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Comparison of two methods of encapsulation of an oligonucleotide into poly(D,L-lactic acid) particles

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Abstract

The aim of this study was to compare two methods to encapsulate a 25-mer-phosphorothioate oligonucleotide (ODN) into poly(D,L-lactic acid) (PLA) particles. Antisense oligonucleotides belong to a new therapeutic class especially attractive for the treatment of cancers and viral diseases. The development of these new drugs suffers, however, from poor stability in biological media and very low cellular uptake. Polymeric particulate systems display interesting features for ODN delivery. ODN are highly hydrophilic and most encapsulation methods are inappropriate for such molecules. Using poly(D,L-lactide) polymer, two methods of encapsulation were compared. First, a double emulsion technique was used to prepare nano- and microparticles. Secondly, the ODN was combined with a quaternary ammonium, the cethyltrimethyl-ammonium bromide (CTAB), to enhance the hydrophobicity of the molecule before entrapment by the emulsification–diffusion method. Both methods led to the formation of individualized and spherical particles loaded with a significant amount of ODN. Similar entrapment efficiencies were obtained for the nanoparticles prepared by both methods (approx. 27% of the theoretical loading) whereas 45% of entrapment efficiency was observed for the microparticles. Seventy five percent of the ODN were released in 60 min with the particles prepared by the emulsification–diffusion method, whereas only 7% were released in 60 h when using the double emulsion method. A viability test on U-937 cells showed better survival rates with the particles prepared by the double emulsion technique. The results suggest that the location of the ODN in the polymeric matrix is affected by the encapsulation method. Particles containing CTAB appeared more toxic than the ones obtained by the double emulsion technique, however, these particles can still be used for antisense activity since high oligonucleotide loading can be achieved.

Keywords: Nanoparticle; Antisense therapy; Poly(D,L-lactic acid); Oligonucleotide delivery; Double emulsion; Emulsification–diffusion

1. Introduction

Oligonucleotides are single stranded pieces of nucleic acids ranging from 15 to 25 bases with a sequence specifically designed to bind an appropriate sequence of intracellular DNA or RNA

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according to the Watson and Crick base pair hybridization. The targeted mRNA translation is therefore blocked and thus, the synthesis of a specific protein is inhibited (Monia et al., 1996; Wagner and Fanagan, 1997; McKay et al., 1999). This leads to the interruption of normal cell cycle or virus replication. Antisense oligonucleotides are therefore a very attractive approach for anticancer and antiviral therapy. From a physicochemical standpoint, oligonucleotides (ODN) are characterized by a high hydrophilicity as well as a global negative charge that both will have consequences in bioavailability and drug delivery design.

Development of antisense ODNs as drugs is seriously limited by their insufficient bioavailability. Indeed, oligonucleotides are poorly stable in biological environment and very sensitive to extra- and intracellular nuclease hydrolysis. ODN, especially phosphorothioates, have a high affinity with proteins with the consequence of decreasing the availability of free ODN at the target site. Furthermore, ODN–protein interaction has been described for some ODN as responsible of side effects by interaction with coagulation and complement systems (Shaw et al., 1997; Yu et al., 1999). Finally, they suffer from a very poor intracellular penetration. Therefore, a reliable delivery system is needed to improve the availability of these new entities. Association with liposomes, cationic lipids, peptides, proteins, dendrimers, polymeric particles and viruses have been developed as ODN delivery strategies to promote stability and enhance cellular uptake (for more information, the reader is referred to a recent review by Lebedeva et al., 2000). Among other systems, polymeric particles present several advantages for ODN delivery. Indeed, entrapment in a polymeric matrix should shield the ODN from the external environment, reducing nuclease hydrolysis and preventing protein binding. Furthermore, phagocytic cell tropism observed with small particles is an advantage to target macrophages and treat intracellular infections such as AIDS.

Polyalkylcyanoacrylate particles have been suggested as ODN carriers (Chavany et al., 1992; Schwab et al., 1994; Lambert et al., 1998), however, the potential toxicity of the polymer raises some concerns (Lherm et al., 1992). Therefore, more acceptable poly(lactide) particles appear more suitable for drug delivery purposes.

The preparation of poly(D-L-lactic acid) (PLA) nanoparticles is commonly based on the formation of an emulsion with an inner phase made of an organic solvent in which the polymer is dissolved and an external phase consisting in a nonmiscible solvent, usually water with or without a surfactant. However, these methods based on an O/W emulsion give very poor entrapment efficiencies for hydrophilic molecules. With the development of new drugs such as proteins, peptides and nucleic acids, lots of efforts have been put in generating alternatives such as the multiple emulsion process (Ogawa et al., 1988; Blanco-Prieto et al., 1994), compound derivatization (Berton et al., 1999a), non-aqueous emulsion (Herrmann and Bodmeier, 1998), or nanocapsules with hydrophilic core (Vranckx et al., 1996; Fattal et al., 2000). In this report, the double emulsion technique and the hydrophobization of the oligonucleotide have been compared based on the entrapment of a 25-mer-phosphorothioate oligonucleotide designed to bind the initiation site of the gag-m RNA of the HIV-1, protein that is essential for virus packaging (Agrawal and Radhakrishnan, 1995).

2. Materials and methods

PLA (Medisorb® 100 DL, MW: 100 kD) was obtained from Medisorb Technology International L.P. (Cincinnati, OH). Poly(vinyl alcohol) (PVAL Mowiol® 4-88, MW: 26 000, Hoescht, Franckfurt am Main, Germany) was used as a stabilizer. Benzyl alcohol, methylene chloride, and cethyltrimethyl-ammonium bromide (CTAB) were purchased from Fluka (Buchs, Switzerland), phorbol 12-myristate 13-acetate (PMA) and the MTT test reagent were obtained from Sigma (Buchs, Switzerland), and the 25-mer-FITC-labelled oligonucleotide from Genset (Paris, France).

For the double emulsion technique, particles were prepared as follows. The oligonucleotide was solubilized in 0.1% PVAL aqueous solution

(100 μl) and the inner aqueous phase was emulsified by sonication (Vibracell, Sonics and Materials, Danbury, CT, USA) in 1 ml of methylene chloride containing 150 mg of PLA. The primary emulsion was then poured into 2 ml of a 2% PVAL solution to form the multiple emulsion. Depending on the method used to produce the secondary emulsion, the size of the final particles varied. Indeed, with sonication, nanoparticles were obtained whereas after vortexing, microparticles were formed. After solvent evaporation at room temperature under stirring in 50 ml of water, particles were washed by centrifugation, lyophilized and characterized.

The preparation method for the precipitation/emulsification–diffusion technique was based on the publications by Berton et al. (1999a,b). Briefly, separate aqueous solutions of oligonucleotide and CTAB were combined to form a water insoluble precipitate. The precipitate and the polymer (50 mg) were solubilized in 1.1 ml benzyl alcohol. An emulsion was formed in 2 g of an aqueous solution of 12.5% PVAL. Solvent was removed by diffusion after adding dropwise 40 ml of distilled water. Particles were then washed by centrifugation and lyophilized before characterization.

Particle size and morphology were assessed by photon correlation spectrometry measurements (Zetasizer 5000, Malvern, UK) as well as scanning electron microscopy (SEM JSM-6400 JEOL, Japan).

Entrapment efficiency was determined by spectrofluorometry, excitation 488 nm, emission 510 nm, with a LS-50B luminescence spectrometer (Perkin Elmer, Rotkreuz, Switzerland) after PLA hydrolysis in 1 N NaOH.

Release kinetics were performed in phosphate buffer (PBS) at 37°C, the released oligonucleotide was measured by the emitted fluorescence in the supernatant after centrifugation. Measurements were done in triplicate.

Effect of particles on cell viability was assessed on a human histiocytic cell line, U-937 (ATTC, Rockville, MD, USA). These cells are able to differentiate into macrophage-like cells after addition of phorbol 12-myristate 13-acetate (PMA). U-937 cells were grown on RPMI 1640 supplemented with glutamine 2 mM, FBS 10%, glucose 4.5 g/l, pyruvate 1 mM, and penicillin and streptomycin (50 000 U, μg/l) at 37°C under 5% CO_2. Cells were seeded on 96 well plates at the density of 10^4cells/well in 200 μl of medium containing 50 nM PMA. After 48 h incubation, the medium was removed and replaced by particle suspensions in fresh medium. Cell survival rates were then assessed by a MTT assay after 24 h incubation. The results are expressed as a percentage of the control cells incubated with medium.

3. Results and discussion

Since the development of new hydrophilic molecules such as peptides, proteins and nucleic acids, as drug candidates there has been a need in terms of drug delivery in which poly(lactide) particles have taken a large place. However, most encapsulation techniques are based on an O/W emulsion and lead to low entrapment efficiency for these compounds. Alternatives have, therefore, been developed to enhance encapsulation rates (Ogawa et al., 1988; Vranckx et al., 1996; Herrmann and Bodmeier, 1998; Berton et al., 1999a). In this report, two techniques, the double emulsion and the precipitation/emulsification–diffusion, have been compared to investigate whether preparation methods would affect oligonucleotide incorporation and final particle characteristics.

Both methods led to the formation of nanoparticles (Table 1). With the double emulsion process, nanoparticles were obtained only when sonication was used for the two emulsification steps. When the first emulsification step was followed by vortexing, microparticles were formed. In terms of entrapment efficiency, no difference was seen between nanoparticles prepared either by emulsification–diffusion or double emulsion (approx. 27%). Higher entrapment efficiency (49%) was obtained with microparticles.

As observed by scanning electron microscopy (Fig. 1), nanoparticles prepared by the emulsification–diffusion process were smooth, well individualized and homogeneous in size. On the other hand, nanoparticles prepared by the double emul-

Table 1
Oligonucleotide-loaded PLA particle characteristics

Method	Size (nm)	Theoretical drug loading (μg/mg)	Actual drug loading (μg/mg)	Entrapment efficiency (%)
Emulsification–diffusion	390	50	19	28
Double emulsion, two sonication steps	450	10	3.5	26
Double emulsion, sonication and vortexing	3100	10	6.5	49

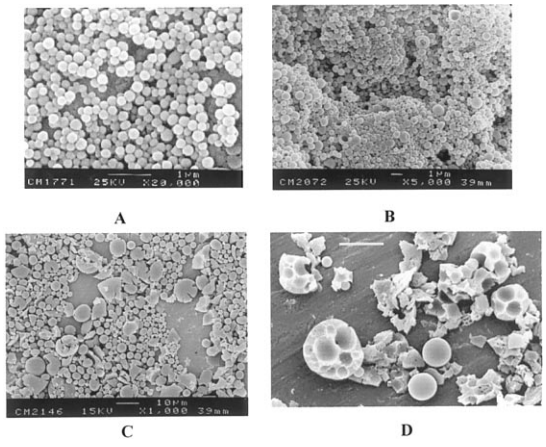

Fig. 1. SEM pictures of the PLA particles prepared by the precipitation/emulsification–diffusion process (A), or the double emulsion technique; nanoparticles (B), and microparticles (C and D, bar = 10 μm).

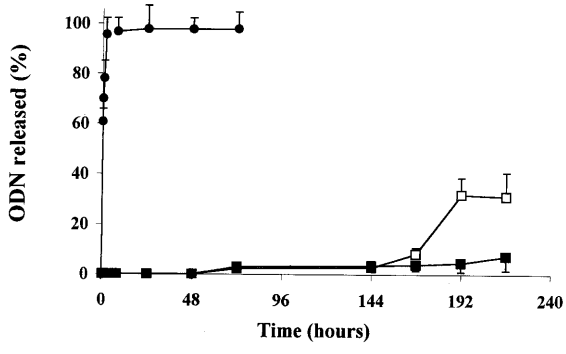

Fig. 2. Release profiles of FITC-labelled ODN from PLA particles (medium: PBS at 37°C, $n = 3$). P-Nano (●): nanoparticles prepared by precipitation/emulsification–diffusion process, DE-Nano (□), Micro (■): nano- and microparticles prepared by double emulsion, respectively.

sion process had a broader size distribution. They appeared more aggregated and some were invaginated, representative of a capsular morphology. Microparticles had a large size distribution and many of the particles were fractured showing the characteristic alveolar morphology of double emulsion cavities (Fig. 1D).

The release profiles were significantly different whether the particles were prepared by the emulsification–diffusion process or by the double emulsion technique (Fig. 2). With the emulsification–diffusion process, a very rapid release was observed (75% in 1 h), pointing out that the ODN is located close to the particle surface. On the contrary, a very slow release profile, starting after 2 days was observed with particles prepared by the double emulsion process suggesting that the ODN is located inside the polymeric matrix and the release presumably involves polymer erosion. Although it is difficult to anticipate the ideal release profile for a substance such as oligonucleotide, it seems, however, that kinetics displayed by the different batches prepared in this study are either too fast or too slow. Therefore, it would be interesting to change either the precipitation agent for the emulsification–diffusion method or the nature of the polymer for the double emulsion technique.

Survival rates of the U-937 cells (Fig. 3) show that particles prepared with the double emulsion technique are better tolerated than particles obtained with the precipitation/emulsification–diffusion technique. The toxicity induced by these particles is most likely related to the presence of

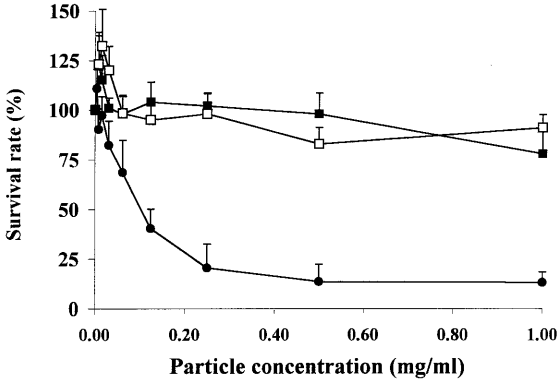

Fig. 3. Survival rates of the different batches of particles on U-937 cells, MTT test (mean of six wells). P-Nano (●): nanoparticles prepared by precipitation/emulsification–diffusion process, DE-Nano (□), Micro (■): nano- and microparticles prepared by double emulsion, respectively.

CTAB since unloaded particles prepared in the same conditions and the free ODN in solution were well tolerated. However, since high loading can be achieved with the emulsification–diffusion method, this does not appear as a limitation to the use of these particles for further experiments on cells.

In conclusion, the results obtained show that particle preparation does affect particle characteristics especially in terms of drug release profile and cellular tolerance. Particles with satisfying oligonucleotide loading were obtained. Very different release profiles were observed and it will be interesting to study how the antisense activity is affected with the different preparations.

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