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Encapsulation of mono- and oligo-nucleotides into aqueous-core nanocapsules in presence of various water-soluble polymers

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Abstract

In a previous study, we have shown that cidofovir (CDV) and azidothymidine-triphosphate (AZT-TP) were poorly encapsulated in poly(*iso*-butylcyanoacrylate) (PIBCA) aqueous-core nanocapsules. This was attributed to the rapid leakage of these small and hydrophilic molecules through the thin polymer wall of the nanocapsules. In the present study, we have selected various water-soluble polymers as increasing Mw adjuvants and investigated their influence on the entrapment of mononucleotides (CDV, AZT-TP) as well as of oligonucleotides (ODN) into these PIBCA aqueous-core nanocapsules. We show here that the presence of cationic polymers (i.e. poly(ethyleneimine) (PEI) or chitosan) in the nanocapsule aqueous compartment allowed successful encapsulation of AZT-TP and ODN.

Keywords: Poly(alkylcyanoacrylate); Nanocapsules; Azidothymidine-triphosphate; Cidofovir; Oligonucleotides

1. Introduction

Mono- and oligo-nucleotides are of major importance in the current or forthcoming treatments of cancer and viral infections. Mononucleotides include monophosphorylated forms of nucleoside analogues like the antiviral phosphonate cidofovir (CDV), but also triphosphorylated forms like azidothymidine-triphosphate (AZT-TP), the active form of the anti-HIV compound AZT, while oligonucleotides (ODN) are short sequences of nucleotides.

The clinical use of nucleotide analogues is limited by their poor stability in biological medium, often resulting in short half-lives and low bioavailabilities (Li and Chan, 1999). The important hydrophilic character of nucleotides also strongly limits their intracellular penetration, especially in the case of ODN and triphosphate nucleotide analogues, due to the low membrane permeability of these susbstances (Loke et al., 1989; Kukhanova et al., 2000).

Among the nanotechnologies developed to address theses issues, poly(alkylcyanoacrylate) (PACA) nanoparticles are of particular interest. Indeed, ODN have been efficiently loaded in PACA nanoparticles, either through adsorption onto nanospheres in presence of hydrophobic cations (Chavany et al., 1992, 1994), or by encapsulation within the internal cavity of aqueous-core nanocapsules (Lambert et al., 2000a,b). Additionally, PACA nanospheres have been shown to be promising carriers for unphosphorylated nucleoside analogues like azidothymidine (AZT) (Lobenberg and Kreuter, 1996; Lobenberg et al., 1998). However, the association of phosphorylated nucleoside analogues like AZT-TP or CDV to PACA nanoparticles remains a challenge. Indeed, we have recently shown in a previous study published in this journal that CDV as well AZT-TP were poorly encapsulated in poly(*iso*-butylcyanoacrylate) (PIBCA) aqueous-cored nanocapsules. This was attributed to the rapid leakage of these small and hydrophilic molecules through the thin polymer wall of the nanocapsules (Hillaireau et al., 2006).

In this study, we selected various water-soluble polymers as increasing Mw adjuvants and investigated their influence on the entrapment of mononucleotides (CDV, AZT-TP) as well as of ODN into PIBCA aqueous-core nanocapsules. We show here that the presence of cationic polymers (i.e. poly(ethyleneimine)

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(PEI) or chitosan) in the nanocapsule aqueous compartment allows successful encapsulation of AZT-TP and ODN, making nanocapsules promising versatile carriers for mono- and oligonucleotides.

2. Materials and methods

2.1. Materials

AZT-TP was purchased from Trilink Biotechnologies (San Diego, USA). The CDV used was the pharmaceutical Vistide® solution for perfusion (Pharmacia & Upjohn, Belgium). [Methyl-^{3}H]-AZT-TP and [2-^{14}C]-cidofovir were from Moravek (Brea, USA). Phosphorothioate 20-mer ODN (TCCGGAGCCAGACTTCATTC) was from Eurogentec (Belgium). Radiolabelling was performed using ^{32}P-ATP and T4 polynucleotide kinase. *Iso*-butylcyanoacrylate (IBCA) was a gift of Loctite (Ireland). PEI (25 kDa, branched), dextran (38 kDa) and heparin were from Sigma (France). Chitosan (30 kDa) was obtained by depolymerization of medium molecular weight chitosan from Fluka as described by (Bertholon et al., 2006). Caprylic/capric triglycerides (Crodamol GTCC™), sorbitan mono-oleate (Crill 4™) and polysorbate 80 (Crillet 4™) were a gift of Croda (France). Caprylic/capric mono/diglycerides (Capmul MCM™) was a gift of Abitec (France). The scintillation liquid Hionic Fluor™ and Soluene™ were from Perkin-Elmer (USA). All solvents were of analytical grade. In the following, demineralised water refers to MilliQ® water (Millipore, France), PBS to PBS without $CaCl_2$ and $MgCl_2$ from Dulbecco (Invitrogen), and 1/10 PBS refers to PBS diluted to the tenth with demineralised water.

2.2. Polymer-to-drug ratios

In order to facilitate comparisons, nucleotide drug concentrations were expressed using their phosphor molar concentration [P] and water-soluble polymer concentrations using the corresponding monomer unit molar concentration [MU]. Polymer-to-drug ratios were expressed using the [MU]/[P] ratio.

2.3. Preparation of nanocapsules

An aqueous phase containing the drug (AZT-TP, CDV or ODN, see Table 1) at a concentration of [P] = 0.3 mM, and a water-soluble polymer (PEI, chitosan, dextran or heparin) at [MU]/[P] ratios ranging from 0 to 10, was prepared in 1/10 PBS (pH 7). 100 μL of the resulting solution were added to 0.76 g of the oil blend (Crodamol GTCC/Capmul MCM 3:1 w/w) and to 0.14 g of the surfactant blend (Crillet 4/Crill 4 3:1 w/w). After this mixture was vortexed and allowed to equilibrate at room temperature, a microemulsion was obtained. Fifteen milligrams of IBCA monomer were then added dropwise under vigorous vortex stirring. After overnight stirring, an oily suspension of aqueous-cored nanocapsules was obtained. The recovery of the nanocapsules in an aqueous medium was achieved after centrifugation (30 min, 20,000 × *g*) of 250 mg of the oily suspension over a layer of 1 mL 1/10 PBS ('washing' step). After careful removing of the supernatant, nanocapsules were dispersed in 1 mL 1/10 PBS by sonication (water bath).

Table 1
Final composition of nanocapsules, as a function of the entrapped drug, using PEI (or Chitosan, in italics) as adjuvants

	AZT-TP	CDV	ODN
Drug characteristics			
MW (g/mol)	504	279	6381
Number of negative charges at pH 7	4	2	19
Nanocapsule composition			
Drug (μg)	0.20 (*0.20*)	<0.04 (*<0.04*)	0.43 (*0.53*)
PEI (*Chitosan*) (μg)	34 (*113*)	0–34 (*0–113*)	34 (*113*)
PIBCA (mg)	1.0 (*1.0*)	1.0 (*1.0*)	1.0 (*1.0*)

2.4. Nanocapsule characterization

Nanocapsule mean size was determined after 1/20 dilution in demineralised water, using dynamic laser light scattering (Zetasizer Nano ZS, Malvern, UK). The morphology of the nanocapsules was visualised by transmission electron microscopy (TEM); practically, 10 μL of the aqueous suspension were deposited on a thin copper grid, allowed to evaporate for 2 min, and observed using a Philips EM208 electron microscope operating at 80 kV.

2.5. Drug entrapment efficiency

Oily suspensions of nanocapsules were prepared as described above using isotopic dilutions of ^{3}H-AZT-TP and ^{14}C-CDV (30 nCi). To determine the encapsulation efficiency and drug loading before washing, the oily suspension was centrifuged (30 min, 20,000 × *g*). The supernatant was withdrawn and the pellet was dissolved in 1 mL Soluene. The radioactivity associated to the supernatant and to the Soluene pellet solution was counted in Hionic Fluor with a liquid scintillation system LS6000TA (Beckman, USA). To determine the encapsulation efficiency after washing, 250 mg of the oily suspension were centrifuged over a layer of an aqueous phase as described above. Radioactivity associated to the supernatant (oily and aqueous phases) and to the pellet was also counted. Encapsulation efficiency was calculated as follows:

$$\text{encapsulation efficiency (\%)} = \frac{100 \times \text{pellet radioactivity}}{(\text{pellet radioactivity} + \text{supernatant radioactivity})}$$

2.6. In vitro release studies

An aqueous suspension of nanocapsules containing an isotopic dilution of AZT-TP (750 nCi) and PEI or chitosan at a [MU]/[P] ratio of 10 was prepared as described above. After dilution (1/20) in PBS, the nanocapsule suspension was stirred at 37 °C. Samples (1 mL) were removed at various time intervals and centrifuged (30 min, 20,000 × *g*). The supernatant was

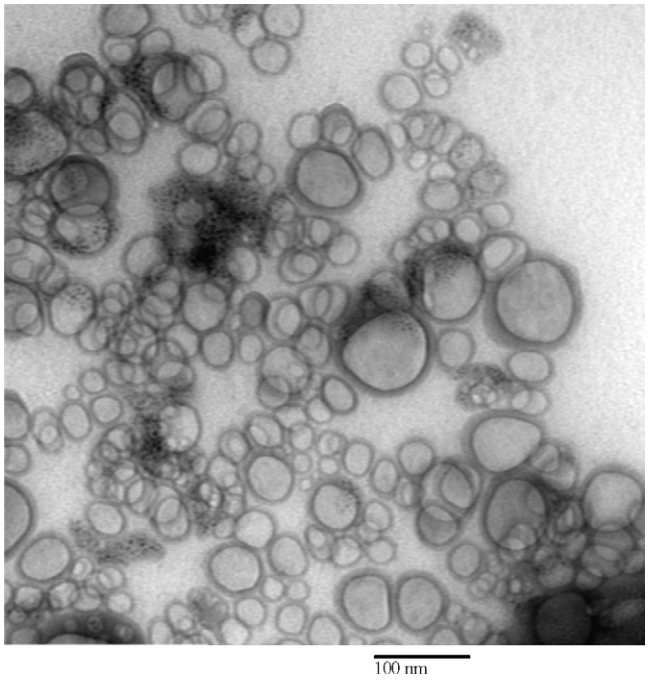

Fig. 1. Drug-unloaded nanocapsules observed in TEM (bar = 100 nm).

withdrawn and the pellet was dissolved in 1 mL Soluene. The radioactivity associated to the supernatant and to the Soluene pellet solution was counted as described previously by scintillation counting. The drug release was calculated as follows:

$$\text{drug release (\%)} = \frac{100 \times \text{supernatant radioactivity}}{(\text{pellet radioactivity} + \text{supernatant radioactivity})}$$

3. Results

3.1. Nanocapsule size and morphology

Oily and aqueous suspensions of nanocapsules were obtained through the interfacial polymerization as described in materials and methods section. Dynamic laser light scattering measurements performed on both oily and aqueous suspensions showed that all nanocapsules were in the 20–250 nm range. TEM observations (Fig. 1) confirmed this distribution and showed the core/shell structure of these nanocapsules. Fig. 1 is representative of drug-unloaded nanocapsules. The encapsulation of water-soluble polymers and drugs did not influence the morphology of the capsules.

3.2. Drug entrapment

The encapsulation efficiency of the mononucleotides AZT-TP and CDV is shown on Fig. 2. The encapsulation of CDV (Fig. 2a) was not affected by the nature nor by the concentration of water-soluble polymer used in the preparation, amounting 10–15%, in sharp contrast to AZT-TP. Indeed, the entrapment of the latter (Fig. 2b) was remarkably enhanced when increasing

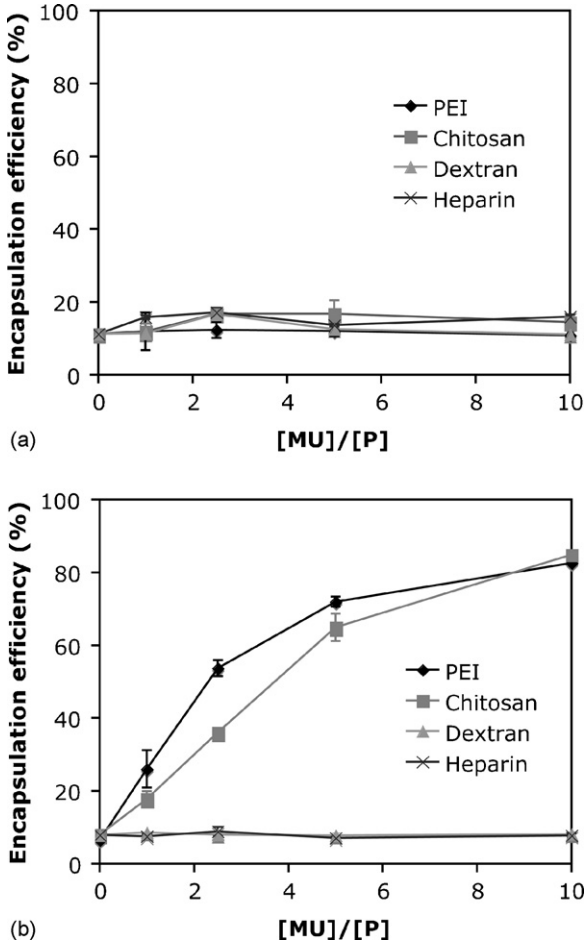

Fig. 2. Encapsulation efficiency of nucleotide analogues CDV (a) and AZT-TP (b) in PIBCA nanocapsules ([P] = 0.3 mM) in the presence of various water-soluble polymers.

amounts of PEI or chitosan were used in the preparation method, reaching 80% for [MU]/[P] ratios of 10. On the contrary, adding dextran or heparin to the water phase of nanocapsules did not affect the encapsulation efficiency, remaining around 7–8%.

High encapsulation values of AZT-TP obtained with PEI and chitosan were maintained after the washing step of nanocapsules, with only 8–9% loss (Fig. 3).

Encapsulation pattern of ODN (Fig. 4) shows that this molecule was more efficiently entrapped (20%) into nanocapsules than the mononucleotides, in the absence of water-soluble polymers. PEI and chitosan increased the encapsulation efficiency of ODN (up to 90%) similarly to AZT-TP. However, contrary to what was observed with the mononucleotides, ODN encapsulation was slightly increased in the presence of dextran and slightly decreased with heparin.

3.3. Drug release

Drug release was measured on nanocapsules entrapping AZT-TP with PEI or chitosan ([MU]/[P] = 10) (Fig. 5). After a slight burst effect (8% with PEI, 15% with chitosan), a zero-order release was observed. Ninety percent of AZT-TP was released after 12 h.

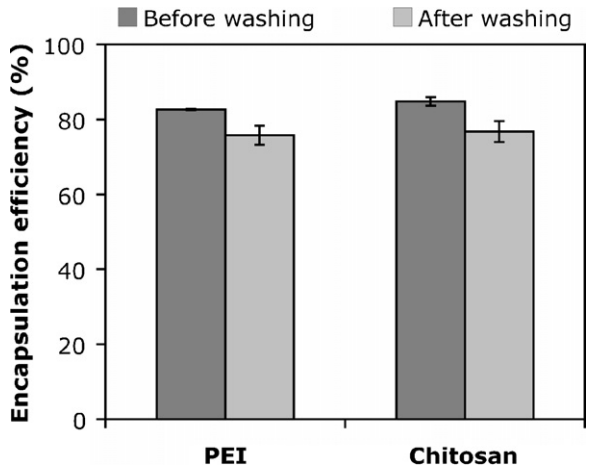

Fig. 3. AZT-TP encapsulation efficiency in PIBCA nanocapsules using water-soluble polymers PEI or chitosan at [MU]/[P] ratios of 10.

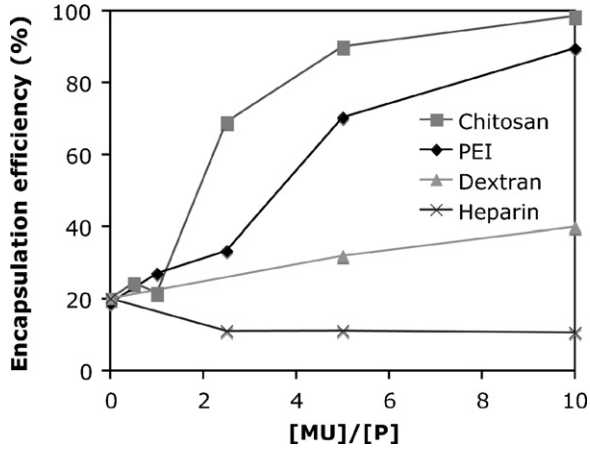

Fig. 4. Encapsulation efficiency of a 20-mer ODN in PIBCA nanocapsules ([P] = 0.3 mM) in the presence of various polymers.

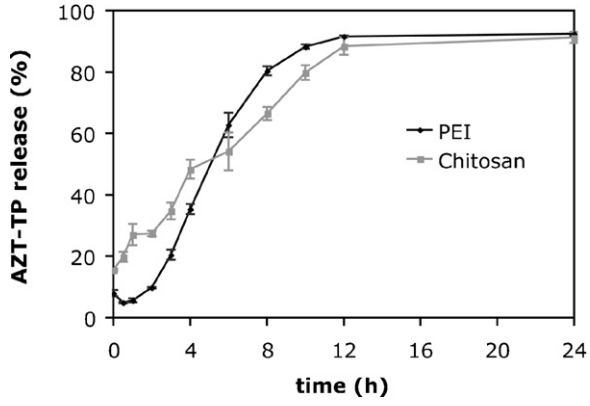

Fig. 5. Release of AZT-TP from PIBCA nanocapsules (prepared using water-soluble polymers PEI or chitosan at [MU]/[P] ratios of 10) after incubation in PBS at 37 °C, expressed as a fraction of the initial AZT-TP loading.

4. Discussion

PIBCA aqueous-core nanocapsules have been shown to be efficient drug delivery systems for hydrophilic substances such as insulin (Watnasirichaikul et al., 2000, 2002) or nucleic acids (Lambert et al., 2000a,b; Toub et al., 2006). However, encapsulation of mono-nucleotides like AZT-TP and CDV in these nanocarriers has failed, because such small and hydrophilic molecules rapidly leaked out of the nanocapsules, resulting in poor encapsulation efficiency and fast release upon dilution (Hillaireau et al., 2006). To address this issue, we investigated the capacity of various water-soluble polymers to retain the nucleotides within the aqueous compartment during the preparation of the nanocapsules, in order to improve the drug entrapment efficiency and slow down the drug release rate.

The data obtained for the triphosphate nucleotide analogue AZT-TP (Fig. 2a) show that the cationic polymer PEI as well as chitosan increased entrapment efficiency of this molecule by 10-fold (reaching up to 80%), in contrast to heparin (a negatively charged polymer) and dextran (neutral). As water-soluble polymers were already known to be efficiently entrapped in such nanocapsules (irrespective of their electrical charge) (Pitaksuteepong et al., 2002), our results suggest that AZT-TP could be encapsulated due to the formation of ionic complexes between the anionic triphosphate nucleotide analogue and the cationic polymer chains of PEI or chitosan. In contrast to AZT-TP, encapsulation of unphosphorylated AZT was overcome several years ago. Indeed, the lower hydrophilicity of AZT allowed its successful encapsulation in PACA nanospheres (Lobenberg and Kreuter, 1996; Lobenberg et al., 1998).

Similarly, ODN encapsulation was significantly increased when PEI or chitosan were used (Fig. 4). Here again, the formation of ionic polyplexes between negatively-charged ODN oligomers and cationic PEI or chitosan may account for this result. Interestingly, dextran also increased ODN encapsulation efficiency, but to a lesser extent, which could result from a reduced permeability of the cyanoacrylate membrane due to the presence of dextran chains and/or to slight interaction between dextran and ODN by hydrogen bonds. On the contrary, addition of heparin decreased ODN encapsulation, probably because of a competitive entrapment of ODN and heparin molecules into the nanocapsules.

Noteworthy, encapsulation of CDV was not affected by any of the above-mentionned water-soluble polymers, probably because the electrical charge of this monophosphate nucleotide analogue was not sufficient compared to AZT-TP and ODN.

After encapsulation, washing the nanocapsules in an aqueous phase is needed to remove residual oil and surfactant for biological applications (Lambert et al., 2000b). Interestingly, high encapsulation of AZT-TP in nanocapsules prepared with PEI or chitosan as adjuvants, was maintained after this step (Fig. 3), whereas in the absence of the cationic polymers, the nucleotides were not retained inside the nanocapsules (Hillaireau et al., 2006). The final compostion of the nanocapsules formulations in summarized in Table 1. These formulations also displayed *in vitro* a limited burst effect and a zero-order release for up to

12 h, being thus relevant formulation for intracellular delivery of nucleotides.

In conclusion, the use of the cationic polymers PEI and chitosan has been shown to overcome the issue of the encapsulation of the triphosphate nucleotide analogue AZT-TP in PIBCA nanocapsules, this strategy being also promising for ODN nanocapsules formulations. Further studies are now in progress to investigate the efficacy of these formulations on experimental viral infections.

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