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Encapsulation of antiviral nucleotide analogues azidothymidine-triphosphate and cidofovir in poly(*iso*-butylcyanoacrylate) nanocapsules

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Abstract

Nucleoside analogues are widely used in the treatment of various viral infections. However, the poor *in vivo* conversion of the nucleoside analogues like azidothymidine (AZT) into their active triphosphate nucleotide counterpart limits their pharmacological efficacy. This could be overcome by the direct administration of azidothymidine triphosphate (AZT-TP), but it requires an appropriate drug delivery approach.

Besides nucleoside analogues, nucleotide analogues like cidofovir (CDV) are also used in the treatment of viral infections. CDV has raised recent interest because of its promising activity against smallpox, but its use is limited by its poor bioavailability and nephrotoxicity. Here again, a proper drug delivery system should address these issues.

In this study, we investigated the encapsulation of the nucleotide analogues AZT-TP and CDV into poly(*iso*-butylcyanoacrylate) aqueous core nanocapsules, known to efficiently entrap oligonucleotides. We show here that the encapsulation of these mono-nucleotides is less efficient than with oligonucleotides and that a rapid release of AZT-TP from the nanocapsules occurred *in vitro*. This highlights the importance of the molecular weight of the entrapped molecules which, if they are too small, are diffusing through the thin polymer membrane of the nanocapsules. On the other hand, a good protection of the encapsulated AZT-TP was observed.

Keywords: Poly(alkylcyanoacrylate); Nanocpasules; Azidothymidine triphosphate; Cidofovir

1. Introduction

Nucleoside analogues are widely used in the treatment of various viral infections, including HIV, herpes virus and cytomegalovirus. These molecules combat virus replication by inhibiting viral DNA synthesis occurring in infected cells. However, they are not active by themselves since they need to be phosphorylated by cellular kinases into their active triphosphate nucleotide form (Van Rompay et al., 2003). Thus, the main limitation in the use of nucleoside analogues, like azidothymidine (AZT), in anti-HIV therapies lies in the poor efficiency of *in vivo* conversion (Balzarini et al., 1989; Kukhanova et al., 2000). This raises the problem of side effects (Tornevik et al., 1995) and emergence of resistances (Larder, 1993). The direct administration of the triphosphate form (e.g. azidothymidine triphosphate, AZT-TP, Fig. 1a) could bypass the metabolic bottleneck, but is currently not yet efficient, due to the excessive hydrophilic character of these molecules, which prevents their intracellular penetration. Since poly(alkylcyanoacrylate) (PACA) nanoparticles encapsulating AZT have been shown, after iv administration, to efficiently deliver this molecule to macrophages, an important reservoir of the infection (Schafer et al., 1992; Lobenberg and Kreuter, 1996; Lobenberg et al., 1998), they may be considered as a relevant drug carrier candidate for the intracellular delivery of AZT-TP by this route.

Besides nucleoside analogues, antiviral nucleotide analogues like cidofovir (CDV, Visitde®, Fig. 1b) already incorporate a phosphonate group, and thus require only two *in vivo* phosphorylation steps. CDV is clinically used for the treatment of cytomegalovirus (De Clercq, 1996). It has recently raised new interest because it holds some promise for the treatment of poxvirus infections, including smallpox, a major bioterrorist threat (De Clercq, 2002). Unfortunately, the use of CDV is limited by its poor bioavailability and nephrotoxicity. A new drug

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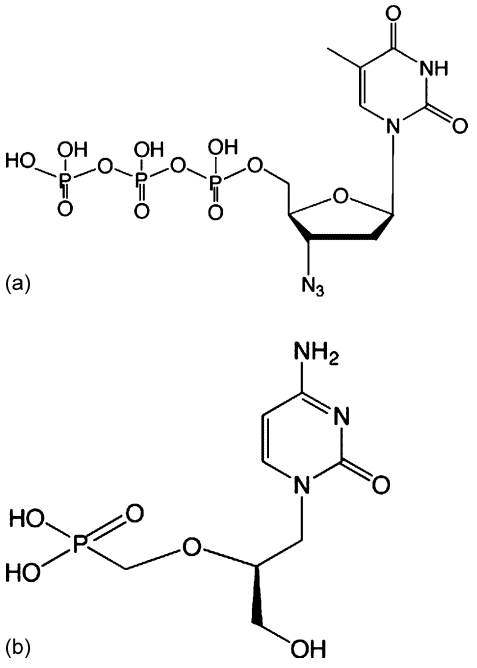


Fig. 1. Chemical structures of: (a) AZT-TP and (b) CDV.

delivery approach is thus needed to achieve efficient delivery of CDV. Here again, PACA nanoparticles may be taken into consideration for this purpose.

Among PACA nanoparticles, poly(*iso*-butylcyanoacrylate) (PIBCA) aqueous-core nanocapsules have been shown to efficiently entrap hydrophilic molecules like oligonucleotides (Lambert et al., 2000a,b; Watnasirichaikul et al., 2002). This is the reason why the present study investigates the encapsulation of the nucleotide analogues AZT-TP and CDV in those PIBCA nanocapsules. We show here that, although appealing, this approach has to address the issue of the size of the molecule to be entrapped.

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). IBCA was a gift from Loctite (Ireland). Caprylic/capric triglycerides (Crodamol GTCC™), sorbitan mono-oleate (Crill 4™) and polysorbate 80 (Crillet 4™) were a gift from Croda (France). Caprylic/capric mono/diglycerides (Capmul MCM™) was a gift from Abitec (France). The scintillation liquid Hionic Fluor™ and Soluene™ were from Perkin-Elmer (USA). All solvents were of analytical grade. In the following, PBS refers to PBS without $CaCl_2$ and $MgCl_2$ from Dulbecco (Invitrogen), and demineralised water to MilliQ® water (Millipore, France).

2.2. Preparation of nanocapsules

An aqueous phase containing AZT-TP (0.1 mM) or CDV (0.1–80 mM) in demineralised water or PBS was first prepared. A 100 μL of this 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. A 15 mg of IBCA were then added dropwise under vigorous stirring. After overnight stirring, an oily suspension of aqueous-cored nanocapsules was obtained. The recovery of the nanocapsules in an aqueous medium was achieved through ultracentrifugation (30 min, 37,000 × *g*) of 250 mg of the oily suspension over a layer of 1 mL demineralised water or PBS ('washing' step). After careful removing of the supernatant, nanocapsules were dispersed in 1 mL PBS by sonication. Drug unloaded nanocapsules could be prepared from a microemulsion containing only demineralised water as aqueous phase. For zeta potential experiments, nanocapsules were dispersed in demineralised water.

2.3. Nanocapsules characterisation

The yield of polymerisation was calculated using the mass of polymer recovered after freeze-drying of the aqueous nanocapsule suspension. Nanocapsules' mean size was determined after 1/20 dilution in demineralised water, using dynamic laser light scattering (Zetasizer Nano ZS, Malvern, UK) (when several populations of nanocapsules were measured for a sample, their relative amounts were given by the intensity distribution). Their zeta potential was determined after 1/20 dilution in 1 mM NaCl solution. The morphology of the nanocapsules was visualised by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). For SEM observations, 2 μL of the 1/8 diluted suspension were deposited on supports, air-dried, coated with a Pt/Pd layer (Cressington, 208 HR) under an argon atmosphere and observed (LEO 9530, France) with a Gemini Column. For TEM observations, 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 operating at 80 kV.

2.4. Determination of encapsulation efficiency and drug loading

The encapsulation efficiency (%) was expressed as the percentage of the total amount of drug used in the process, which was associated to the nanocapsules. The drug loading (% w/w) was expressed as the percentage of the w/w drug-to-polymer ratio.

Oily suspensions of nanocapsules were prepared as described above using isotopic dilutions of AZT-TP and CDV. 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 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 and drug loading were calculated as follows:

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

$$\text{drug loading (\%)} = \frac{\text{initial drug weight} \times \text{encapsulation efficiency}}{\text{polymer weight}} \times 100$$

2.5. In vitro release studies

An aqueous suspension of nanocapsules containing an isotopic dilution of AZT-TP was prepared as described above. After dilution in PBS (1/3), the nanocapsule suspension was stirred at 37 °C. Samples (1 mL) were removed at various time intervals and centrifuged (30,000 × *g*, 15 min). The supernatant was withdrawn and the pellet was dissolved in 1 mL Soluene. The radioactivity associated to the supernatant and to the Soluene solution was counted as described previously by scintillation counting. The drug release was calculated as follows:

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

2.6. HPLC analysis

The HPLC analyses were performed on a Waters liquid chromatograph equipped with a photodiode array detector (Waters 2996). The Novapack C_{18} column (Interchim, France), 3.9 mm × 150 mm, 4 μm particle size, was guarded with a μBondapack C_{18} precolumn (Waters). The method was adapted from Molema et al. (1992). The eluent consisted in a 100 mM sodium dihydrogenphosphate–disodium hydrogenphosphate buffer supplemented with 8 mM TBAS pH 7.5 and acetonitrile in a ratio of 95:5 (v/v). The flow rate was 1 mL/min. Results were acquired and processed using Empower™ software (Waters). AZT-TP was detected at a retention time around 16 min. The calibration curve was linear ($R^2 > 0.99$) between 0.1 and 5 μg/mL.

3. Results

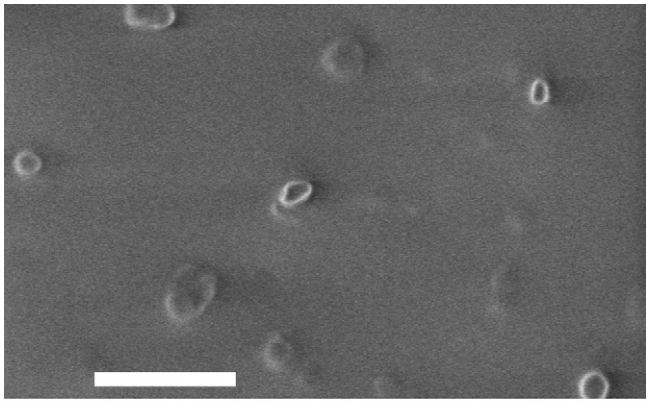
3.1. Preparation of nanocapsules

The aqueous suspension of PIBCA nanocapsules was obtained through the interfacial polymerisation as described in materials and methods section. The polymerisation yield was 63% and, according to the size measurements, two populations of nanocapsules were identified, one around 150 nm and another of 20–30 nm (Table 1). Zeta potential was close to −10 mV. Noteworthy, the encapsulation of the nucleotides AZT-TP and CDV did not substantially affect neither the size nor the zeta potential of PIBCA nanocapsules.

Table 1
Size and zeta potential of drug unloaded PIBCA nanocapsules (NC), NC loaded with AZT-TP and NC loaded with CDV

	Size[a] (nm)	Zeta potential[b] (mV)
Drug unloaded NC	136 ± 24 (88%); 22 ± 3 (12%)	−9.9 ± 4.1
NC AZT-TP	171 ± 30 (70%); 26 ± 5 (30%)	−10 ± 6.8
NC CDV	169 ± 40 (84%); 31 ± 7 (16%)	−8.2 ± 5.7

[a] The two different values refer to the two different nanocapsule populations obtained by the preparation procedure (their relative amount is given in parentheses).
[b] The two populations of nanocapsules exhibited the same zeta potential value.


(a)

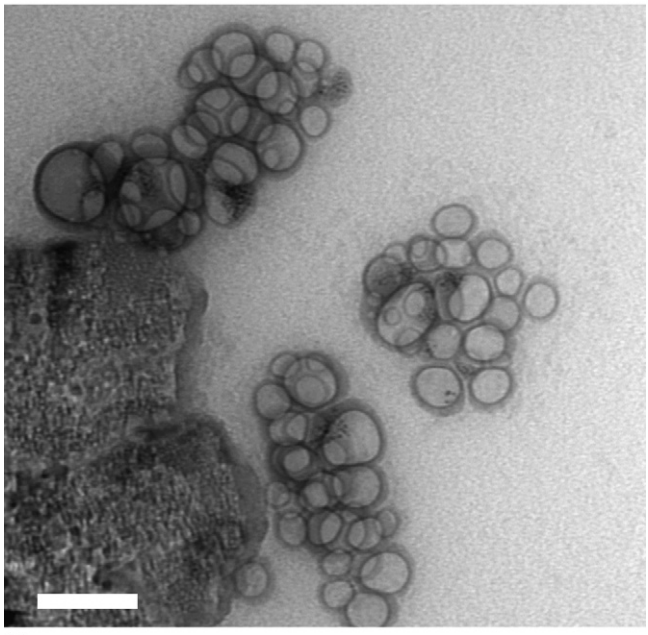

(b)

Fig. 2. Nanocapsules observed by: (a) SEM (bar = 1 μm) and (b) TEM (bar = 100 nm).

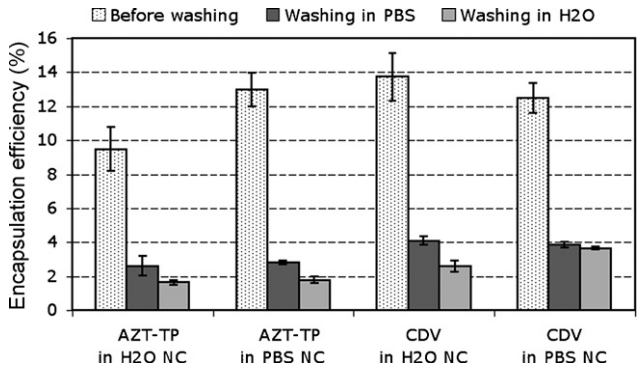


Fig. 3. Encapsulation efficiency (%) of AZT-TP and CDV in PIBCA nanocapsules before and after washing, as a function of the nature of the drug-containing nanocapsule aqueous core (demineralised water or PBS) and of the nature of the aqueous medium employed in the washing step (demineralised water or PBS).

SEM and TEM observations confirmed the presence of two nanocapsules populations. Although the smallest population seemed predominant, some pictures showed also clusters of 100–200 nm formed by the aggregation of several smaller particles (Fig. 2b). The morphology of the nanocapsules were further visualised by TEM, showing capsules with a central cavity surrounded by a thin polymer membrane (Fig. 2b), indicating a core/shell structure. Moreover, SEM pictures showed collapsed particles, which was consistent with a rapid evaporation of the aqueous core due to vacuum drying during the metal coating step (Fig. 2a).

3.2. AZT-TP and CDV encapsulation

The drug encapsulation efficiency of AZT-TP and CDV before washing (see Section 2) was found between 10 and 14% whatever the nature of the nanocapsule aqueous core (demineralised water or PBS) (Fig. 3). Increasing the amount of CDV used in the preparation of the nanocapsules did not alter the encapsulation efficiency (Fig. 4a), thus increasing the drug loading up to 2% (Fig. 4b). After washing, a dramatic decrease of the encapsulation efficiency was observed both for AZT-TP and CDV. Since this decrease was slightly less pronounced with PBS (Fig. 3), this medium was chosen for further experiments.

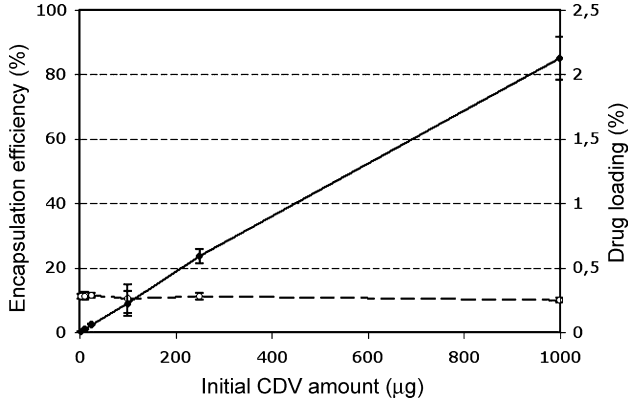


Fig. 4. (a) Encapsulation efficiency (%) (◯) and (b) drug loading (%) (●) of CDV in PIBCA nanocapsules as a function of the amount of CDV used for the preparation.

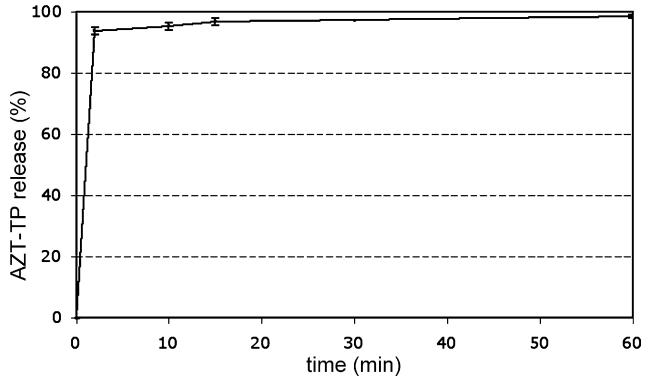


Fig. 5. Release of AZT-TP from nanocapsules after incubation in PBS at 37 °C, expressed as a fraction of the initial AZT-TP loading.

3.3. In vitro release

The release of AZT-TP from the nanocapsules was very fast: 95% of the drug was released after a few minutes, as assayed with radiolabelled AZT-TP (Fig. 5). The chemical integrity of the released drug was investigated by means of HPLC analysis, using a photodiode array detector. The chromatogram performed at 267 nm displayed a peak at a retention time (RT) of 16.0 min, corresponding to the reference AZT-TP (RT = 16.1 min) (Fig. 6a) and amounting 92% of AZT-TP as determined by scintillation counting. The UV spectrum of the sample corresponding to the peak at RT = 16.0 min (Fig. 6b) was exactly the same as that of the reference AZT-TP at RT = 16.1 min (Fig. 6c). This shows that AZT-TP was released from nanocapsules without any chemical degradation.

4. Discussion

Aqueous core PIBCA nanocapsules have been previously shown to efficiently encapsulate oligonucleotides (up to 70%) without chemical degradation (Lambert et al., 2000b). Using the same range of concentration of nucleoside entities, we observed here that the encapsulation of AZT-TP and CDV was less efficient (10–20%) and independent of the initial nucleotide concentration, suggesting a partitioning-type equilibrium between nanocapsule-bound and unbound nucleotides. In addition, the encapsulation efficiency was found to dramatically decrease after washing with an aqueous medium. It is hypothesized that mononucleotides leaked out from nanocapsules by a single diffusion process through the pores of the polymeric membrane, which didn't occur with oligonucleotides having a much higher molecular weight. This is consistent with previous observations by Pitaksuteepong et al. (2002) showing that small hydrophilic model molecules are poorly encapsulated into poly(ethylcyanoacrylate) nanocapsules. Thus, the molecular weight of the entrapped drug appears to be a key point for efficient encapsulation. Despite low nanocapsule entrapment, nucleotides were kept intact after encapsulation as shown by HPLC and UV spectrometric investigations on the released molecules after incubation in PBS at 37 °C. This is an important point since modified nucleotides like AZT-TP are poorly sta-

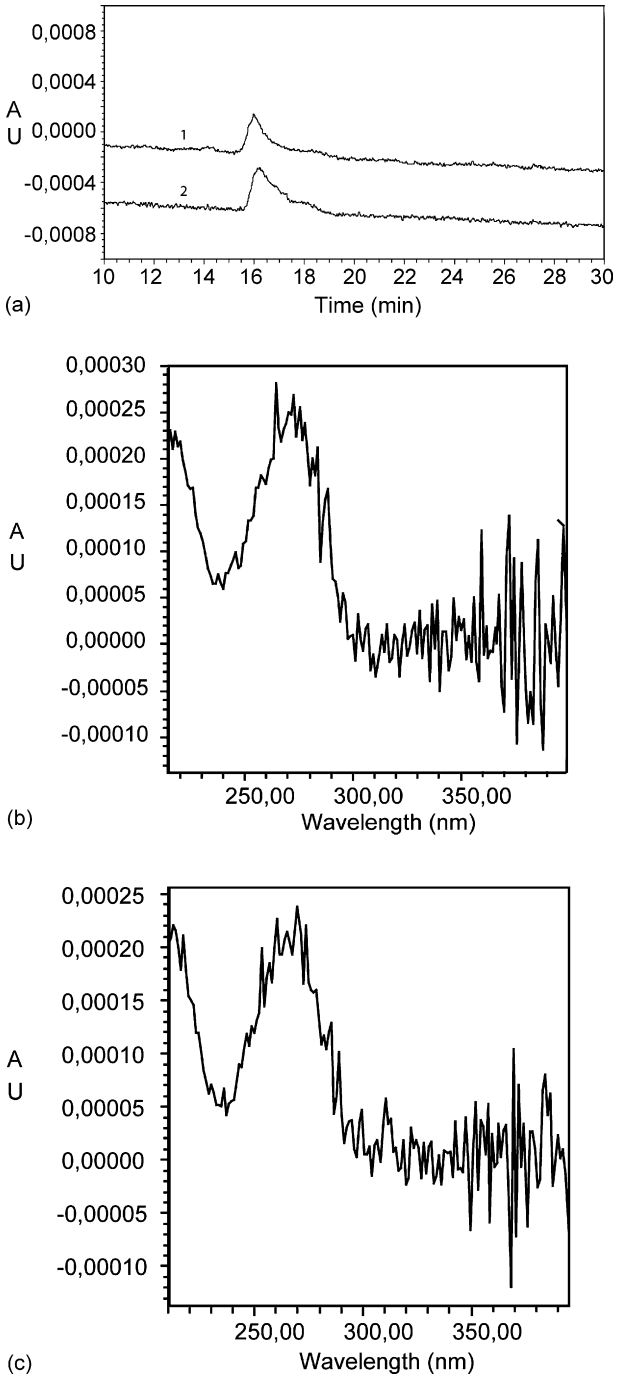


Fig. 6. HPLC analysis of the release medium sample after incubation of AZT-TP loaded nanocapsules. (a) Chromatograms at 267 nm: sample (1) compared to reference AZT-TP (2). (b) UV spectrum of the sample at RT = 16.0 min. (c) UV spectrum of the reference AZT-TP at RT = 16.1 min.

ble. Moreover, some nucleoside analogues have been reported previously to initiate ionic or zwitterionic polymerisation of cyanoacrylate monomers, leading to the inactivation of the drug (Guise et al., 1990; Simeonova et al., 2003, 2004).

In conclusion, if the encapsulation of modified nucleotides in aqueous core PIBCA nanocapsules is a promising strategy to design new drug delivery systems for improved antiviral treatments, the association of such small and hydrophilic molecules to hydrophobic polymers remains a challenge that should be addressed with new approaches.

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