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Note

Biodegradable microparticles for the controlled delivery of oligonucleotides

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Abstract

Microspheres allowing the controlled release of the model oligonucleotide pdT16 were designed. The oligonucleotide, alone or associated with polyethylenimine (PEI) at different nitrogen/phosphate ratios, was encapsulated within poly(lactide-co-glycolide) microspheres prepared by the multiple emulsion–solvent evaporation technique. The introduction of PEI in the internal aqueous phase resulted in a strong increase of the oligonucleotide encapsulation efficiency. PEI affected also microsphere morphology inducing the formation of very porous particles and yielding to an accelerated release of pdT16. However, when incubated with HeLa cells, microspheres encapsulating pdT16/PEI complexes allowed an improvement of the intracellular penetration of the released oligonucleotide. The developed strategy appears to be a very interesting tool to obtain a sustained release system for oligonucleotides with an efficient cellular delivery. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Oligonucleotide; Polyethylenimine; Microparticles; Cell delivery

Antisense oligonucleotides are short DNA fragments with high therapeutic potentialities in the treatment of major diseases such as cancer and viral infections. However, their short in vivo half-life and a limited cellular uptake hamper the development of oligonucleotides-based therapeutics (Akhtar et al., 2000).

The use of systems based on biodegradable polymers such as poly(lactide-co-glycolide) (PLGA) allow the achievement of a sustained pharmacological activity of oligonucleotides and avoid repeated administrations without affecting oligonucleotide biological stability as well as its hybridisation capability (Lewis et al., 1995). However this approach does not improve oligonucleotide cellular uptake. The cationic polymer polyethylenimine (PEI) has shown to provide an important capability for the in vitro and in vivo intracellular delivery of nucleic acids (Godbey et al., 1999). Therefore, we have designed a new delivery system for oligonucleotides combining a sustained release effect and an improved intracellular penetration of the oligonucleotide. PLGA

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microspheres entrapping the model oligodeoxythymidilate pdT16 (3′-TTT TTT TTT TTT TTT T-5′) and pdT16/PEI complexes were produced and characterized. The subcellular distribution of pdT16 released from the particles was also tested on HeLa cells.

Microspheres were prepared by a modified multiple emulsion–solvent evaporation method (Rojas et al. 1999). Briefly, 0.4 ml of an aqueous solution containing 308 nmol of oligodeoxythymidilate pdT16 or 3′-rhodamine-labelled pdT16, in free form or in association with PEI (25 000 Da, branched) at the Nitrogen to Phosphate (N/P) ratios of about 15 or 45, were emulsified into 2.5 ml of methylene chloride containing 250 mg of poly(D,L-lactide-co-glycolide) (Resomer RG 756, Mw 98 000 Da). This 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 batch was prepared in triplicate. The mean diameter and size distribution of the microspheres were determined by laser light scattering while the morphology was analysed by scanning electron microscopy (SEM). For the determination of the oligonucleotide content in the microspheres, a weighted amount of lyophilised particles was dispersed in NaOH 0.5 N under stirring at 37 °C. When a limpid solution was obtained, oligonucleotide concentration was determined by UV spectrophotometry at 260 nm. In vitro release studies of the oligonucleotide from microspheres were carried out by suspending the particles in 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. Moreover, PEI release from particles was analysed spectrophotometrically, after addition of a $CuSO_4$ aqueous solution to the release medium to form a coloured Cu^{2+}/PEI complex.

In order to follow intracellular trafficking of the oligonucleotide, microspheres containing rhodamine-labelled pdT16 or pdT16/PEI complexes were added on HeLa cells suspension on uncoated 24-well plates containing one cover glass. 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. After incubation for 24 h at 37 °C, cells were washed with PBS and fixed with 4% paraformaldehyde. After cells washing with PBS and a SlowFade™—Light Antifade kit was used to avoid fluorescence quenching. Cover glasses were mounted on micro-slides and observations were carried out with a confocal microscope.

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 and pdT16 actual loading.

The encapsulation of pdT16/PEI complexes resulted in porous microspheres displaying a larger diameter at higher N/P ratios (Fig. 1). Such surface porosity as well as the higher size of micro-

Table 1
Characteristics of microspheres encapsulating pdT16 or pdT16/PEI complexes

N/P ratio	Particle size (μm)	Oligonucleotide actual loading[a]	Encapsulation efficiency[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

[a] μg of pdT16/mg microspheres ± S.D.

[b] (oligonucleotide actual loading/oligonucleotide theoretical loading) × 100 ± S.D. The theoretical loading was 5.9 μg of pdT16/mg of microspheres.

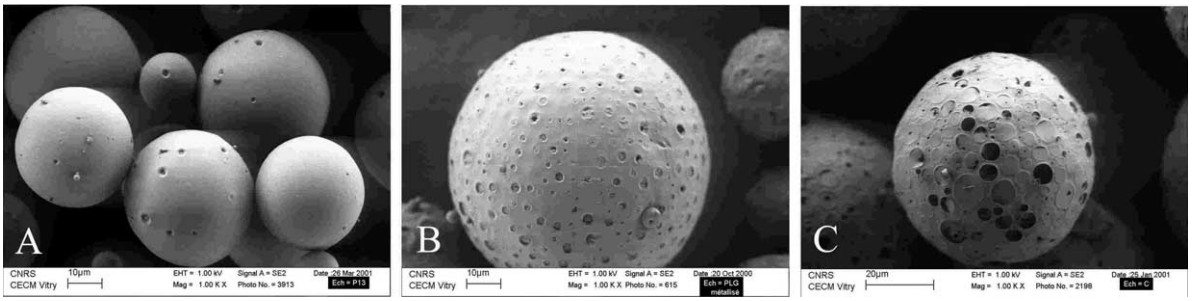

Fig. 1. SEM analysis of microspheres containing pdT16 (A), pdT16/PEI complex (N/P = 15) (B), pdT16/PEI complex (N/P = 45) (C).

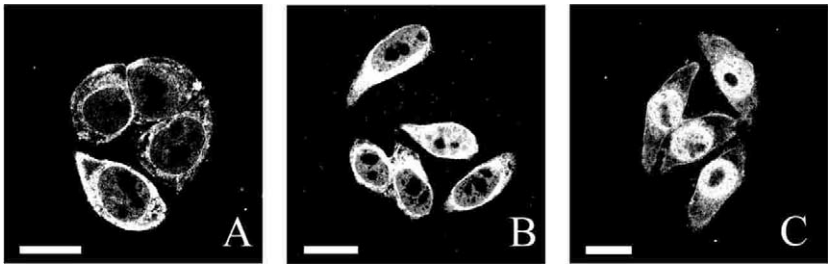

Fig. 2. Confocal microscopy of HeLa cells after 24 h of incubation with: (A) microspheres containing pdT16; (B) microspheres containing pdT16/PEI complex (N/P = 15); (C) microspheres containing pdT16/PEI complex (N/P = 45). Scale bar = 20 μm.

spheres containing complexes could be explained by the presence of the osmotically active polycation added in the internal aqueous phase. The addition of PEI to the internal aqueous phase increased the encapsulation efficiency, this effect being more evident at the higher N/P ratio (Table 1). It is suggested that a possible ionic interaction between pdT16/PEI and PLGA occurred. Such interaction can contribute to effectively increase the entrapment of the oligonucleotide inside the microspheres.

Microspheres containing free pdT16 were characterised by a *burst effect* of about 20% followed by a slow in vitro release profile. 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 N/P ratio of 45. In this last case, a very rapid oligonucleotide release corresponding to about 80% of the total content was observed after 4 days. The fast release profile of particles containing pdT16/PEI complexes could be ascribed to both the presence of a porous structure and the hydrophilisation of polymer matrix exerted by the cationic polymer. Moreover, for each formulation release profile of PEI and pdT16 showed that the oligonucleotide and the cationic polymer were released in a complexed form. However the N/P ratio was lower than the initial one varying from about 6 to 14, indicating that a part of the PEI in excess was lost during the preparation of the microspheres. After being released from microspheres, the oligonucleotide penetrated inside HeLa cells but when microspheres containing oligonucleotide/PEI complexes were incubated with cells, a preferential localization in the nucleus was observed. In particular, when the higher N/P ratio was used oligonucleotide was exclusively located inside the nucleus (Fig. 2). It has been reported that the accumulation of the oligonucleotide in the cytoplasm can promote the nuclear localization of the oligonucleotide (Leonetti et al., 1991). These findings are coherent with our results since a more efficient nuclear uptake of pdT16 was observed when high

cytoplasmic concentration of the oligonucleotide was achieved.

In this paper a new type of PLGA microspheres able to deliver oligonucleotide/polycation complexes was designed. The encapsulation of oligonucleotide/PEI complexes induced a strong increase of microsphere loading, a more rapid oligonucleotide release profile and an improved intracellular penetration of the released oligonucleotide with a localization in the nucleus especially when high PEI concentrations were used.

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