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 (HSV1 and HSV2) (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).

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 intra-vitreally (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, Ludwigshafen, Germany). 1, 2-distearoyl-sn-glycero-3 Phosphatidylethanolamine-N-[Poly(ethylene glycol)-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' phosphorolylated oligothymidy late (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 [32P] ATP (Isotopheim, France). To this mixture, 12 μl of distilled water were added and the whole was incubated for 1h30 at 37°C. The T4pnk 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 oligothymidy late were composed of PC: CHOL (molar ratio 70:30) or PC:CHOL:
PEG-DSP (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 radio-labelled oligohydroxymide and unlabelled oligohydroxymide 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-DSP) 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 oligonucleotide 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 oligohydroxymide 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 cryoprotector 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 33PpdT16 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 (15000g: +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 samples 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:

\[ \ln(M/M_0) = -kt \]

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:

\[ T_{1/2} = \ln 2/k \]