxamer gels suggested that the dissolution of the copolymer was the major factor controlling pdT16 release from the gel and from the liposomes dispersed within the gel (Fig. 4a). Indeed, for liposomes dispersed within the 27% poloxamer gel, the percentage of pdT16 released (free or still encapsulated) was directly correlated with the weight percentage of poloxamer gel dissolved ($T1/2_{\text{poloxamer dissolution}}$ = 5.13 hours and $T1/2_{\text{pdT16 released}}$ = 4.81 hours). In the case of free pdT16 within a 27% poloxamer gel without liposomes, the release kinetic of pdT16 ($T1/2_{\text{pdT16 released}}$ = 2.45 hours) was, however, more rapid than the dissolution kinetic of the gel. This indicated that in the case of gel devoid of liposomes, pdT16 release was also affected by the diffusion of the oligonucleotide through the aqueous channels within the gel, in addition to gel erosion (Fig 4a). It is obvious that in the case of liposomes dispersed within the gel, the simple diffusion of the vesicles through the aqueous channels of the copolymer was not possible. In the case of 20% poloxamer gel, the dissolution (T1/2 = 1.45 hours) was more rapid, thus explaining why it was the only factor affecting pdT16 release (Fig. 4b).

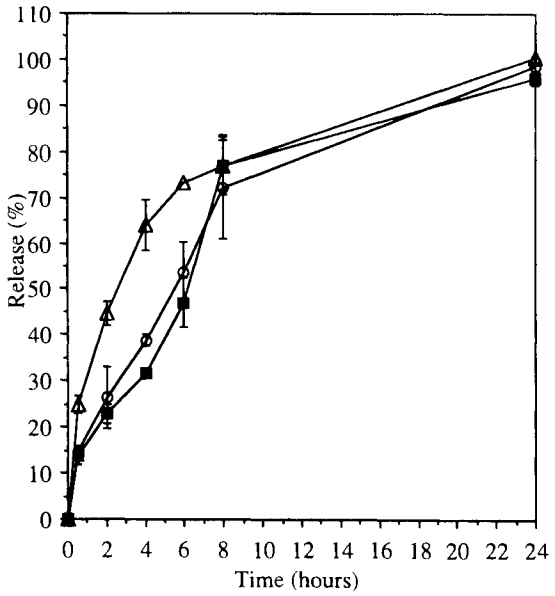

Fig. 4a. Comparison between the kinetics of dissolution of 27% poloxamer gels (—■—), and the release of pdT16 from 27% poloxamer gels without liposomes (—△—) or from liposomes dispersed within 27% poloxamer gels (—○—). (n = 3).

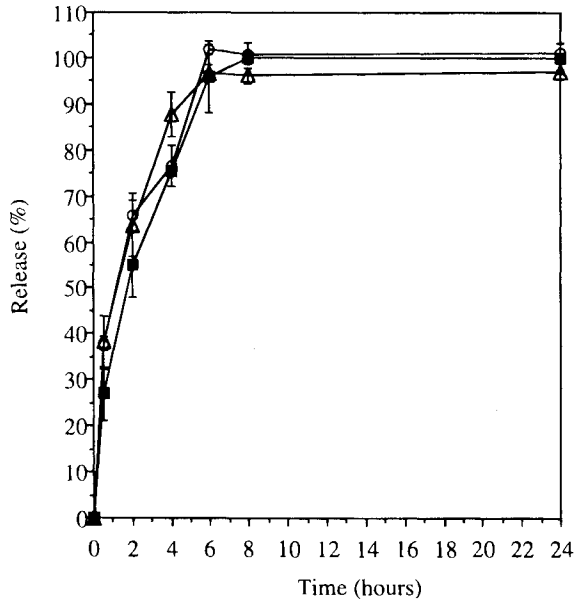

Fig. 4b. Comparison between the kinetics of dissolution of 20% poloxamer gels (—■—), and the release of pdT16 from 20% poloxamer gels without liposomes (—△—) or from liposomes dispersed within 20% poloxamer gels (—○—). (n = 3).

Measurement of the amount of free or encapsulated pdT16 released showed that free pdT16 was predominant in comparison with pdT16 released still encapsulated within liposomes. The presence of liposomes in the release medium could be explained by the fact that the dissolution process of gels was controlling the release. However, the erosion of the gel also causes the release of soluble polyoxyethylene-polyoxypropylene into the incubation medium. After complete dissolution of the 27% gel, the poloxamer concentration in the medium was 2%. Under these conditions, due to its surfactant properties, this poloxamer would induce destabilization of the phospholipid vesicles, thus explaining the leakage of pdT16 from the liposomes released from the 27% poloxamer gel. The same conclusion may be drawn concerning liposomes dispersed within 20% poloxamer gels: during the first 6 hours of incubation, the amount of the liposomal form of pdT16 increased in the release medium in parallel with the dissolution of the copolymer, but after this time point the 20% gel was completely dissolved, so all the vesicles were in contact with a dilute poloxamer solution, thus leading to liposome destabilization and release of the oligonucleotide. However, when liposomes were dispersed within a 27% poloxamer gel, the amount of pdT16 still encapsulated within liposomes remained constant (around 25%) between 8 hours and 24 hours, whereas the amount of free pdT16 increased (Fig. 3a). This may be explained by the complete dissolution of 27% poloxamer gel occurring between 8 hours and 24 hours, thus causing pdT16, still encapsulated within liposomes, to be released in the aqueous medium during this period.

In conclusion, we have developed a new delivery system based on the dispersion of sterically stabilized liposomes within a thermosensitive gel. Whereas the liposomes can be considered to prevent the diffusion of oligonucleotides in the gel, poloxamer dissolution was found to control the release process. As a result of these properties, this new delivery system may have great potential for ocular administration of nucleic acids.

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REFERENCES

1. C. Hélène and J. J. Toulmé. Specific regulation of gene expression by antisense, sense and antigene nucleic acids. *Biochim. Biophys. Acta.* **1049**:99–125 (1990).
2. R. M. Crooke, G. D. Hoke, and J. E. E. Shoemaker. In vitro toxicological evaluation of ISIS 1082, a phosphorothioate oligonucleotide inhibitor of herpes simplex virus. *Antimicrobiob. Agents. Chemother.* **36**:527–32 (1992).
3. R. F. Azad, V. B. Driver, K. Tanaka, R. M. Crooke, and K. P. Anderson. Antiviralactivity of a phosphorothioate oligonucleotide complementary to RNA of the human cytomegalovirus major immediate-early region. *Antimicrob. Agents. Chemother.* **37**:1945–1954 (1993).
4. P. S. Eder, R. J. Devine, J. M. Dagle, and J. A. Walder. Substrate specificity and kinetics of degradation of antisense oligonucleotides by a 3′ exonucleases in plasma. *Antisense Res. Dev.* **1**:141–151 (1991).
5. C. Ropert, C. Malvy, and P. Couvreur. Inhibition of the friend retrovirus by antisense oligonucleotides encapsulated in liposomes: Mechanism of action. *Pharm. Res.* **10**:1427–1433 (1993).
6. U. Pleyer, S. Lutz, W. Jusko, K. Nguyen, M. Narawane, D. Rückert, B. J. Mondino, and V. H. Lee. Ocular absorption of topically applied FK506 from liposomal and oil formulations in rabbit eye. *Invest. Ophthalmol. Vis. Sci.* **34**:2737–2742 (1993).
7. C. Tremblay, M. Barza, F. Szoka, M. Lahav, and J. Baum. Reduced toxicity of liposome-associated amphotericin B injected intravitreally in rabbits. *Invest. Ophthalmol. Vis. Sci.* **26**:711–718 (1985).
8. T. A. Mc Calden and M. Levy. Retention of topical liposomal formulations on the cornea. *Experientia.* **46**:713–715 (1990).
9. V. H. L. Lee and J. R. Robinson. Mechanistic and quantitative evaluation of precorneal pilocarpine disposition in albino rabbits. *J. Pharm. Sci.* **68**:673–784 (1979).
10. D. A. Japs. Treatment of cytomegalovirus retinitis in patients with aids. *Ann. Intern. Med.* **125**:144–145 (1996).
11. I. R. Schmolka. A review of block polymer surfactants. *J. Am. Oil Chemists' Soc.* **54**:110 (1977).
12. B. Baleux. Dosage colorimétrique d'agents de surface non ioniques polyoxyéthylènes à l'aide d'une solution iodo-iodurée. *Cr. Acad. Sc. Paris.* **274**:1617–1620 (1972).
13. M. Jamshaid, S. J. Farr, P. Kearney, and I. W. Kellaway. Poloxamer sorption on liposomes: comparison with polystyrene latex and influence on solute efflux. *Int. J. Pharm.* **48**:125–131 (1988).
14. M. C. Woodle, M. S. Newman, and F. J. Martin. Liposome leakage and blood circulation: comparison of adsorbed block copolymers with covalent attachment of PEG. *Int. J. Pharm.* **88**:327–334 (1992).
15. V. P. Torchilin, V. G. Omelyanenko, M. I. Papisov, A. A. Bogdanov, V. S. Trubetskoy, J. N. Herron, and C. A. Gentry. Poly (ethylene glycol) on the liposome surface: on the mechanism of polymer-coated liposome longevity. *Biochim. Biophys. Acta.* **1195**:11–20 (1994).