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Encapsulation of gemcitabine lipophilic derivatives into polycyanoacrylate nanospheres and nanocapsules

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Abstract

The aim of this study was to develop both a physical and a chemical protection of the anticancer drug gemcitabine, which suffers from a rapid plasmatic metabolization. For this purpose, we used a series of lipophilic derivatives of gemcitabine in which an acyl chain is covalently coupled to the 4-amino group of gemcitabine; moreover, a physical protection of the drug was attempted by incorporating these lipophilic derivatives into poly(H_2NPEGCA-*co*-HDCA) nanospheres and nanocapsules. Nanoparticles were prepared by nanoprecipitation of the poly(H_2NPEGCA-*co*-HDCA) copolymer and their size, zeta potential and encapsulation efficiency were further characterized. These results have been relied on lipophilicity and flexibility studies. Data showed that only the more lipophilic derivative, 4-(*N*)-stearoylgemcitabine, was incorporated with a high yield. Thus, 4-(*N*)-stearoylgemcitabine-containing nanospheres and nanocapsules were further analyzed by differential scanning calorimetry. Their cytotoxicity was tested on two human cancer cell lines and compared to that of gemcitabine and free 4-(*N*)-stearoylgemcitabine.

Keywords: Polycyanoacrylate; Nanospheres; Nanocapsules; Gemcitabine; Cytotoxicity

1. Introduction

Colloidal drug delivery systems have received increasing attention as possible means to obtain a higher therapeutic effect, a lower toxicity and a protection from *in vivo* metabolization of incorporated drugs (Moghimi et al., 2001). From a structural point of view, nanoparticles comprise different systems, including nanospheres and nanocapsules (Couvreur et al., 2002). Among the different polymers used for nanoparticles preparation, poly(alkylcyanoacrylates) (PACA) have been extensively studied to obtain biodegradable drug carriers (Vauthier et al., 2003). We have previously synthesized and characterized a new amphiphilic PACA copolymer, the poly[aminopoly(ethylene glycol)cyanoacrylate-*co*-hexadecyl cyanoacrylate] [poly(H_2NPEGCA-*co*-HDCA)], which allows to prepare stable and small-sized nanospheres without any surfactant (Stella et al., 2000). These nanoparticles can be considered as suitable colloidal carriers for many drugs, such as the antitumoral gemcitabine, (2′,2′-difluoro-2′-deoxycytidine, dFdC). This molecule, structurally similar to cytarabine (Ara-C), is a nucleoside analogue of deoxycytidine in which two fluorine atoms are placed in the geminal configuration at the 2′ position of the sugar ring (Heinemann et al., 1988; Hertel et al., 1988). Gemcitabine has a therapeutic activity against a variety of solid malignancies, including colon, lung, pancreatic, breast, bladder and ovarian cancers (Hertel et al., 1990; Plunkett et al., 1995a). Once transported into the cell, gemcitabine needs to be phosphorylated by deoxycytidine kinase into its active form, the 5′-triphosphategemcitabine, which is incorporated into the DNA strand, halting its elongation and causing cell death. Moreover, gemcitabine action involves also ribonucleotide reductase inhibition (Plunkett et al., 1995b, 1996). At the same time, gemcitabine is rapidly metabolized in the blood, liver and kidneys by deamination by cytidine deaminase into the inactive derivative 2′,2′-difluoro-2′-deoxyuridine (Heinemann et al., 1992; Bouffard et al., 1993; Matsuda and Sasaki, 2004). Thus, when administered intravenously, gemcitabine has a very short plasma half-life, which represents a major limitation of this

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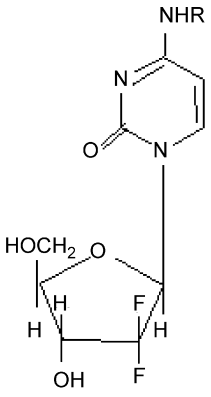


R=H	gemcitabine
R=CO-$(CH_2)_3$-CH_3	4-(*N*)-valeroylgemcitabine
R=CO-$(CH_2)_{10}$-CH_3	4-(*N*)-lauroylgemcitabine
R=CO-$(CH_2)_{16}$-CH_3	4-(*N*)-stearoylgemcitabine

Fig. 1. Chemical structure of gemcitabine and of 4-(*N*)-acylgemcitabine derivatives.

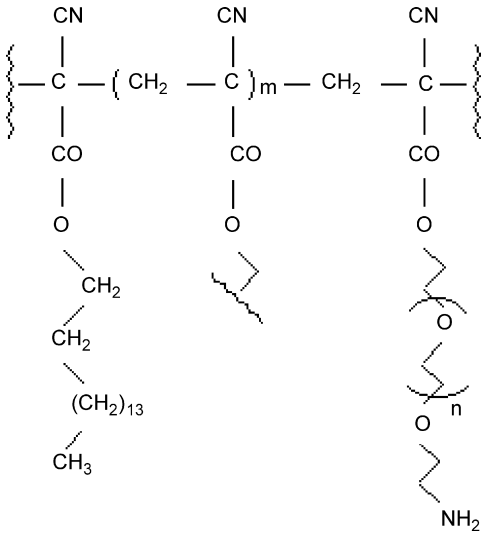


Fig. 2. Chemical structure of the poly(H_2NPEGCA-*co*-HDCA) copolymer.

anticancer compound (Abbruzzese et al., 1991; Grunewald et al., 1992; Storniolo et al., 1997). In the literature, the synthesis of various gemcitabine derivatives was attempted in order to chemically protect the 4-amino group of the drug from the metabolic inactivation. In particular, saturated and monounsaturated 18–20 carbon atom chains have been conjugated to the 3′- and/or 5′-OH and/or the 4-amino group, giving esters or amides of gemcitabine (Myhren et al., 2002). Although some of these derivatives showed a higher cytotoxic activity, the chemical derivatization of gemcitabine strongly diminishes its water solubility, constituting a disadvantage for its intravenous administration. In order to overcome this problem, we recently synthesized a series of lipophilic derivatives of gemcitabine, in which an acyl chain of increasing length was covalently coupled to the 4-amino group of gemcitabine (Fig. 1). The aim of the present study was to prepare polymeric nanospheres and nanocapsules containing gemcitabine and its lipophilic derivatives.

2. Materials and methods

2.1. Materials

The poly(H_2NPEGCA-*co*-HDCA) copolymer (Fig. 2) was synthesized as previously described (Stella et al., 2000). Briefly, *t*-Boc-HNPEG 3400 cyanoacetate was condensed with *n*-hexadecyl cyanoacetate (molar ratio, 1:5) in ethanol in the presence of formalin and dimethylamine. The copolymer was then reacted with trifluoroacetic acid in dichloromethane in order to selectively deprotect the amino group of *t*-Boc-HNPEG. Miglyol 812N was a gift from Sasol (Witten, Germany). 4-(*N*)-valeroyl-, 4-(*N*)-lauroyl- and 4-(*N*)-stearoylgemcitabine were synthesized as described elsewhere (Immordino et al., 2004). All other reagents and solvents used were of analytical grade.

2.2. Cell culture

KB3-1 cells, a human cervix carcinoma cell line and human breast adenocarcinoma MCF-7 cells were obtained from ATCC (Marne la Vallée, France). All cells were cultured as monolayers at 37 °C in a 5% CO_2/95% humidified atmosphere with Dulbecco's Modified Eagle's Medium (DMEM) (Biological Industries) supplemented with 10% fetal calf serum (FCS), 50 U/ml penicillin, 50 μg/ml streptomycin and 2 mM L-glutamine.

2.3. Partition coefficients and flexibility indexes

In order to determine the partition coefficients of gemcitabine and 4-(*N*)-valeroylgemcitabine, traditional shake-flask experiments have been performed both in *n*-octanol/water ($\log D_{oct}$) and in Miglyol 812N/water ($\log D_{mig}$) at pH 7.4. *n*-octanol, Miglyol 812N and water were mutually saturated; then, each derivative was added to the aqueous phase. After a vigorous agitation, the phases were separated and the optical density was measured in the aqueous phase at 266 nm for gemcitabine and 248 nm for 4-(*N*)-valeroylgemcitabine. Because of the low-water solubility of 4-(*N*)-lauroyl- and of 4-(*N*)-stearoylgemcitabine, their partition coefficients values have been calculated. Kier's flexibility descriptors have been calculated by Dragon software.

2.4. Preparation of nanoparticles

For the preparation of poly(H_2NPEGCA-*co*-HDCA) nanospheres containing gemcitabine and 4-(*N*)-acylderivatives, the nanoprecipitation technique was employed (Fessi et al., 1989). Practically, for each preparation, to 40 mg of the copolymer poly(H_2NPEGCA-*co*-HDCA) dissolved in warm acetone, an aliquot of an ethanolic stock solution of each gemcitabine derivative was added until a total volume

of 4 ml. This organic solution was then poured into 8 ml of MilliQ® water under magnetic stirring. Precipitation of particles occurred spontaneously. After solvent evaporation by Rotavapor®, an aqueous suspension of nanospheres was obtained. For nanocapsules preparation, the same method was used, except that Miglyol 812N (32 μl) was added to the organic solution of the polymer in order to form the inner oily cavity. To purify the nanoparticles from non-incorporated drug, gemcitabine- and 4-(*N*)-valeroylgemcitabine-containing nanospheres or nanocapsules were extensively dialyzed against MilliQ® water at 4 °C (Spectra/Por® 3500 MWCO dialysis membrane, Spectrum, Huston, TX). 4-(*N*)-lauroyl- and 4-(*N*)-stearoylgemcitabine-nanoparticles (both nanospheres and nanocapsules) were purified by filtration through 0.45 μm hydrophilic syringe filter (Sartorius AG, Goettingen, Germany). The absence of solubilized or nanoprecipitated free 4-(*N*)-lauroyl- and 4-(*N*)-stearoylgemcitabine after purification was checked both by UV and QELS analysis of a polymer-free filtered drug sample. The particles were then stored in the dark at 4 °C.

2.5. Nanoparticles characterization

The mean particle size and polydispersity index of poly(H_2NPEGCA-*co*-HDCA) nanoparticles were determined at 20 °C by quasi-elastic light scattering (QELS) using a nanosizer (Coulter® N4MD, Coulter Electronics Inc., Hialeah, FL). The selected angle was 90° and the measurement was made after dilution of the nanoparticles suspensions in MilliQ® water. Each measure was performed in triplicate. The stability of the formulations was evaluated by measuring the size of the particles after 2 weeks of storage at 4 °C in MilliQ® water. The surface charge of the nanoparticles was evaluated by zeta potential measurements after dilution of the suspensions in 1 mM KCl using a zetasizer (Zeta Potential Analyzer Ver. 2.17, Brookhaven Inst. Corp., Holtsville, NY). The amount of drugs incorporated into nanospheres and nanocapsules was determined by HPLC (Merck Hitachi HPLC System, Milan, Italy). For this, an aliquot of particles suspension was diluted by adding water and acetonitrile (60:40) to a total volume of 0.5 ml. Extraction of gemcitabine or 4-(*N*)-acylderivatives was accomplished by adding 4.0 ml of *tert*-butyl methyl ether and vortex mixing the sample for 30 s. The mixture was then centrifuged for 15 min at 300 × *g* after which 3.0 ml of the organic layer was transferred and evaporated to dryness under nitrogen (Crosasso et al., 2000; Sharma et al., 1994). The residue was reconstituted with 100 μl of methanol before HPLC analysis. Forty μl of the solutions were injected into a Symmetry C18 column, 5 μm (Merck) equipped with a C18 column guard (Merck). The column was eluted with methanol–water (90:10) for gemcitabine, 4-(*N*)-valeroyl- and 4-(*N*)-lauroylgemcitabine (flow rate 0.8 ml/min) and with methanol for 4-(*N*)-stearoylgemcitabine (flow rate 1 ml/min). Detection was performed by UV absorption measurement at 266 and 248 nm for gemcitabine and 4-(*N*)-acylderivatives, respectively. Peak heights were recorded and processed on a CBM-10A Shimadzu interface. The drug concentration was calculated from standard curves. The assay was linear over the tested concentration range (20–1000 ng).

The concentration of the nanoparticles in the suspension was based on dry weight analysis.

2.6. Differential scanning calorimetry (DSC)

4-(*N*)-stearoylgemcitabine encapsulating poly(H_2NPEGCA-*co*-HDCA) nanospheres and nanocapsules were analyzed by differential scanning calorimetry (DSC) using a differential scanning calorimeter DSC 7 (Perkin-Elmer) equipped with an instrument controller Tac 7/DX (Perkin-Elmer). The instrument was calibrated daily with indium (ΔH = 28.4 J/g, m.p. 156.6 °C) for melting point and heat of fusion. A second standard, naphthalene (ΔH = 149.0 J/g, m.p. 80.3 °C) was also used because it has a melting point closer to that of polymeric nanoparticles. A heating rate of 10 °C/min was employed throughout the analyses to determine the melting point and the heat of fusion of nanoparticles. Thermograms were recorded in the temperature range 30–100 °C. The enthalpy of fusion (ΔH_m) was calculated by a computer interfaced with the DSC from the peak area of the melting endotherm. The program estimated the onset temperature by extrapolating the leading edge of the peak back to the baseline. Analyses were performed under a nitrogen purge; standard aluminum sample pans (Perkin-Elmer) were used and an empty pan was used as reference. Empty poly(H_2NPEGCA-*co*-HDCA) nanospheres and nanocapsules were also tested. Triple runs were carried out on each sample to check the reproducibility.

2.7. Cytotoxicity assay

The cytotoxicity of 4-(*N*)-stearoylgemcitabine-loaded poly(H_2NPEGCA-*co*-HDCA) nanoparticles (both nanospheres and nanocapsules) was determined by assessing cell viability on KB3-1 and MCF-7 cell lines, using the 3-[4,5-dimethylthiazol-2-yl]-3,5-diphenyl tetrazolium bromide (MTT) test (Mosmann, 1983), measuring mitochondrial dehydrogenase cell activity. KB3-1 and MCF-7 cells were seeded in DMEM into 96-well plates at a density of 1×10^4 cells/well. Different dilutions of gemcitabine, 4-(*N*)-stearoylgemcitabine and 4-(*N*)-stearoylgemcitabine encapsulated into poly(H_2NPEGCA-*co*-HDCA) nanospheres or nanocapsules were added to the cells in the culture medium. Each dilution was tested in triplicate. After 72-h incubation at 37 °C, 20 μl of a MTT solution in DMEM (5 mg/ml) was added to each well. After incubation for 2 h at 37 °C, culture medium was removed and formazan crystals were dissolved in 100 μl DMSO. The absorbance of converted dye, which correlates with the number of viable cells, was measured at 570 nm, with background subtraction at 650 nm using a microplate reader (Metertech Σ 960, Fisher Bioblock, Illkirch, France). The percentage of survival cells was calculated as the absorbance ratio of treated to untreated cells. The cytotoxic activity of empty poly(H_2NPEGCA-*co*-HDCA) nanospheres and nanocapsules was also tested.

Table 1
Partition coefficients and flexibility indexes of gemcitabine and its lipophilic derivatives

	Molecular weight	log D_{oct}[a]	log D_{mig}[b]	Flexibility index
Gemcitabine	263	−1.24	<−3	2.54
4-(*N*)-Valeroylgemcitabine	347	1.10	−0.97	4.67
4-(*N*)-Lauroylgemcitabine	445	4.74*	–	8.58
4-(*N*)-Stearoylgemcitabine	529	7.91*	–	12.49

[a] Logarithm of the distribution coefficient at pH 7.4 in the system *n*-octanol/water.
[b] Logarithm of the distribution coefficient at pH 7.4 in the system Miglyol 812N/water.
* Calculated values.

2.8. Statistical analysis

They were performed by the use of the Student's *t*-test. Values with $P < 0.05$ and $P < 0.01$ were considered as significantly different.

3. Results

3.1. Partition coefficients and flexibility indexes

As shown in Table 1, the flexibility of the molecules increases with the length of the lateral chain linked in the 4 position of gemcitabine.

Since gemcitabine derivatives tested are neutral at physiological pH, the absence of protonated species at pH 7.4 for all compounds allows to consider log D_{oct} as log P. As shown in Table 1, the values increase starting form the more hydrophilic compound, gemcitabine, till to 4-(*N*)-stearoylgemcitabine. Interestingly, the lipophilicity of gemcitabine and of 4-(*N*)-valeroylgemcitabine in the Miglyol 812N/water system was very much lower than in *n*-octanol/water. This may be in relation with solvatochromic properties of Miglyol and the gemcitabine derivatives. Even if it was not possible to calculate the partition coefficients of 4-(*N*)-lauroyl- and 4-(*N*)-stearoylgemcitabine in the Miglyol 812N/water system, we can expect the same trend as in *n*-octanol/water.

3.2. Nanoparticles characterization

The poly(H_2NPEGCA-*co*-HDCA) nanospheres containing gemcitabine and its lipophilic prodrugs were prepared in a single step by nanoprecipitation, using acetone as organic solvent. For nanocapsules preparation, a medium chain triglyceride, Miglyol 812N, was added to acetone before pouring the organic solution into water. Nanoparticles have been purified by dialysis or by filtration according to the water solubility of the encapsulated compound.

As found by QELS analysis, the poly(H_2NPEGCA-*co*-HDCA) nanospheres containing the gemcitabine lipophilic derivatives showed a homogenous mean diameter ranging between 150 and 190 nm with an unimodal size distribution (polydispersity index < 0.1) (Table 2). The efficiency of drug incorporation into nanospheres was strongly dependent on drug polarity; in particular, only with 4-(*N*)-stearoylgemcitabine it was possible to reach a drug concentration of 1 mg/ml.

For poly(H_2NPEGCA-*co*-HDCA) nanocapsules, the mean diameter was higher than for nanospheres, with a unimodal size distribution only for 4-(*N*)-stearoylgemcitabine-loaded nanocapsules (Table 3). Although statistical analysis using the Student's *t*-test revealed no significant differences in the nanocapsules diameters (as well as in those of nanospheres, see Table 2), the size of gemcitabine derivative-containing nanocapsules has some tendency to diminish with the lipophilicity of the encapsulated compound. What was observed with nanospheres (Table 2) remained true for nanocapsules: the encapsulation efficiency increased with gemcitabine derivative's lipophilicity.

Gemcitabine could not be either associated to nanospheres or encapsulated into nanocapsules (data not shown).

After 2-week storage at 4 °C in water, no appreciable nanoparticles size change was detected by QELS and no drug precipitation or nanoparticles aggregation was observed.

Concerning 4-(*N*)-stearoylgemcitabine, the mean diameter and the zeta potential of drug loaded nanospheres and nanocapsules have been evaluated as a function of increas-

Table 2
Characteristics of gemcitabine derivative-containing poly(H_2NPEGCA-*co*-HDCA) nanospheres obtained by nanoprecipitation (polymer concentration: 5 mg/ml) ($n = 3$)

Drug encapsulated into poly(H_2NPEGCA-*co*-HDCA) nanospheres	Mean diameter ± S.D. (nm)	Polydispersity index	Encapsulation efficiency (%)[a]	Drug loading (%)[b]	Drug conc. (mg/ml)
4-(*N*)-Valeroylgemcitabine	165 ± 65	0.097	6	0.4	0.02
4-(*N*)-Lauroylgemcitabine	187 ± 72	0.084	67	1.3	0.07
4-(*N*)-Stearoylgemcitabine	154 ± 47	0.077	100	17	1

[a] *Encapsulation efficiency*: ratio between drug/polymer ratio after nanoparticle purification and drug/polymer ratio after nanoparticle preparation × 100.
[b] *Drug loading*: ratio between drug and (drug + polymer) × 100 in the formulation after purification.

Table 3
Characteristics of gemcitabine derivative-containing poly(H_2NPEGCA-*co*-HDCA) nanocapsules obtained by nanoprecipitation (polymer concentration: 5 mg/ml) ($n = 3$)

Drug encapsulated into poly(H_2NPEGCA-*co*-HDCA) nanocapsules	Mean diameter ± S.D. (nm)	Polydispersity index	Encapsulation efficiency (%)[a]	Drug loading (%)[b]	Drug conc. (mg/ml)
4-(*N*)-Valeroylgemcitabine	301 ± 107	0.222	6	0.3	0.02
4-(*N*)-Lauroylgemcitabine	235 ± 130	0.278	100	1.6	0.15
4-(*N*)-Stearoylgemcitabine	182 ± 42	0.086	100	10	1

[a] *Encapsulation efficiency*: ratio between drug/polymer ratio after nanoparticle purification and drug/polymer ratio after nanoparticle preparation × 100.
[b] *Drug loading*: ratio between drug and (drug + polymer + oil) × 100 in the formulation after purification.

ing the drug concentration (Fig. 3). No significant variation could be observed concerning the diameter of both nanocapsules and nanospheres when the 4-(*N*)-stearoylgemcitabine concentration was increased. Concerning zeta potential, for nanospheres it increased only at a drug concentration of 250 μg/ml, while for nanocapsules it increased at 125 μg/ml of 4-(*N*)-stearoylgemcitabine and after that it diminished until a drug concentration of 1000 μg/ml.

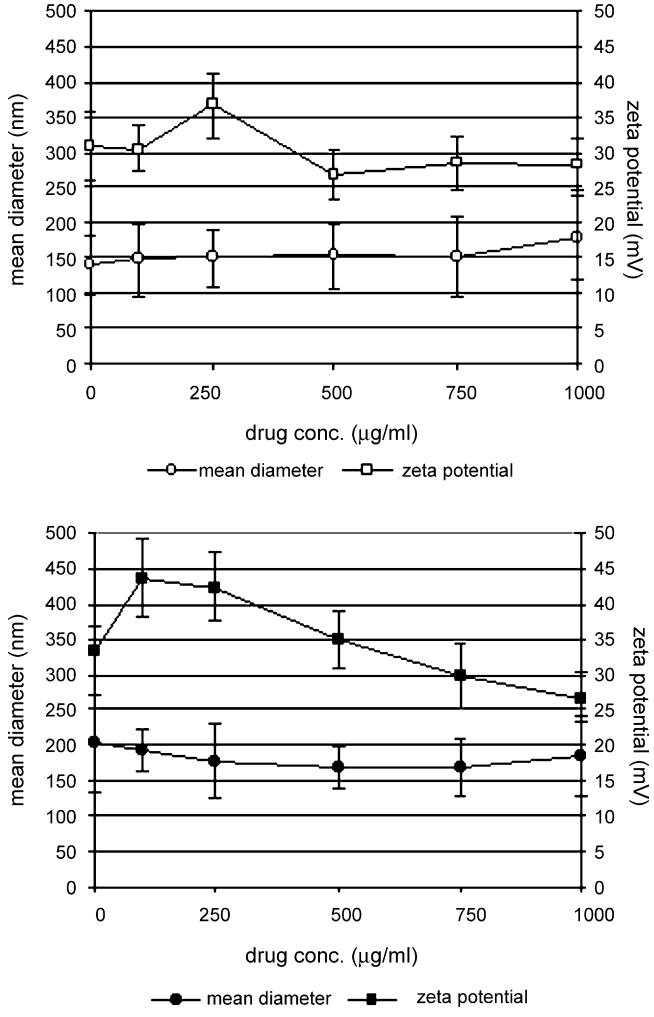
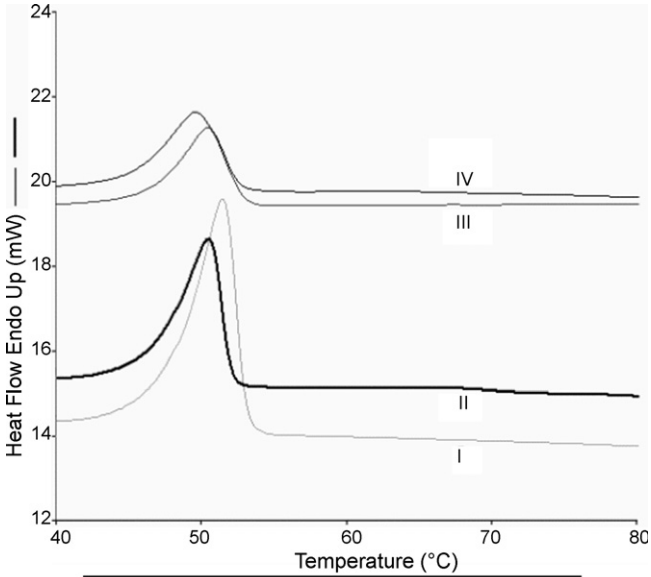


Fig. 3. Mean diameter and zeta potential of 4-(*N*)-stearoylgemcitabine-loaded nanospheres (white symbols) and nanocapsules (black symbols) as a function of drug concentration.

3.3. Differential scanning calorimetry (DSC)

DSC thermograms for empty and 4-(*N*)-stearoylgemcitabine-loaded poly(H_2NPEGCA-*co*-HDCA) nanoparticles are shown in Fig. 4. Unloaded nanospheres and nanocapsules showed a sharp main endothermic peak at 47.41 and 46.55 °C (T_m) with ΔH_m values of 66.93 and 39.39 J/g, respectively. The difference between nanospheres and nanocapsules is related to the presence of the Miglyol in the nanocapsule system. Incorporation of 4-(*N*)-stearoylgemcitabine into poly(H_2NPEGCA-*co*-HDCA) nanoparticles decreased the melting temperature and the enthalpy value of the T_m peaks, showing the presence of the gemcitabine prodrug in both the nanoparticulate systems. In particular, the transition peak of 4-(*N*)-stearoylgemcitabine nanospheres was shifted to 46.51 °C (ΔH_m 54.85 J/g), while for loaded nanocapsules the transition peak was at 45.17 °C (ΔH_m 37.74 J/g). Analysis in triplicate was reproducible.



	Onset (°C)	H (J/g)
nanospheres (I)	47.41	66.93
4-(*N*)-stearoylgemcitabine nanospheres (II)	46.51	54.85
nanocapsules (III)	46.55	39.39
4-(*N*)-stearoylgemcitabine nanocapsules (IV)	45.17	37.74

Fig. 4. DSC thermograms of empty nanospheres (I) and nanocapsules (III) and of 4-(*N*)-stearoylgemcitabine-containing nanospheres (II) and nanocapsules (IV).

Table 4
Cytotoxicity (IC_{50}) of gemcitabine, 4-(*N*)-stearoylgemcitabine and 4-(*N*)-stearoylgemcitabine-containing nanoparticles on human tumor cell lines KB3-1 and MCF-7 as determined by MTT assay (72-h incubation), mean ± S.D. ($n = 3$)

	IC_{50} (μM)	
	KB3-1	MCF-7
Gemcitabine	50.8 ± 5.0	29.0 ± 1.4
4-(*N*)-Stearoylgemcitabine	12.7 ± 5.1**	19.5 ± 4.3*
4-(*N*)-Stearoylgemcitabine nanospheres	10.2 ± 4.9**	12.3 ± 5.4**
4-(*N*)-Stearoylgemcitabine nanocapsules	14.0 ± 7.1**	17.7 ± 6.5*

* $P < 0.05$.
** $P < 0.01$.

3.4. Cytotoxicity assay

The cytotoxic activity of poly(H_2NPEGCA-*co*-HDCA) nanoparticles loaded with 4-(*N*)-stearoylgemcitabine was evaluated on KB3-1 and on MCF-7 human cancer cell lines after 72-h incubation at 37 °C using the MTT method. Free gemcitabine, 4-(*N*)-stearoylgemcitabine and empty nanoparticles were also tested as controls. The results reported in Table 4 show that 4-(*N*)-stearoylgemcitabine was more toxic than gemcitabine on both cell lines. Moreover, incorporation of 4-(*N*)-stearoylgemcitabine in nanospheres or nanocapsules did not change the IC_{50} values of this compound. Unloaded poly(H_2NPEGCA-*co*-HDCA) nanospheres and nanocapsules did not show any cytotoxic activity at the considered concentration range.

4. Discussion

The lipophilicity of gemcitabine and its derivatives was measured or calculated to identify a relationship between lipophilicity-related descriptors and encapsulation efficiency in poly(H_2NPEGCA-*co*-HDCA) nanospheres and nanocapsules. Our data clearly show that the more lipophilic the derivatives were, the more efficient was the encapsulation both in poly(H_2NPEGCA-*co*-HDCA) nanospheres and nanocapsules. Moreover, the drug/carrier association was stable during at least 2 weeks. In fact, the stability of the encapsulated drug within the inner core of the nanoparticles depends also on the transfer rate by diffusion in the aqueous medium, which is critically dependent on the partition coefficient of the drug between the particle core and the water medium; the higher the partition coefficient, the slower was the transfer rate. For 4-(*N*)-stearoylgemcitabine, even if its partition coefficient in the Miglyol 812N/water is lower than that in *n*-octanol/water, its lipophilicity assures a stable incorporation into poly(H_2NPEGCA-*co*-HDCA) nanoparticles. Moreover, high molecule flexibility probably facilitates the disposition of the drug within the inner core of the nanospheres too.

Since 4-(*N*)-stearoylgemcitabine showed the best results in terms of encapsulation efficiency both in nanospheres and nanocapsules, the next steps of nanoparticles characterization were focused on this compound. In particular, the modification of nanoparticles mean diameter was evaluated as a function of 4-(*N*)-stearoylgemcitabine concentration; the results may suggest that at least in nanocapsules 4-(*N*)-stearoylgemcitabine is not only located in the oily inner nanocapsule's core but also distributed at the outer surface. The mean diameter, in fact, tends to diminish with drug concentration, suggesting that 4-(*N*)-stearoylgemcitabine may also act as a surfactant at the interface with water. Further investigations on particle surface chemical composition will allow identifying the functional groups of gemcitabine derivatives on the surface of nanospheres and nanocapsules in order to find the exact location of these compounds. The association of the 4-(*N*)-stearoylgemcitabine to the poly(H_2NPEGCA-*co*-HDCA) nanospheres and nanocapsules was confirmed by DSC analysis, which showed a decrease in the melting temperature and in the enthalpy when the prodrug is encapsulated into nanoparticulate systems. The evaluation of the cytotoxicity of 4-(*N*)-stearoylgemcitabine-nanoparticles towards human cancer cells confirmed the interest in these formulations, since poly(H_2NPEGCA-*co*-HDCA) could efficiently encapsulate this compound, preserving its cell toxicity, which was higher than that of the parent drug gemcitabine. The cytotoxic activity of 4-(*N*)-stearoylgemcitabine was not modified by the encapsulation into poly(H_2NPEGCA-*co*-HDCA) nanoparticles; in fact, the nanoprecipitation technique allowed to preserve the prodrug structure and, thus, did not diminish its cytotoxicity. Moreover, the nanoparticles allowed the complete liberation of the gemcitabine prodrug after 72-h incubation at 37 °C. Nevertheless, the cytotoxic activity of the encapsulated 4-(*N*)-stearoylgemcitabine did not increase, as poly(H_2NPEGCA-*co*-HDCA) copolymer was not cytotoxic at these concentrations. Moreover, these nanoparticles are an efficient system to solubilize this strong lipophilic derivative of gemcitabine.

5. Conclusion

The encapsulation of gemcitabine and of its lipophilic derivatives into poly(H_2NPEGCA-*co*-HDCA) nanoparticles opens interesting perspectives to improve the administration of these compounds.

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References

Abbruzzese, J.L., Grunewald, R., Weeks, E.A., Gravel, D., Adams, T., Nowak, B., Mineishi, S., Tarassoff, P., Satterlee, W., Raber, M.N., Plunkett, W., 1991. A phase I clinical, plasma, and cellular pharmacology study of gemcitabine. J. Clin. Oncol. 9, 491–498.

Bouffard, D.Y., Laliberté, J., Momparler, R.L., 1993. Kinetic studies on 2′,2′-difluorodeoxycytidine (gemcitabine) with purified human deoxycytidine kinase and cytidine deaminase. Biochem. Pharmacol. 45, 1857–1861.

Couvreur, P., Barratt, G., Fattal, E., Legrand, Ph., Vauthier, C., 2002. Nanocapsule technology: a review. Crit. Rev. Ther. Drug Carrier Syst. 19, 99–134.

Crosasso, P., Ceruti, M., Brusa, P., Arpicco, S., Dosio, F., Cattel, L., 2000. Preparation, characterization and properties of sterically stabilized paclitaxel-containing liposomes. J. Control. Release 63, 19–30.

Fessi, H., Puisieux, F., Devissaguet, J.Ph., Ammoury, N., Benita, S., 1989. Nanocapsule formation by interfacial polymer deposition following solvent displacement. Int. J. Pharm. 55, R1–R4.

Grunewald, R., Kantarjian, H., Du, M., Faucher, K., Tarassoff, P., Plunkett, W., 1992. Gemcitabine in leukemia: a phase 1, clinical, plasma, and cellular pharmacology study. J. Clin. Oncol. 10, 406–413.

Heinemann, V., Hertel, L.W., Grindey, G.B., Plunkett, W., 1988. Comparison of the cellular pharmacokinetics and toxicity of 2′,2′-difluorodeoxycytidine and 1-β-D-arabinofuranosylcytosine. Cancer Res. 48, 4024–4031.

Heinemann, V., Xu, Y.Z., Chubb, S., Sen, A., Hertel, L.W., Grindey, G.B., Plunkett, W., 1992. Cellular elimination of 2′,2′-difluorodeoxycytidine 5′-triphosphate: a mechanism of self-potentiation. Cancer Res. 52, 533–539.

Hertel, L.W., Kroin, J.S., Misner, J.W., Tustin, J.M., 1988. Synthesis of 2-deoxy-2,2-difluoro-D-ribose and 2-deoxy-2,2-D-ribofuranosyl nucleosides. J. Org. Chem. 53, 2406–2409.

Hertel, L.W., Boder, G.B., Kroin, J.S., Rinzel, S.M., Poore, G.A., Todd, G.C., Grindey, G.B., 1990. Evaluation of the antitumor activity of gemcitabine (2′,2′-difluoro-2′-deoxycytidine). Cancer Res. 50, 4417–4422.

Immordino, M.L., Brusa, P., Rocco, F., Arpicco, S., Ceruti, M., Cattel, L., 2004. Preparation, characterization, cytotoxicity and pharmacokinetics of liposomes containing lipophilic gemcitabine prodrugs. J. Control. Release 100, 331–346.

Matsuda, A., Sasaki, T., 2004. Antitumor activity of sugar-modified cytosine nucleosides. Cancer Sci. 95, 105–111.

Moghimi, S.M., Hunter, A.C., Murray, J.C., 2001. Long-circulating and target-specific nanoparticles: theory to practice. Pharmacol. Rev. 53, 283–318.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.

Myhren, F., Borretzen, B., Dalen, A., Sandvold, M.L., 2002. Gemcitabine derivatives. US Patent US2,002,042,391.

Plunkett, W., Huang, P., Gandhi, V., 1995a. Preclinical characteristics of gemcitabine. Anticancer Drugs 6, 7–13.

Plunkett, W., Huang, P., Searcy, C.E., Gandhi, V., 1996. Gemcitabine: preclinical pharmacology and mechanism of action. Semin. Oncol. 23, 3–15.

Plunkett, W., Huang, P., Xu, Y.Z., Heinemann, V., Grunewald, R., Gandhi, V., 1995b. Gemcitabine: metabolism, mechanism of action, and self-potentiation. Semin. Oncol. 22, 3–10.

Sharma, A., Conway, W.D., Straubinger, R.M., 1994. Reversed-phase high-performance liquid chromatographic determination of taxol in mouse plasma. J. Chromatogr. B Biomed. Appl. 655, 315–319.

Stella, B., Arpicco, S., Peracchia, M.T., Desmaële, D., Hoebeke, J., Renoir, M., d'Angelo, J., Cattel, L., Couvreur, P., 2000. Design of folic acid-conjugated nanoparticles for drug targeting. J. Pharm. Sci. 89, 1452–1464.

Storniolo, A.M., Allerheiligen, S.R.B., Pearce, H.L., 1997. Preclinical, pharmacologic, and phase I studies of gemcitabine. Semin. Oncol. 24, S7-2–S7-7.

Vauthier, C., Dubernet, C., Fattal, E., Pinto-Alphandary, H., Couvreur, P., 2003. Poly(alkylcyanoacrylates) as biodegradable materials for biomedical applications. Adv. Drug Deliv. Rev. 55, 519–548.