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Note

A new delivery system for antisense therapy: PLGA microspheres encapsulating oligonucleotide/polyethyleneimine solid complexes

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Abstract

Microspheres for the controlled release of an antisense oligonucleotide against the Transforming growth factor β_1 were designed. Free oligonucleotide or its solid complexes with polyethylenimine (PEI) at different nitrogen/phosphate (N/P) ratios, were encapsulated within poly(lactide-co-glycolide) (PLGA) microspheres prepared by the multiple emulsion–solvent evaporation technique. The encapsulation of the oligonucleotide in form of solid complexes, the N/P ratio, as well as the PLGA type affected microspheres characteristics in term of loading, morphology, oligonucleotide distribution inside matrix and in vitro release profile. The designed microspheres allow the encapsulation and slow release of oligonucleotide/PEI solid complexes that should be effectively internalized inside cells.

Keywords: Oligonucleotides; Microparticles; Polyethylenimine; Poly(lactide-co-glycolide)

Techniques for the regeneration of injured spinal cord involve the use of peripheral nerve grafts to reconnect the damaged tissue to denervated skeletal muscles. (Horvat, 1991). However, the regeneration is limited by the action of extracellular matrix proteins (especially chondroitin sulfate proteoglycans). The use of an antisense therapy designed to transiently block the expression of either these inhibitory proteins or factors that control their production (TGFβ_1) could favour the supraspinal input restoration after peripheral nerve implantation in the spinal cord (Clark and Coker, 1998). A family of cytokines involved in the proliferation of inhibitory matrix proteins are Transforming growth factors β (TGFβ). Unfortunately, oligonucleotide-based therapies are strongly limited by poor biological stability, short in vivo half-lives and limited cellular uptake of nucleic acids (Akhtar et al., 2000). Modified oligonucleotides such as phosphorothioate are more stable to nuclease digestion although their membrane permeability is still very poor. Nevertheless, an improved cellular penetration of nucleic acids can be obtained by using cationic

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polymers such as polyethylenimine (Godbey et al., 1999).

We have recently demonstrated that the encapsulation of oligonucleotides or oligonucleotide/PEI complexes in PLGA microspheres offers advantages such as a sustained release of the oligonucleotide, low toxicity and improved cellular penetration (De Rosa et al., 2002). A similar approach was applied to a phosphorothioate antisense oligonucleotide (5′-GAA GCA ATA GTT GTT GTC CA-3′) against the transforming growth factor β_1 (*anti-TGF*β_1) (Merrilees and Scott, 1994). The goal of the present study was to design microparticles for the delivery of an antisense oligonucleotide against the Transforming growth factor β_1. PLGA microparticles were designed and characterized for loading, morphology, oligonucleotide distribution inside matrix and in vitro release profile.

Anti-TGFβ_1 or *anti-TGF*β_1/PEI complexes at the nitrogen to phosphate (N/P) ratios of about 13 or 35 were encapsulated inside microspheres by a modified multiple emulsion/solvent evaporation method (Rojas et al., 1999). Briefly, 0.4 ml of an aqueous solution containing 308 nmol (2 mg) of *anti-TGF*β_1 in free form or in association with polyethylenimine (25,000 Da, branched), were emulsified into 2.5 ml of methylene chloride containing 250 mg of PLGA. The addition of PEI to *anti-TGF*β_1 resulted in the formation of a solid complex. The w/o emulsion was dispersed in 20 ml of a 0.5% (w/v) poly(vinylalcohol) aqueous solution and the resulting w/o/w emulsion was stirred at room temperature for 3 h to allow solvent evaporation. Microspheres were then collected by centrifugation, washed with distilled water and freeze-dried. The final product was stored at 4 °C. Each formulation was prepared in triplicate. Microspheres were prepared from two different PLGA types: Resomer RG 756 (lactide/glycolide ratio 75:25, Mw 98,000 Da, end capped) or Resomer RG 503H (lactide/glycolide ratio 50:50, Mw 22,000 Da, non-end capped). Mean diameter and size distribution of the microspheres were determined by laser light scattering while the morphology was analysed by Scanning Electron Microscopy (SEM). To investigate oligonucleotide distribution inside the polymeric matrix, microspheres encapsulating a mixture of *anti-TGF*β_1 and 3′-rhodamine labelled *anti-TGF*β_1 were analysed by confocal microscopy. The amount of oligonucleotide loaded inside microspheres was determined after particle and complex dissolution in 0.5 N NaOH under stirring at 37 °C. Oligonucleotide concentration in the resulting solution was determined by UV spectrophotometry at 260 nm. In vitro release studies of *anti-TGF*β_1 were carried out at 37 °C by suspending the particles in PBS at pH 7.4 under stirring (500 rpm). In the case of particles containing *anti-TGF*β_1 by itself, the suspension was centrifuged at predetermined time intervals and 1.5 ml of the release medium were withdrawn and replaced with fresh medium. *Anti-TGF*β_1 concentration in the supernatant was determined by UV spectrophotometry at 260 nm. In the case of microspheres encapsulating *anti-TGF*β_1/PEI complexes, microspheres and complexes released from microspheres were separated by a gradient-based technique (Vauthier et al., 1999). Briefly, equal volumes of sucrose solutions at concentrations of 50, 60, 65 and 70% (w/w), were superimposed to form successive layers of decreasing concentrations. The layers were allowed to diffuse for 6 h at room temperature before use. The release medium containing microspheres (total volume of 1 ml) was placed on the top of the sucrose gradient and centrifuged at 20 °C (27,000 rpm—1 h). PLGA microspheres (middle height of the tube) and released complexes (bottom of the tube) were separately withdrawn; *anti-TGF*β_1 content was measured after complex dissolution in 0.5 M NaOH. Fig. 1 shows SEM images of all the microsphere formulations. Microspheres prepared from Resomer RG 756 containing the free oligonucleotide were spherical with a smooth surface (Fig. 1A), whereas microspheres encapsulating *anti-TGF*β_1/PEI complexes were very porous (Fig. 1B and C). Particles morphology strongly changed in the case of Resomer RG 503H. When *anti-TGF*β_1 was encapsulated by itself, particles were irregular and with large pores (Fig. 1D) whereas microspheres encapsulating *anti-TGF*β_1/PEI complexes displayed numerous clefts on the surface (Fig. 1E and F). An increase of size was observed for microspheres prepared from Resomer RG 756, as in the case *anti-TGF*β_1/PEI complexes were encapsulated at the N/P ratio of 35 (Table 1).

The actual loading and encapsulation efficiency of microspheres are reported in Table 1. The use of Resomer RG 756 resulted in encapsulation efficiencies higher than those obtained with Resomer RG 503H (Table 1). Furthermore, the encapsulation of

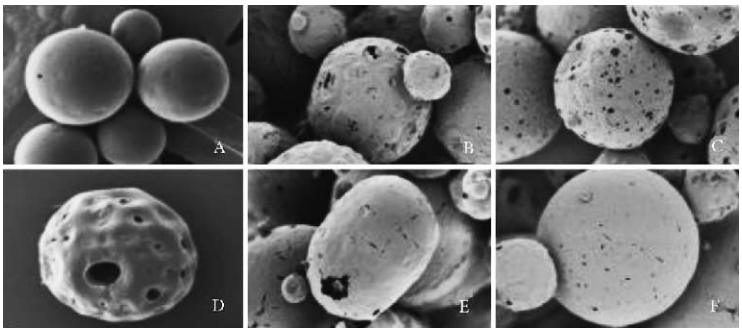

Fig. 1. SEM analysis of microspheres encapsulating *anti-TGFβ_1*. Microspheres prepared from Resomer RG 756 and encapsulating *anti-TGFβ_1* (A), *anti-TGFβ_1*/PEI complexes at the N/P ratio of 13 (B) and 35 (C). Microspheres prepared from Resomer RG 503H and encapsulating *anti-TGFβ_1* (D), *anti-TGFβ_1*/PEI complexes at the N/P ratio of 13 (E) and 35 (F).

anti-TGFβ_1/PEI complexes in the internal aqueous phase increased oligonucleotide encapsulation efficiency for both PLGA types, especially at the initial N/P ratio of 13 (Table 1).

Confocal microscopy images showed a very different oligonucleotide distribution depending on the presence of PEI (Fig. 2). When *anti-TGFβ_1* was encapsulated as PEI complex, the oligonucleotide was localized in round structures, probably the internal cavities within the polymeric matrix (Fig. 2A and B). In the case of microspheres encapsulating the complex at the N/P ratio of 13, the oligonucleotide was well distributed in the matrix and localized in nanoscopic spots (Fig. 2C and D). In all the cases, the use of Resomer RG 756 resulted in a more homogeneous distribution of the oligonucleotide inside matrix.

The higher viscosity of Resomer RG 756, which results in the formation of a more stable primary emulsion, could contribute to increase the encapsulation efficiencies, yielding to a more homogeneous distribution of the encapsulated drug in the polymeric matrix.

In vitro release profiles of *anti-TGFβ_1* from microspheres encapsulating the free oligonucleotide are reported in Fig. 3 . Particles prepared with Resomer RG 756 showed a triphasic release, characterized by a very low *burst effect* (about 10%) followed by a phase of slow release. Then, the release rate strongly increased resulting in an amount of oligonucleotide released of about 70% after 20 days. In the case of particles prepared from Resomer RG 503H, 80% of the entrapped oligonucleotide were immediately released and the release was complete after about 8 days. In this case, the large pores observed on particles surface could be responsible for the fast overall release rate. For particles containing *anti-TGFβ_1*/PEI complexes, the UV dosage of *anti-TGFβ_1* did not show any oligonucleotide release up to 60 days. Presumably, *anti-TGFβ_1* was released as solid complex with PEI

Table 1
Characteristics of microspheres encapsulating *anti-TGFβ_1*

PLGA type (Resomer)	N/P ratio	Mean diameter (μm ± S.D.)	*Anti-TGFβ_1* actual loading (nmoles of *anti-TGFβ_1*/100 mg PLGA ± S.D.)[a]	Encapsulation efficiency (% ± S.D.)[a]
RG 756	–	35 ± 4	0.29 ± 0.03	36 ± 3.7
RG 756	13	45 ± 4	0.73 ± 0.04	91 ± 0.5
RG 756	35	64 ± 4	0.44 ± 0.01	55 ± 0.6
RG 503 H	–	23 ± 4	0.17 ± 0.01	14 ± 1.0
RG 503 H	13	26 ± 5	0.42 ± 0.01	53 ± 0.6
RG 503 H	35	59 ± 5	0.38 ± 0.01	48 ± 1.3

[a] *Anti-TGFβ_1* actual loading/theoretical loading × 100.

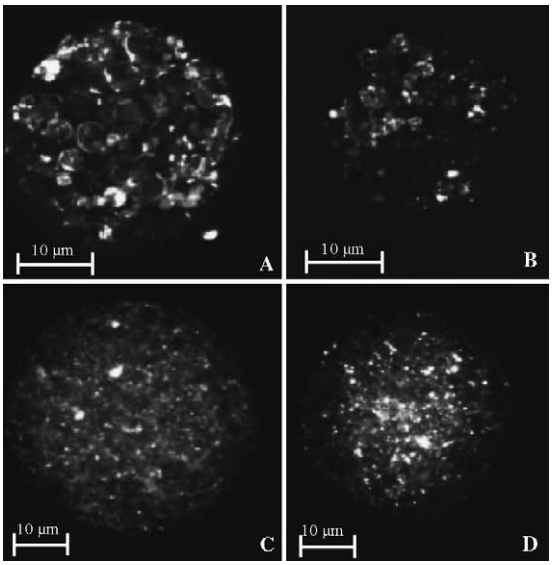

Fig. 2. Confocal microscopy images of microspheres encapsulating *anti-TGFβ_1*. Microspheres encapsulating *anti-TGFβ_1* and prepared from Resomer RG 756 (A) or Resomer RG 503H (B). Microspheres encapsulating *anti-TGFβ_1*/PEI complexes and prepared from Resomer RG 756 (C) or Resomer RG 503H (D).

and hence had to be separated from microspheres by sucrose gradient and then decomplexed before assay. The percentages of *anti-TGFβ_1* released as complex have been reported in Table 2. After 15 days, microspheres prepared with Resomer RG 756 released a similar amount of complex at both N/P ratios. On the contrary, at 30 and 45 days, particles prepared at the N/P ratio of 35 showed a progressive release with more than 40% of *anti-TGFβ_1* released after 45 days, while at the N/P ratio of 13 the amount of released complex did not significantly increase with time. This effect could be attributed to the presence of PEI inside microspheres, which can account for a hydrophilization of the polymeric matrix thus resulting in a faster release. The influence of complex N/P ratio on release rate was different in the case of microspheres prepared from Resomer RG 503H. After 45 days, only a very low percentage of *anti-TGFβ_1* was released from microspheres prepared at the N/P ratio of 13. For particles with a N/P ratio of 35, the percentage of released complex even decreased with time. In the case of microspheres prepared from Resomer RG 503H, an interaction between complexes and free carboxylic end-groups of PLGA could occur. This effect probably results in the absorption of complexes on particles surface, giving a slow and incomplete release.

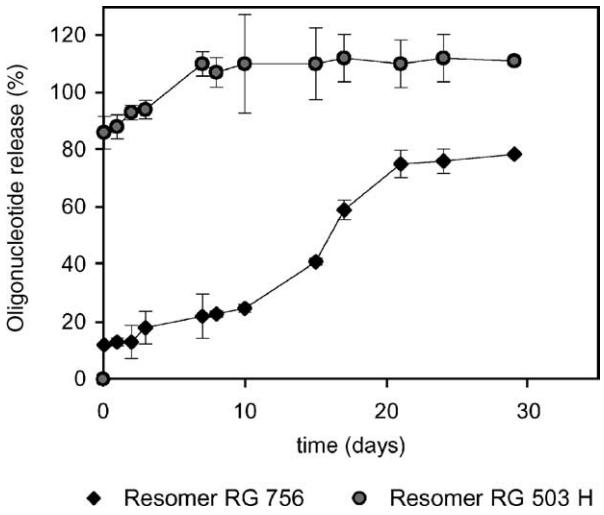

Fig. 3. In vitro release profiles of *anti-TGFβ_1* from microspheres encapsulating naked oligonucleotide.

Table 2
In vitro release of *anti-TGFβ_1* from microspheres encapsulating complexes

PLGA type (Resomer)	N/P ratio	*Anti-TGFβ_1* released (% ± S.D.)[a]		
		15 days	30 days	45 days
RG 756	13	15.8 ± 8.0	11.9 ± 5.2	9.5 ± 7.0
RG 756	35	15.0 ± 8.3	20.7 ± 7.0	43.8 ± 19.7
RG 503H	13	1.1 ± 1.4	3.2 ± 5.3	4.4 ± 3.8
RG 503H	35	52.2 ± 13.1	20.0 ± 13.1	38.6 ± 4.0

[a] The amount of oligonucleotide was determined after separation of the complex from microspheres by sucrose gradient and complex solubilization in 0.5 N NaOH.

In conclusion, the encapsulation of oligonucleotides within PLGA microspheres open up interesting perspectives for the controlled delivery of *anti-TGFβ_1* oligonucleotides in the regeneration of injured spinal cord.

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