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Gel and solid matrix systems for the controlled delivery of drug carrier-associated nucleic acids

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Abstract

In order to achieve a sustained pharmacological activity of oligonucleotides (ODNs) and avoid repeated administrations, we have developed a new concept of delivery system that combine sustained release and improved intracellular penetration. These systems are designed for the intravitreal delivery of antisense ODNs. The first concept consisted in using liposomes dispersed in a thermosensitive gel (poloxamer 407). After intravitreal administration in a rabbit model, liposomes and liposomes-gel formulations provided, 1-day postinjection, significantly higher drug levels than the control solution of the oligothymidilate pdT16. In addition, there was no significant difference in the amounts of pdT16 found in the vitreous humor between the liposomes and liposomes-gel. Nevertheless, because of their better stability in the absence of poloxamer, liposomes alone allowed to a larger extent to control the delivery of ODNs as compared to liposome-gel formulations since 37% of the ODNs were still found in the vitreous 15 days after administration. In addition, the ODNs found in the vitreous humor were protected against degradation by their encapsulation within liposomes. The second approach consisted in designing microspheres allowing to release in a controlled fashion pdT16. The ODN was encapsulated within poly(lactide-co-glycolide) microspheres alone or associated with polyethylenimine (PEI) at different nitrogen/phosphate (N/P) ratios. The introduction of PEI in the internal aqueous phase resulted in a strong increase of the ODN encapsulation efficiency. PEI affected microsphere morphology inducing the formation of very porous particles yielding to an accelerated release of pdT16. Porosity and controlled delivery was prevented by introducing sodium chloride in the external preparation medium. When incubated with HeLa cells, microspheres encapsulating pdT16/PEI complexes allowed an improvement of the intracellular penetration of the released ODN. Both liposomes and microspheres are suitable for local delivery of ODNs.

Keywords: Oligonucleotide; Thermosensitive gel; Microparticles; Polyethylenimine

1. Introduction

Antisense ODNs are short DNA fragments that have been proved to selectively modulate the expression of a specific gene. Many recent studies achieved in cell cultures and animal models have shown that ODNs can potentially be used in the treatment of major diseases such as cancer and viral infections (Myers and Dean, 2000). Nevertheless, the development of ODNs-based therapeutics is hampered by several problems among which the necessity of repeating the number of administrations. This drawback is mainly due to the short in vivo half-life of the ODNs (Akhtar et al., 1991; Agrawal et al., 1991) and their limited cellular uptake (Akhtar and Juliano, 1992).

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Chemically modified ODNs have improved stability and increased their cellular uptake, but this approach has often yielded to a reduction of their biological activity and the appearance of side effects (Levin, 1999; Agrawal, 1999). In order to achieve a sustained pharmacological activity of ODNs and avoid repeated administrations, we suggest to design new delivery systems for ODNs combining a sustained release effect, and an improved intracellular penetration.

2. Material and methods

2.1. Liposomes in thermosensitive gel for oligonucleotide delivery

Blank PC:CHOL:DSPE-PEG2000 (molar ratio 64/30/06) liposomes were produced by the lipid hydration method followed by an extrusion through polycarbonate membranes with diameters of 0.4 and 0.2 μm, respectively. A radiolabeled model oligonucleotide, ^{33}pdT16, was encapsulated in the liposomes by the freeze and thawing technique (Bochot et al., 1998a). Free pdT16 was removed from the supernatant after ultracentrifugation of the vesicles.

Poloxamer 407 gel was prepared by the cold process described by Schmolka et al. (1972). Liposomes were dispersed within poloxamer 407 gels under stirring at +4 °C to get a final poloxamer concentration of 27% (w/v).

The distribution of pdT16 in several ocular tissues (cornea, conjunctiva, sclera, iris, lens, aqueous, and vitreous humors) was determined in the rabbit after instillation of various dosage forms. Radiolabeled pdT16 was applied as a simple solution, a 27% poloxamer 407 gel, a suspension of liposomes or liposomes dispersed within a 27% poloxamer 407 gel. pdT16 concentrations were measured in the tissues and fluids by radioactivity counting at time intervals of 10 min, 2 h, and 24 h.

Ocular distribution and clearance from the vitreous humor of pdT16 were evaluated during 30 days in the rabbit by radioactivity measurements after intravitreal injection of either a solution or liposomes containing pdT16 ODN or liposomes dispersed within poloxamer gel. The integrity of pdT16 was investigated using a competitive hybridization assay (De Oliveira et al., 2000).

2.2. Biodegradable microparticles for the controlled delivery of oligonucleotides

Microparticles were prepared by the multiple emulsion/solvent evaporation method (De Rosa et al., 2002). Briefly, 0.4 ml of an aqueous solution containing 1.5 mg of pdT16 were complexed with PEI at the nitrogen/phosphate (N/P) ratios of 15 or 45. In both cases, a limpid solution was obtained. The pdT16/PEI solution was emulsified into 2.5 ml of methylene chloride containing 250 mg of PLGA (75:25, M_w 98,000 Da). Emulsification was achieved using an Ultraturrax homogenizer operating at 13,500 rpm for 2 min. The w/o emulsion was rapidly added into 20 ml of a 0.5% (w/v) PVA aqueous solution, and homogenized at 8000 rpm for 1 min. Alternatively, NaCl at the concentrations of 1 and 3% (w/v) was added to the external aqueous phase. The resulting w/o/w emulsion was stirred at 1000 rpm at room temperature for 3 h to allow solvent evaporation. Microparticles were then collected by centrifugation (4000 rpm for 15 min), washed with distilled water and freeze-dried for 48 h. The final product was stored at 4 °C. Each batch was prepared in triplicate.

Freeze-dried microspheres were dispersed in distilled water and the mean diameter and size distribution were determined by laser light scattering. Microsphere shape and morphology were analyzed by Scanning Electron Microscopy (SEM). Each sample was coated with a Pt/Pd layer under argon atmosphere and micrographs were obtained using a scanning electron microscope equipped with a Gemini Column.

Encapsulation efficiency was determined as followed: for each batch, 10 mg of microspheres were dissolved in 3 ml of NaOH 0.5N under stirring (500 rpm) at 37 °C until a limpid solution was obtained. Samples were centrifuged at 3000 rpm for 15 min and the supernatant was analyzed by UV spectrophotometry at 260 nm. Each sample was assayed in triplicate.

In vitro release studies of ODN from microspheres were carried out by suspending 20 mg of microspheres in 2 ml of 0.01 M PBS pH 7.4, at 37 °C under stirring (500 rpm). At predetermined time intervals, the suspension was centrifuged and 1.5 ml of the release medium were withdrawn and replaced with the same volume of fresh medium. The pdT16 concentration in the supernatant was determined by UV

spectrophotometry at 260 nm. Each experiment was performed in triplicate.

HeLa (human epitheloid carcinoma cells, ATCC, USA) cells were grown in DMEM medium with sodium pyruvate, pyridoxine, and 1000 mg/l glucose supplemented with 10% of heat-inactivated fetal calf serum (FBS), 100 μg/ml of streptomycin and 100 U/ml of penicillin. Cells were incubated at 37 °C in 5% of CO_2. The culture media, trypsin and PBS were pre-warmed at 37 °C before use.

Approximately 6000 cells suspended in 100 μl of growth media were added to each well in a 96-well plate. After 24 h of incubation at 37 °C to allow cell attachment to the plates, pdT16, pdT16/PEI complexes, unloaded microspheres and microspheres encapsulating pdT16 alone or pdT16/PEI complexes were added to the wells. All experiments were carried out using pdT16 concentrations ranging from 0.0025 to 10 nmol/ml. HeLa cell viability was assessed by the MTT test after 24 h of incubation.

In order to follow intracellular trafficking of the ODN, rhodamine-labeled pdT16 was used. Briefly, 1 ml of a HeLa cells suspension (about 25,000 cells/ml) was seeded on uncoated 24-well plates containing one cover glass in each well and incubated at 37 °C for 24 h. The following samples (0.5 ml) were added to the wells and incubated at 37 °C for 24 h: a solution containing pdT16 or pdT16/PEI complexes (N/P ratio of 15 or 45), or a suspension of microspheres containing pdT16 or pdT16/PEI complexes (N/P ratio of 15 or 45). The concentration of pdT16 for solutions was 0.1 nmol/ml. The amount of microspheres used in the experiment was adjusted in order to release from all the batches 0.1 nmol/ml of pdT16 after 24 h.

Hereafter, cells were washed with PBS and fixed with 4% paraformaldehyde. Cell membranes were stained with *N*-(fluorescein-5-thiocarbamoyl)-1,2-diexadecanoyl-*sn*-glycero-3-phosphoethanolamine (fluoresceine DHPE, Molecular Probes, Netherlands). Cells were washed with PBS and a SlowFade™-Light Antifade kit was used to avoid fluorescence quenching. Cover glasses were mounted on micro-slides and stored at 4 °C. Observations were carried out with a confocal microscope equipped with a Zeiss 40X/1.20 NA water immersion objective lens. Fluorescence images were acquired with argon (wavelength 488 nm) and helium neon (wavelength 543 nm) lasers. Simultaneous images corresponding to fluoresceine and rhodamine were obtained using the multi-tracking function of the microscope. Moreover, differential interference contrast (Nomarski technique) was used to visualize individual cells.

For control experiments, rhodamine alone, rhodamine in association with PEI or rhodamine-labeled pdT16 digested by DNAse for 30 min at 37 °C were added to cells. These experiments allowed us to check that the fluorescence within the cells was due to the ODN uptake and not to the uptake of the free-probe after rhodamine-ON hydrolysis.

3. Results and discussion

3.1. Liposomes in thermosensitive gel for oligonucleotide delivery

Electron microscopy studies (not shown) demonstrated that liposomes were intact when dispersed 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 what would happen in the in vivo situation after dissolution of the gel in the vitreous humor) resulted in a considerable leakage of pdT16 from liposomes (not shown). Release studies were performed using membrane-free release conditions that present the advantage of having the formulations in direct contact with the release medium, thus mimicking the ocular administration into the vitreous humor. We have observed a dissolution of the gels in time. The total dissolution of gel was obtained after 8 h. Poloxamer dissolution was found to control the release process of pdT16 release, whereas the dispersion of liposomes within 27% poloxamer gel was shown to slow down the diffusion of pdT16 out from the gel (Fig. 1).

In a first step, the distribution of pdT16 after instillation was performed. When the pdT16 solution was used, the highest concentrations were observed in the conjunctiva and the cornea, while a substantial amount of drug was also present in the sclera. Low concentrations were measured in the iris. Using the same treatment protocol, the two liposomal formulations (liposomes dispersed in buffer or liposomes

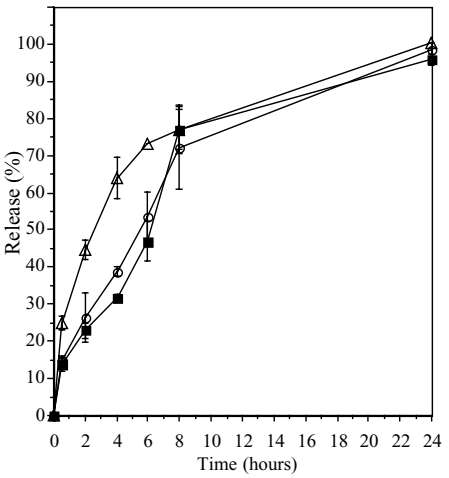

Fig. 1. Comparison between the kinetics of dissolution of 27% poloxamer gels (■), and the release of pdT16 from either 27% poloxamer gels without liposomes (△) or from liposomes dispersed within 27% poloxamer gels (○).

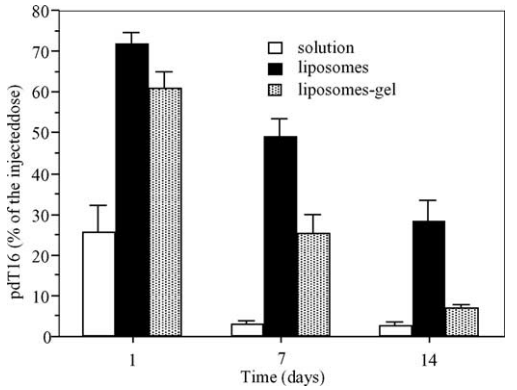

Fig. 2. Distribution of $^{(33P)}$pdT16 in the vitreous humor.

dispersed within the poloxamer gel) delivered low drug amounts of pdT16 to all ocular tissues, and particularly to the conjunctiva and the cornea. The poloxamer gel provided higher tissue concentrations of pdT16 than liposomes but lower than those observed with the solution. These findings indicated that liposomal forms may not be considered useful delivery systems for topical administration of ODNs in superficial ocular diseases (Bochot et al., 1998b).

The residual concentration of pdT16 ODN within the ocular tissues was significantly increased after intravitreal administration of the liposomal suspension instead of a single solution (Fig. 2). Fourteen days postinjection, pdT16 within the vitreous humor using the liposomal suspension in buffer, was 9.3-fold higher than that obtained with pdT16 in solution and 4-fold higher than that measured with the liposomes dispersed within the gel (Fig. 2). In the vitreous humor, pdT16 administered with liposomes dispersed within poloxamer gel was cleared faster than pdT16 injected within a simple liposomal suspension, although this difference was only visible after 1 day (Bochot et al., 2002). The shorter pdT16 residence time within the vitreous obtained with the liposomes-gel was probably due to a faster pdT16 release from the vesicles induced by the presence of the poloxamer 407 forming the gel. Indeed, we have previously demonstrated that poloxamer 407, exhibited a different behavior with respect to liposomes depending on its concentration in the formulations (Bochot et al., 1998a). In dilute solution, poloxamer 407 exhibited surfactant properties able to destabilize the liposomes, which resulted in a considerable leakage of encapsulated pdT16 whereas the same liposomes dispersed within a high poloxamer concentration (27%) were stable. Moreover, we have observed that 27% poloxamer gels could be dissolved in aqueous media. As a consequence, the gel state disappeared to form a dilute poloxamer solution.

In conclusion, administration of liposome-encapsulated pdT16 ODN resulted in a sustained release into the vitreous and the retina-choroid comparatively to the solution and in a reduced distribution in non-relevant tissues (sclera, lens). In addition, liposomes have allowed the protection of the phosphodiester ODN against degradation (Bochot et al., 2002). This was not obtained after administration of the ODN free. These results represent a potential for the treatment of intravitreal diseases (Bochot et al., 2000).

3.2. Biodegradable microparticles for the controlled delivery of oligonucleotides

Mean diameter, actual loading, and encapsulation efficiency of PLGA microspheres containing pdT16 as such or associated with PEI at different N/P ratios are reported in Table 1. The presence of PEI within the internal aqueous phase affected microsphere size, morphology (not shown) and pdT16 actual loading. The mean diameter of microspheres was increased upon PEI addition, this effect being more evident with the

Table 1
Mean diameter and encapsulation efficiency of pdT16-containing PLGA microparticles

N/P ratio	% w/v NaCl in W2	Mean diameter (μm)	Actual loading (μg ON/mg Ms ± S.D.)[a,b]	Encapsulation efficiency (% ± S.D.)[b]
–	–	30.3 ± 2.3	2.4 ± 0.4	40 ± 6
15	–	45.3 ± 2.4	4.0 ± 0.3	67 ± 5
45	–	57.4 ± 1.2	4.5 ± 0.5	83 ± 9
15	1	28.4 ± 4.0	4.3 ± 0.2	73 ± 2
45	1	31.0 ± 3.4	4.1 ± 0.1	69 ± 3
45	3	33.1 ± 3.8	3.4 ± 0.2	58 ± 2

S.D.: standard deviation.
[a] Actual loading is expressed as μg of encapsulated pdT16 per mg of microparticles (Ms).
[b] Encapsulation efficiency is the ratio between the oligonucleotide actual and theoretical loading percent. The theoretical loading was 5.9 μg of pdT16/mg of microparticles.

higher amount of polycation used to complex pdT16. When pdT16 was encapsulated as such the actual loading was 2.4 μg of pdT16/mg of microspheres, corresponding to an encapsulation efficiency of about 40%. The addition of PEI in the internal aqueous phase increased the encapsulation efficiency up to 67 and 83% at the N/P ratios of 15 and 45, respectively (Table 1). However, SEM observations (not shown) show that microparticles containing PEI display very large pores. The increase in the osmotic pressure of the internal aqueous phase due to the presence of PEI was suggested to be the main factor responsible for the high porosity of microparticles. PEI in the internal aqueous phase could act as an osmotically active agent that induces a massive influx of water toward the internal aqueous droplets, thus leading to an increase of the inner droplet volume, giving rise to very porous structure (De Rosa et al., 2002). However, microparticles prepared with NaCl in the external aqueous phase were spherical, with a smooth surface and only very few pores (not shown). In particular, particles containing the complex at the highest N/P ratio (45) and prepared with 1% of NaCl displayed a higher porosity (not shown), than particles encapsulating the complex at the lower N/P ratio and the same amount of NaCl. The porosity of microparticles encapsulating the complex at the highest N/P ratio was slightly reduced by using larger amounts of NaCl (3%) (not shown) although the presence of NaCl did not modify particle diameter (De Rosa et al., 2003) (Table 1). Only a slight decrease of the encapsulation efficiency was observed at high concentration of NaCl (3%). This effect could be due to the diffusion of water droplets across the oil layer.

Microspheres containing free pdT16 were characterized by a triphasic release profile. The initial release, the so-called *burst effect*, which corresponded to about 20% of the total ODN loaded, was followed by a phase which lasted about 15 days characterized by a very slow pdT16 liberation. In the third phase, a more rapid ODN release rate was observed. When PEI was present, pdT16 was more rapidly released. However, the *burst effect* was lower at the N/P ratio of 15 as compared to the higher N/P ratio. In this last case, a very rapid ODN release corresponding to about 80% of the total content was observed after 4 days. In the presence of NaCl, microparticles encapsulating complexes exhibited a *burst effect* dependent on the salt concentration used during preparation. For instance, in the case of N/P ratio of 45, the increase of NaCl in the external aqueous phase from 1 to 3% resulted in *burst release* of about 30 and 7%, respectively.

ON penetration inside cells and its intracellular localization were also investigated (De Rosa et al., 2003). As control experiments, rhodamine alone or associated with PEI was added to cells. In this case, only a negligible fluorescence inside cells was observed (not shown). When rhodamine-labeled pdT16 was administered to the cells, the ODN was located into the cytoplasm (not shown). After incubation of the cells with microspheres containing pdT16 alone, a significant localization inside cytoplasm and a weak presence inside nucleus of the ODN were observed (not shown). When HeLa cells were incubated with pdT16 associated to PEI at the N/P ratio of 15, the ODN was exclusively localized in the cytoplasm (not shown). On the contrary, when the same complex was

released from the microspheres the ODN was present in both cytoplasm and nucleus. This difference was more evident in the case of the complex at the highest N/P ratio (not shown). In fact, free complex slightly penetrated inside cytoplasm, while when the same complex was released from microspheres pdT16 was exclusively localized inside the nucleus (not shown).

4. Conclusion

Thermosensitive gels such as poloxamer 407 when used alone or in combination with the sterically stabilized liposomes allowed to prolong the retention of the ODNs in the vitreous. However, because of their destabilization in the presence of the diluted gel, liposomes alone were improved more efficiently the intraocular retention of the ODNs but intravitreal half-life was around 7 days. This is the reason why we have developed a biodegradable polymer system which permit a more prolonged release of the ODNs. In addition, because it is able to release a PEI/ODNs complexes, this system enhance the intracellular penetration of the nucleic acids.

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