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ORIGINAL ARTICLE

Microencapsulation of cytarabine using poly(ethylene glycol)–poly(ε-caprolactone) diblock copolymers as surfactant agents

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Abstract

Background: The high water solubility and the low molecular weight of cytarabine (Ara-C) are major obstacles against its particulate formulation as a result of its low affinity to the commonly used hydrophobic polymers. *Methods*: Biodegradable cytarabine loaded-microparticles (Ara-C MPs) were elaborated using poly(ε-caprolactone) (PCL) and monomethoxy polyethylene glycol (mPEG)–PCL diblock copolymer in order to increase the hydrophilicity of the polymeric matrix. For this purpose, a series of mPEG–PCL diblock copolymers with different PCL block lengths were synthesized. Compositions and molecular weights of obtained copolymers were characterized by Fourier transform infrared spectroscopy, nuclear magnetic resonance, size exclusion chromatography, and size exclusion chromatography–multi-angle laser light scattering. Ara-C MPs were prepared by double emulsion-solvent evaporation method. The effects of varying PCL block lengths on microparticle encapsulation efficiency, size, and zeta potential were evaluated. *Results*: Increasing the PCL block lengths of copolymers substantially increased the Ara-C encapsulation efficiency and the microparticle size but it decreased their zeta potential. Microparticles were spherical in shape, with a smooth surface and composed of homogenously distributed Ara-C-containing aqueous domains in the polymer matrix. The in vitro drug release kinetics of the optimized microparticles showed a hyperbolic profile with an initial burst release. *Conclusion*: These results showed the important role of the amphiphilic diblock copolymers as stabilizing agent in the encapsulation of Ara-C in PCL microparticles, suggesting their potential use for the microparticulate formulations of other small hydrophilic bioactive molecules.

Key words: *Amphiphilic diblock copolymers; cytarabine; double emulsion; microparticles; poly(ε-caprolactone)*

Introduction

The clinical use of cytarabine (Ara-C) like other nucleoside analogues is potentially limited by its short half-life after intravenous administration and its narrow therapeutic index inducing severe side effects while maintaining a low anticancer activity[1,2]. Consequently, the minimum effective dose is high and has to be regularly increased. In this context, toxicity becomes the main limiting factor to the treatment[2]. Furthermore, the important hydrophilic character of Ara-C strongly limits its intracellular uptake, because of the low membrane permeability of this molecule[3]. On the other hand, its extensive degradation by cytidine deaminases in the liver and kidney and its poor stability in biological media adversely affect its anticancer activity and raise the question of improved formulation for better tumor targeting and drug stability[4–6].

Nano- and microparticulate systems prepared from biocompatible preformed polymers, such as poly(ε-caprolactone) (PCL), poly(lactic acid) (PLA), or poly(lactic-co-glycolic acid) (PLGA), could be promising carriers achieving both a controlled release and a good protection

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of encapsulated Ara-C from enzymatic degradation. Moreover, biodegradable microparticles could be particularly phagocytized by cancerous cells,[7–9] which make these drug vehicles suitable for the intraperitoneal[10], intratumoral,[11,12] or subcutaneous[13,14] anticancer drug delivery.

Unfortunately, the high water solubility and the low molecular weight of Ara-C are major obstacles against its particulate formulation, as a result of its low affinity to the commonly used hydrophobic polymers, such as PCL, PLA, or PLGA[15]. This generally induces the drug loss into the external aqueous phase during the emulsification and hardening of the microparticles[16], whereas the commonly used surfactant agents, such as poly(vinyl alcohol) (PVA) or pluronic, remain disabled to limit the drug migration to the external aqueous phase,[17] yielding low encapsulation efficiencies (EEs) of hydrophilic small molecules[18–20]. This is probably related to the fact that these surfactant agents do not contribute to the microparticle polymeric matrix formation during the solvent evaporation and the subsequent polymer precipitation.

To address this point resulting from the hydrophobic nature of homopolymers, we introduced a hydrophilic block, the monomethoxy poly(ethylene glycol) (mPEG), to form amphiphilic diblock copolymers mPEG–PCL to be used as surfactant agents for the preparation of Ara-C-loaded PCL microparticles.

In fact, diblock copolymers mPEG-polyester have been widely employed in the elaboration of stealth carriers[21,22] and for the encapsulation of some macromolecules such as proteins and peptides using the double emulsion technique[23].

Based on these considerations, we hypothesized that the use of surfactant agents with an anchor segment, such as mPEG–PCL copolymers, should potentially limit the drug partitioning to the continuous aqueous phase. These copolymers contributing to the microparticle polymeric matrix could sequester the small hydrophilic drug in the microparticles because of their hydrophilic segments (mPEG) increasing the hydrophilicity of the delivery system.

In this work, our objective was to elaborate a novel microparticulate system for Ara-C delivery. In this system, both of PCL and its diblock copolymer mPEG–PCL were employed (Figure 1). Indeed, as highlighted earlier, the use of a diblock copolymer composed of PCL and mPEG to encapsulate the Ara-C is of great interest, as it should increase the overall hydrophilicity of the polymeric matrix of the microparticles, leading to an improved EE of the hydrophilic small drug, whereas the homopolymer PCL chains could provide rigidity and a better in vitro stability of the microparticulate system[24]. To the best of our knowledge, no research work has already been reported on the encapsulation of Ara-C in micro- or nanoparticles based on PCL or other biodegradable polymers, such as PLA or PLGA, either with or without their corresponding PEG copolymers.

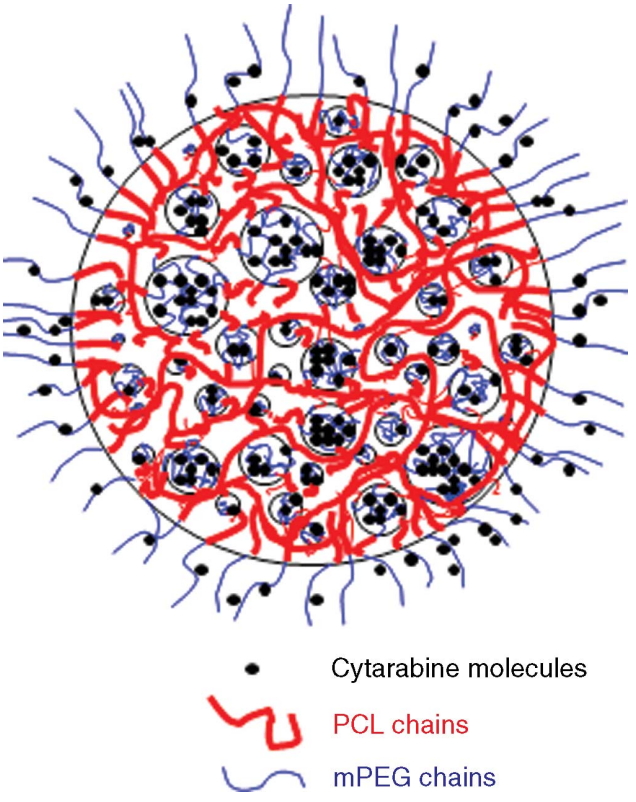

Figure 1. Illustration of PCL/mPEG–PCL composite microparticles loaded with cytarabine. In this schematic we suggest that mPEG blocks are oriented toward both the external aqueous medium and to the aqueous medium in the internal vesicles.

Having this goal in mind, we synthesized a series of mPEG–PCL diblock copolymers with different molecular weight (but with the same mPEG chain length). These copolymers were then used for the preparation of the microparticles using the double emulsion–solvent evaporation method, which has been generally applied to microencapsulate hydrophilic macromolecules, such as proteins and peptides[9,25,26].

The different microparticle batches prepared from different corresponding copolymers were characterized for their size, morphology, and EE to select the appropriate diblock copolymer molecular weight for Ara-C encapsulation. Thereafter, the microparticles prepared with the selected diblock copolymer underwent further physicochemical characterizations and in vitro release studies.

Materials and methods

Synthesis of mPEG–PCL diblock copolymers

Epsilon-caprolactone (ε-CL) (Sigma-Aldrich, Saint Quentin Fallavier, France) was purified by vacuum distillation over CaH_2 (Acros Organics, Geel, Belgium). mPEG with a molecular weight of 5000 g/mol (Sigma-Aldrich) was

dried by an azeotropic distillation with anhydrous toluene (Sigma-Aldrich) under dry nitrogen atmosphere. Metal calcium was used as received. Stannous 2-ethylhexanoate [$Sn(Oct)_2$] (Sigma-Aldrich) was used without further purification. All the other reagents used in this work were of analytic reagent grade and used as received.

The mPEG–PCL diblock copolymers were synthesized by ring-opening polymerization of ε-CL with mPEG as a macroinitiator and stannous 2-ethylhexanoate as a catalyst. A predetermined amount of ε-CL, mPEG, and $Sn(Oct)_2$ (0.1% of ε-CL in molar amount) were weighed into a three-necked glass bottle equipped with a magnetic stirring bar. The bottle was sealed under dry nitrogen and was immersed in an oil bath at 130°C for 12 hours. The resulting copolymer was cooled to room temperature, and then they were precipitated in excess of cold diethyl ether from toluene solution. The obtained copolymers were purified by dissolving them in dichloromethane (DCM) and then precipitated in excess of cold diethyl ether. Finally the mixture was filtered and dried at room temperature under vacuum for 24 hours.

The molar ratio of ε-CL to mPEG was varied to obtain copolymers with different PCL block lengths. The degree of polymerization of PCL block was then calculated from nuclear magnetic resonance (^{1}H NMR) spectra.

In this article, mPEG–PCL diblock copolymers are denoted as mPEG*x*–PCL*y*, where *x* and *y* represent the number average molecular weight M_n $\left[M_n = \sum c_i / \sum (c_i / M_i)\right]$ of mPEG and PCL block, respectively. The obtained copolymers are listed in Table 1.

Characterization of mPEG–PCL diblock copolymers

Fourier transform infrared spectroscopy

Fourier transform infrared spectra were obtained using the KBr technique in the range of 4500–400 cm^{-1} and an infrared spectrophotometer (Unicam Mattson 5000; Unicam, Argenteuil, France) at room temperature.

Nuclear magnetic resonance analysis

^{1}H NMR spectra were recorded by using a Brucker (DRX 300; Brucker, Fremont, CA, USA) spectrometer operating at 300 MHz using the deuterated chloroform ($CDCl_3$) as a solvent. Chemical shifts (δ) were measured in ppm using tetramethylsilane as an internal reference standard.

Size exclusion chromatography

Average molecular weights were determined on a size exclusion chromatography (SEC) system (Waters, Milford, MA, USA) equipped with an isocratic pump (Waters 515) operated at a flow rate of 1 mL/min with tetrahydrofuran (THF) (Sigma-Aldrich), an autosampler (Waters 717 plus), a column oven, and a refractive-index (RI) detector (Waters 410) with integrated temperature controller maintained at 30°C.

Data collection and data process were performed with the software Empower Pro version 5.0 from Waters Corporation. For molecular mass separation a guard column (PL gel 5 μm), three Polymer Laboratories columns [2 × PLgel 5 μm Mixed C (300 × 7.5 mm), and 1 × PLgel 5 μm 500 A (300 × 7.5 mm)] (Agilent, Shropshire, UK) were used in series at 30°C.

Calibration was carried out using narrow distributed polystyrene standards. The mobile phase was THF [high-performance liquid chromatography (HPLC) grade] stabilized with dieter butyl-2,6 methyl-4 phenol (Acros Organics). Polymer samples were dissolved in THF to form a homogeneous solution. Chromatography was carried out after sample filtration through a 0.45-μm cellulose membrane filter.

Absolute molar mass determination by SEC–multiangle laser light scattering/refractive index

Absolute molecular weights of copolymer were determined on a Waters Size Exclusion Chromatography system coupled with

Table 1. The synthesis of mPEG–PCL diblock copolymers with different PCL block lengths.

Copolymer	(ε-CL)/(EO) in feed	(ε-CL)/(EO) calculated from ^{1}H NMR spectra	M_w[a]	M_{NMR}[b]	M_{nSEC}[c]	PI[d]	PD[e]	Solubility in water
mPEG 5K–PCL 1.6K CP1	0.17	0.12	7.3×10^3	6.6×10^3	9.9×10^3	1.10	14	Soluble
mPEG 5K–PCL 2.5K CP2	0.26	0.19	8.4×10^3	7.5×10^3	11.5×10^3	1.14	22	Not soluble
mPEG 5K–PCL 3.4K CP3	0.30	0.25	9×10^3	8.4×10^3	12.6×10^3	1.15	30	Not soluble
mPEG 5K–PCL 3.6K CP4	0.35	0.27	9.5×10^3	8.6×10^3	12.8×10^3	1.23	32	Not soluble
mPEG 5K–PCL 7.4K CP5	0.61	0.57	13×10^3	12.4×10^3	17.3×10^3	1.34	65	Not soluble
mPEG 5K–PCL 11K CP6	0.88	0.81	16.4×0^3	16.1×10^3	22.4×10^3	1.45	97	Not soluble

[a]Theoretical molecular weight as calculated according to the feed ratio.
[b]The molecular weight as calculated according to the integrated area ratio of the resonance peaks because of the PCL block at 4.07 ppm and because of the mPEG block at 3.65 ppm.
[c]The number average molecular weight as measured by SEC analysis (calibrated with polystyrene standards).
[d]Polydispersity index (PI) as measured by the SEC analysis.
[e]Polymerization degree (PD) as calculated from the ^{1}H NMR spectra.

- an RI detector Model (Waters 410) with integrated temperature controller maintained at 30°C,
- a triple-angle light scattering (LS) detector (MiniDAWN Tristar; Wyatt Technology, Santa Barbara, CA, USA).

The two signals were measured simultaneously because of the online multiangle laser light scattering/refractive index (SEC–MALLS/RI) arrangement; so the absolute molecular weight of the copolymers could be deduced.

Data collection and processing were performed using two softwares; ASTRA® version 4.5 from Wyatt Technology and the software Empower Pro version 5.0 from Waters Corporation.

Preparation of PCL/mPEG–PCL microparticles

Different batches of microparticles have been prepared by a double emulsion–solvent evaporation method, as described by Rawat et al.[27], with some modifications (Table 2). The double emulsion was prepared from three phases as follows:

- Internal aqueous phase: 25 mg of Ara-C (HalloChem Pharma, Chongqing, China) was dissolved in aqueous solution of PVA (M_w = 31 kDa, hydrolyzation degree = 88%; Aldrich). PVA was used at 5%.
- Organic phase: the polymer (PCL, M_w = 80 kDa; Aldrich) and one of the synthesized diblock copolymers (mPEG–PCL) that have been dissolved in a nonmiscible organic solvent, the DCM (Laurylab, Saint Fons, France) at a concentration of 5% (w/w) and 10% (w/w), respectively.
- External aqueous phase: PVA aqueous solution at the same percentage used in the internal aqueous phase.

The primary emulsion water/oil (W/O) was prepared by adding the organic phase to the internal aqueous phase under mechanical stirring (Ultraturax® T25; IKA Werke GmbH, Staufen, Germany) at 13,500 rpm for 1 minute. The primary emulsion was then poured into the external aqueous phase under mechanical stirring (Ultraturax® T25; IKA Werke GmbH) at 6500 rpm for 1 minute to form the double emulsion water/oil/water (W/O/W).

After obtaining emulsion, the DCM was evaporated by a rotative evaporator (R-144; Buchi, Flawil, Switzerland) at 100 rpm for 15 minutes. The formed microparticles were separated by ultracentrifugation (Beckman, Miami, FL, USA) at 25,000 rpm for 20 minutes. The obtained microparticles were then dried under compressed air for 24 hours at room temperature.

The selection of the preparation parameter values, including the Ara-C initial load, the volumes of emulsion phases, and the choice of the solvent evaporation rather than the solvent extraction, was based on the results of preliminary investigations.

Table 2. The different microparticle batches prepared using the synthesized diblock copolymers by the double emulsion–solvent evaporation technique and their physico-chemical characterization.

Batch	MP polymeric composition	Encapsulation efficiency (%)[a]	Size (μm)[a]	Zeta potential (mV)[a]
MP0	PCL	1.30 ± 0.18	3.5 ± 1.6	−22.2 ± 0.4
MP1	PCL + CP1	1.57 ± 0.16	4.4 ± 1.8	−20.7 ± 0.4
MP2	PCL + CP2	3.18 ± 0.58	4.3 ± 2.3	−15.6 ± 0.5
MP3	PCL + CP3	8.15 ± 0.11	8.5 ± 4.7	−13.7 ± 0.1
MP4	PCL + CP4	8.94 ± 1.26	9.6 ± 5.1	−13.1 ± 0.7
MP5	PCL + CP5	12.63 ± 1.34	13 ± 5.2	−12.5 ± 0.1
MP6	PCL + CP6	9.32 ± 1.14	13.5 ± 5.7	−13.4 ± 0.7

[a]The data were expressed as means ± SD ($n = 3$).

Encapsulation efficiency determination

Ara-C EE in the microparticles was assessed by HPLC. Briefly, 15 mg of dried loaded microparticles was added to 5 mL DCM under magnetic stirring at 50°C water bath until complete dissolution of microparticles. Then 5 mL of distilled water was added and vortexed for 15 minutes. DCM evaporation was accelerated by a short sonication (around 10 minutes) in an ultrasonic bath at 40°C. After a clear solution has been obtained, the sample volume was made to 10 mL by adding distilled water. All the samples were filtrated (0.45 μm cellulose membrane filter) before being analyzed by HPLC. The drug extraction from different microparticle batches, of the same preparation formula, was performed in triplicate.

The HPLC unit (Thermosystems, Inc., Lombard, IL, USA) consisted in a set of a Spectra System P1000XR pump, a Spectra System AS 300 autosampler, and a Spectra System UV 6000LP diode array detector. The data were recorded and analyzed with the Chromquest® PC software over the Spectra System SN4000 unit. Chromatographic separations were performed at 25°C using a modified method of that employed by Gomez et al.[28]. Twenty microliters of samples or calibration standards were injected directly into the column and were eluted under isocratic conditions through a Spherisorb ODS-2, C18, 5 μm (250 cm × 4.6 mm) (MZ-Analysentechnik GmbH, Mainz, Germany). The mobile phase was 5 mM monobasic potassium phosphate in distilled water containing 5% (v/v) methanol. It was filtered through a 0.45-μm pore size cellulose membrane filter and degassed with helium flow before use. The flow rate was set at 1 mL/min, the total run time was 10 minutes, and the wavelength detector was 272 nm. Each determination was carried out in triplicate.

A calibration curve was performed for Ara-C standards in deionized water, with concentrations ranging from 10 to 100 μg/mL. Ara-C EE in microparticles was then determined by the linear calibration curve obtained from the area under the Ara-C peak in the HPLC chromatogram. A correlation coefficient of 0.9998 was obtained. The sample chromatograms showed a clear single peak (retention time 5 ± 0.4 minutes) belonging to Ara-C.

The drug EE was expressed as the percentage of the encapsulated amount measured in microparticles to the total amount initially used in the formulation as follows:

$$\mathrm{EE} = \frac{\mathrm{encapsulated}_{\mathrm{cytarabine}}}{\mathrm{total}_{\mathrm{cytarabine}}} \times 100\%$$

Microparticle size and zeta potential

The size distribution of microparticles was determined using laser diffraction technique (Beckman Coulter LS 230, Fullerton, CA, USA). Microparticle size was expressed as a volume diameter.

Zeta potential was determined in diluted particles suspensions using Zetasizer 3000 HSa (Malvern; Malvern, England) at 25°C. Each measurement (size or zeta potential) was performed in triplicate.

Transmission electron microscopy

Microparticle suspensions (MP5 formula) were imaged using a transmission electron microscope (TEM) (Philips CM120, Eindhoven, the Netherlands). The suspensions were placed on a carbon-coated copper TEM grid and then dried under atmosphere conditions.

Wet scanning transmission electron microscopy

Wet scanning transmission electron microscopy (Wet-STEM) observations of Ara-C microparticles (MPs) (MP5 formula) were carried out by using environment scanning electronic microscope-field emission gun (Philips XL30, Eindhoven, the Netherlands). A specific device developed for the imaging of wet samples in transmission mode[29] was used. We employed holey carbon-coated TEM copper grids, with the carbon layer down to use copper squares as retention basins. The pressure and temperature were adjusted to evaporate a small amount of water from the microparticle suspension droplet. It allows keeping a water layer thin enough so that electrons both transmitted and scattered pass through it and can be collected to contribute to the formation of the STEM image. The signal was collected by a detector, usually used for the collection of backscattered electrons, but in our case located below the sample.

Confocal laser scanning microscopy

Drug distribution within microparticles was investigated using a hydrophilic molecule model, Fluorescein isothiocyanate-dextran (FD40; Sigma-Aldrich), by a confocal laser scanning microscope (Leica TCS SP2, Bensheim, Germany) with a regular 63× numerical aperture 1.32 oil-immersion objective lens.

Fluorescent microparticles were prepared according to MP5 formula by substituting the Ara-C by the FD40. Then they were re-dispersed in distilled water and placed onto a glass slide before the observations. FD40 was detected using an argon laser with an excitation wavelength of 488 and a 507–567 nm band-pass emission filter. All the images were obtained under the same resolution.

Residual solvent measurement by gas chromatography

Twenty milligrams of Ara-C MPs (MP5 formula) was dissolved in 2 mL of dimethyl sulfoxide (Laurylab) in a 5 mL vial and an appropriate amount of toluene was added as an internal standard. The vial was airtight and kept at 4°C before gas chromatography (GC) analysis to avoid DCM evaporation.

Analysis of the residual solvents was carried out on a gas chromatograph (Model 4890; Agilent Technologies, Santa Clara, CA, USA) equipped with a split/splitless inlet port and a flame ionization detector. Separations were performed on a Bonded FSOT Capillary column [30 m × 0.53 mm (i.d.); Superox-FA; polyethylene glycol ester; Alltech Associates Inc., Deerfield, IL, USA].

The following conditions were used:

- inlet temperature, 250°C
- detector temperature, 280°C
- oven temperature set at 70°C, then increased at a rate of 10°C/min until 220°C. It remains at this final temperature for 2 min
- nitrogen with a flow rate of 13 mL/min was used as a carrier gas
- a volume of 0.1 μL was injected in split mode (split ratio, 1/40).

GC ChemStation Rev. A.08.03 Software from Agilent Technologies was used to acquire and process the data.

A calibration curve was traced for DCM standards diluted in dimethyl sulfoxide, with concentrations ranging from 31 to 500 ppm. A concentration of 100 ppm of toluene was used as an internal standard. Residual DCM levels in MP5 were then determined by the linear calibration curve constructed from the ratio of the AUP (area under the peak in the GC-chromatogram) of DCM standards by the AUP of toluene. A correlation coefficient of 0.9951 was obtained.

Differential scanning calorimetry

Thermal characterization of Ara-C MPs (formula MP5) was performed with a differential scanning calorimeter differential scanning calorimetry (DSC) TA 125 (TA Instrument, New Castle, DE, USA). The equipment was calibrated with indium. All samples (Ara-C MPs, blank microparticles, physical mixture, and raw materials) were accurately weighed (5–10 mg) and sealed in aluminum pans[30]. Each sample was scanned at a speed of 10°C/min in the temperature range of 20–250°C. Nitrogen was used as the purge gas with the flow rate set at 50 mL/min with an empty aluminum pan as reference.

In vitro release study

In vitro release studies of Ara-C MPs (formula MP5) were performed using a dialysis bag (dialysis tubing cellulose membrane, molecular weight cutoff 7402 Da; Sigma-Aldrich Chemie GmbH PO, Taufkirchen, Germany). The dialysis bag was pre-treated during 1 hour with a phosphate-buffered saline (PBS, pH 7.4) to ensure its wetting and sealing. Microparticles containing 5 mg of micro-encapsulated Ara-C were suspended in 1 mL of the PBS (pH 7.4) and placed in the dialysis tube. The dialysis tube was then immersed into 10 mL of PBS (pH 7.4) at 37°C. The total volume of the receptor buffer solution was removed at pre-determined time intervals and replaced with 10 mL of fresh buffer medium. All tests were performed in triplicate. The Ara-C concentrations in the PBS (pH 7.4) were measured using HPLC analysis as described above.

Results and discussion

Synthesis and characterization of mPEG–PCL diblock copolymers

The mPEG–PCL diblock copolymers (shown in Table 1) with different molecular weights and compositions were obtained by changing the feed molar ratio of mPEG/ε-CL in the presence of stannous 2-ethylhexanoate as a catalyst.

Tin compounds are well known as highly effective esterification or *trans*-esterification catalysts and they are also known to be efficient initiators of ring-opening polymerizations of lactones and related heterocycles[31]. Moreover, $Sn(Oct)_2$ has been widely used in mPEG-polyester diblock copolymer synthesis for the elaboration of drug delivery systems[21,23].

Fourier transform infrared spectroscopy

The mPEG5K–PCL7.4K diblock copolymer (CP5), mPEG, and PCL infrared spectra were recorded. The major absorption peaks in the CP5 spectrum were the absorption band at 1725 cm^{-1} (ester C=O stretching vibrations), which is ascribed to PCL blocks and the absorption band at 1110 cm^{-1} (C–O–C stretching vibrations) being attributed to $-OCH_2CH_2$ characteristic of the mPEG blocks. It is obvious that the CP5 exhibits characteristic peaks of both mPEG and PCL segments, confirming the presence of these two moieties. These results are consistent with the data published elsewhere[32].

Nuclear magnetic resonance analysis

To further confirm the formation of mPEG–PCL copolymers, ^{1}H NMR spectra were recorded. Results are shown in Figure 2 and the characteristic resonance peaks are indicated in this figure. Peaks at chemical shifts of 1.39 (multiplet, *f*), 1.65 (multiplet, *e*), 2.31 (multiplet, *d*), and 4.07 ppm (multiplet, *c*) are assigned to the methylene protons of $-(CH_2)_3-$, $-OCCH_2-$, and $-CH_2OOC-$, respectively, in PCL units. The sharp peak at 3.65 ppm (multiplet, *b*) is attributed to methylene protons of $-CH_2CH_2O-$ of mPEG units in the block copolymer. The very weak peaks at 4.23 and 3.82 ppm are, respectively, attributed to the methylene protons of $-O-CH_2-CH_2-$ of mPEG end unit that links with PCL blocks. The singlet (*a*) for monomethoxy in mPEG end groups (–OCH3) appears at 3.40 ppm.

The molecular weight (M_{NMR}) of mPEG–PCL copolymers was calculated from ^{1}H NMR spectra according to the following equations[33,34]:

$$\frac{4m+2}{3} = \frac{\text{Int.}3.65}{\text{Int.}(-OCH_3)} \quad (1)$$

$$\frac{2(n+1)}{3} = \frac{\text{Int.}4.07}{\text{Int.}(-OCH_3)}, \quad (2)$$

where Int. 3.65 is the integral of the intensity of the methylene proton peak of $-OCH_2CH_2-$ belonging to mPEG blocks at 3.65 ppm; Int. 4.07 is the integral of the intensity of the methylene proton peak of $-CH_2OCO-$ belonging to PCL blocks at 4.07 ppm; *m* and *n* are the corresponding polymerization degrees of mPEG and PCL blocks, respectively.

The molecular weight M_{NMR}, calculated according to the integrated area of the resonance peak of the PCL block at 4.07 ppm and that of the mPEG block at 3.65 ppm, is calculated as follows:

$$M_{NMR} + M_{w(\text{PCL block})} + M_{w(\text{mPEG block})} = 44m + 114n. \quad (3)$$

The values 44 and 114 are the molar masses of the repeating units of the mPEG and PCL blocks, respectively.

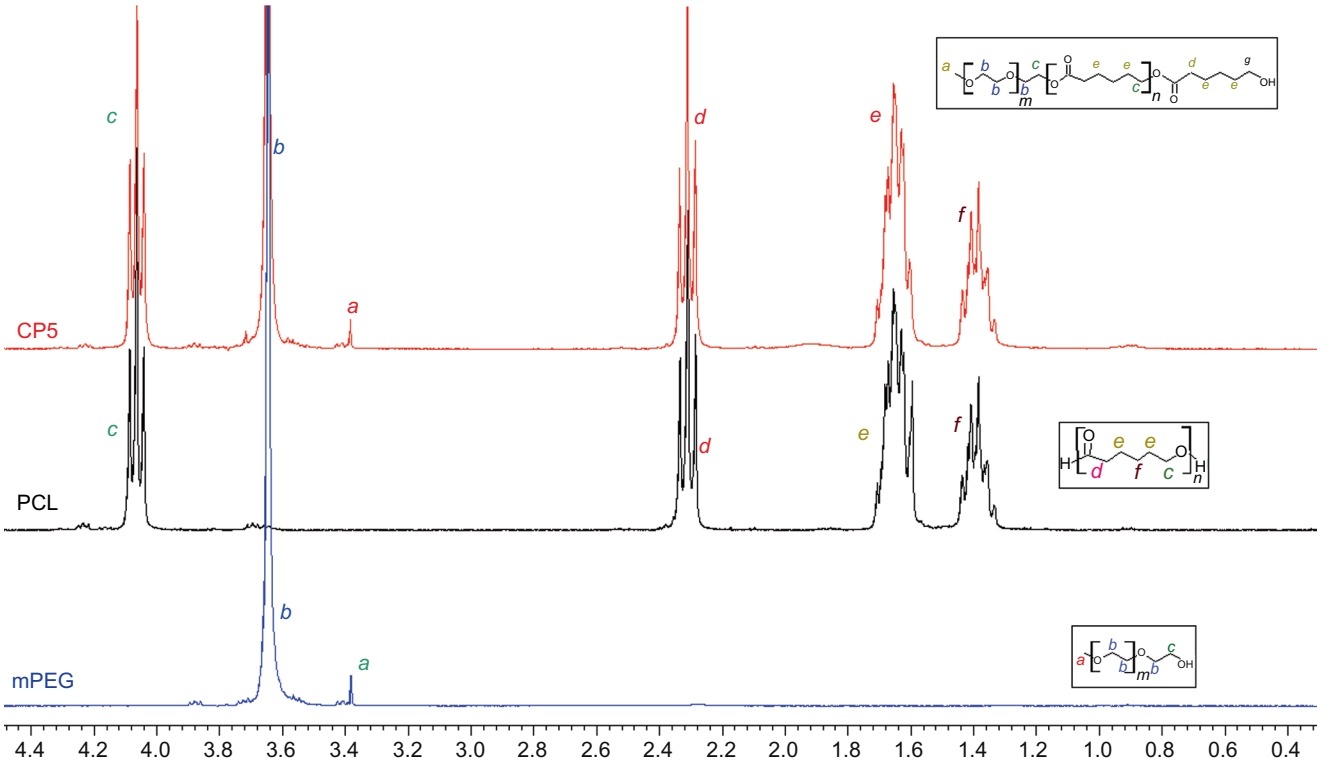

Figure 2. ^{1}H NMR spectra of CP5 (top), PCL (middle), and mPEG (bottom) in $CDCl_3$, and the assignment of the resonance peaks.

The M_{NMR}-obtained values were very close to the theoretical values M_w. In addition, the ^{1}H NMR analysis with regard to the relative contents of both blocks in the copolymer clearly indicates that the experimental data of (ε-CL)/(EO) are consistent with theoretical values.

Size exclusion chromatography

The average molecular weights and polydispersity indexes of the mPEG–PCL diblock copolymers were also measured by SEC using THF as an elution solvent and monodisperse poly(styrene) as standards. As shown in Figure 3, by increasing the ε-CL monomer amount in feed, the elution time of resultant diblock copolymers decreased, indicating the increase of copolymer molecular weights. The diblock copolymers show narrow molecular distributions with polydispersity index values around 1.10–1.45.

SEC analysis of the copolymers indicates that the observed molecular weights are higher than those of the theoretical values, especially in the case of copolymers with high PCL content (Table 1). This is explained as a result of the calibration, which was carried out with polystyrene standards. Similar observations have been reported elsewhere[35].

Absolute molar mass determination by SEC–MALLS/RI

The MALLS coupled with an RI detector allows the absolute molecular weight of polymers to be determined after separation by SEC, without column calibration of the respective polymer. However, this method is difficult to be applied to polymers of low molecular weight (≤30,000 g/mol) even though we tried to use it for the molar mass characterization of CP5 and CP6.

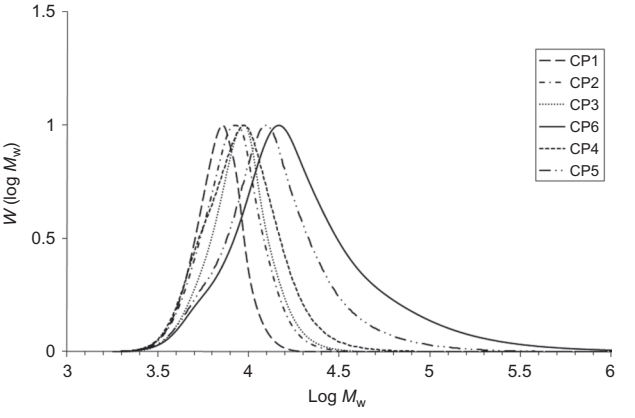

Figure 3. SEC traces of synthesized diblock copolymers. *W* represents the copolymer molar mass.

Figure 4 shows an SEC–MALLS chromatogram of CP5, with the response of the RI concentration detector and the 90° angle view of the LS detector. The vertical

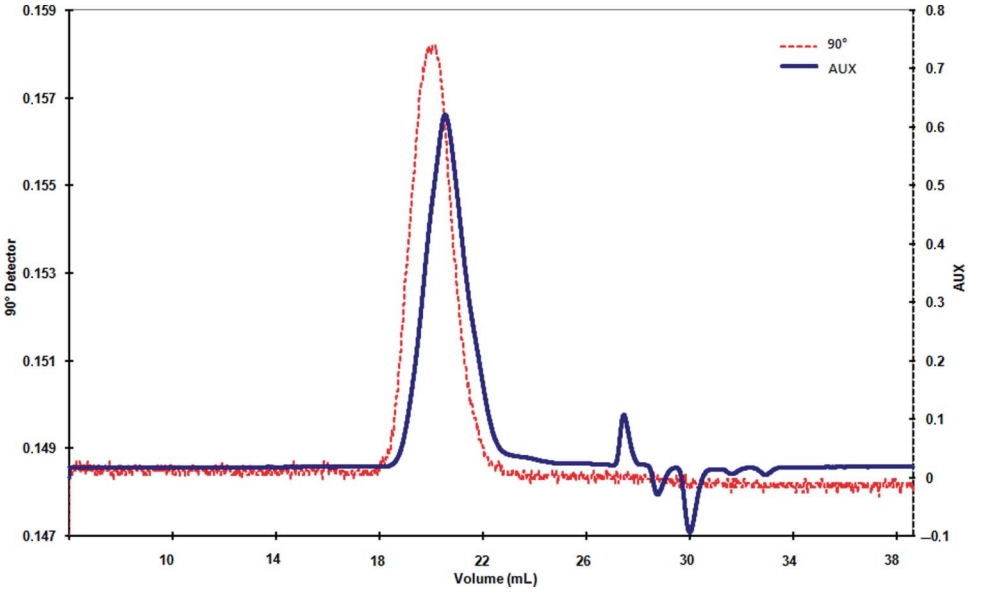

Figure 4. Elution curve of mPEG 5K–PCL7.4K (CP5), mobile phase, THF; detectors, RI (refractive index detector signal in solid line), and LS (light scattering detector signal taken at 90° in dashed line).

Table 3. Comparison of SEC–MALLS and NMR results in terms of absolute molecular weight and polymerization degree.

Copolymer	M_w[a]	$M_{w\ (SEC\text{-}MALLS)}$[b]	M_{NMR}[c]	$PD_{(SEC\text{-}MALLS)}$[d]	PD_{NMR}[e]
CP5	13×10^3	12.8×103	12.4×103	68	65
CP6	16.4×10^3	16.2×103	16.1×103	97	97

[a]Theoretical molecular weight as calculated according to the feed ratio.
[b]The absolute molecular weight as determined by SEC–MALLS method.
[c]The molecular weight as calculated according to the integrated area ratio of the resonance peaks because of the PCL block at 4.07 ppm and because of the mPEG block at 3.65 ppm.
[d]Polymerization degree as calculated from SEC–MALLS data.
[e]Polymerization degree as calculated from ^{1}H NMR data.

lines show the integration limits used in the molar mass calculation by the ASTRA® software.

Values of M_w for CP5 and CP6 determined by the two absolute methods, SEC–MALLS and ^{1}H NMR, are very close, as shown in Table 3. This suggests that the molar mass characterization of mPEG–PCL copolymers (whose M_w > 10,000 g/mol) might be achieved using SEC–MALLS, which appears to be a reliable and rapid method giving results that correlate well with those of ^{1}H NMR.

Formulation of cytarabine in PCL/mPEG–PCL microparticles

Ara-C MPs have been prepared by a modified double emulsion-solvent evaporation method. The different synthesized diblock copolymers mPEG–PCL were used as surfactant agents to stabilize the second emulsion during the microparticle preparation and then to minimize the leakage of Ara-C aqueous droplets to the external aqueous phase, being the main phenomenon leading to the poor drug EE[36]. The diblock copolymer giving rise to the optimal EE value was then selected.

The Ara-C EE, the size, and zeta potential of the microparticles prepared using the different diblock copolymers are shown in Table 2. After preliminary experiments, the preparation conditions have been fixed to study the effect of each diblock copolymer on the drug EE, size, and zeta potential of Ara-C MPs. The mPEG block length in all the copolymers was fixed while the only difference concerned the PCL block length.

Influence of the diblock copolymer molecular weight on the encapsulation efficiency

Obviously, the use of the diblock copolymers has considerably enhanced the Ara-C EE when compared to

PCL microparticles. As previously noted[37], the presence of mPEG blocks oriented toward the inner aqueous phase of the double emulsion decreases the hydrophobic character of the polymeric matrix and so increasing the affinity of the hydrophilic drug to the polymeric core, leading to an improved EE of the drug reaching approximately 13% when CP5 was used (formula MP5) compared with 1.3% without adding the copolymer in the formulation (formula MP0).

It is worth noting that the longer the PCL block chain in the mPEG–PCL copolymer used in the microparticle elaboration, the higher the Ara-C EE, when all the other preparation conditions are fixed. This could be explained by the fact that a diblock copolymer with a longer anchor segment (PCL block) is more able to be adsorbed[38] or introduced in the particle polymeric matrix than a diblock with a relatively shorter PCL block.

Influence of the diblock copolymer molecular weight on the microparticle size and zeta potential

Generally speaking, using the diblock copolymers in the formulation induced a clear increase in the microparticle size. For instance, the PCL-based microparticles were around 3.5 ± 1.6 μm and increased up to 13 ± 5.2 and 13.5 ± 5.27 μm in MP5 and MP6, respectively, prepared with the longest diblock copolymers being used in our study (Table 2). This coincides well with data reported in scientific literature[39] and could be explained by the fact that the copolymer is adsorbed and/or integrated in the microparticle polymeric matrix contributing thereby to the volume of obtained microparticles.

It can be concluded that copolymers of relatively longer PCL chain may be more capable of stabilizing microparticles, as it was previously reported for nanoparticles prepared with linear or star-shaped[40] PCL/mPEG copolymers. In these works, it was found that the molecular weight of PCL block in a copolymer significantly affected the stability of nanoparticles in aqueous solution and nanoparticles with shorter PCL block length degraded faster.

Moreover, the zeta potential values of the microparticles were obviously affected by the presence of the diblock copolymers mPEG–PCL at their surfaces. Indeed, the zeta potential of obtained PCL microparticles (formula MP0) was negative (−22.2 mV). A clear decrease in the surface charge of microparticles was observed in the other formula (Table 2, e.g., −12.5 mV for MP5). This can be attributed to a shift of the shear plane position far from the particle surfaces. Similar observations have been already reported by the literature[41].

Microparticle morphology

Microparticles prepared with copolymer CP5 having the optimal EE have been selected for TEM and Wet-STEM observation to investigate their morphology. TEM micrograph (Figure 5a) shows aggregated microparticles that were almost spherical. Additionally, because of some adherence of microparticles to the copper grid, the shrinkage and collapse of particles during the drying process might cause an irregular shape of some particles. Therefore, we employed the Wet-STEM to visualize the microparticles in a thin water layer and then to address the artefact problem related to the drying process in TEM imaging. As expected, the spherical shape was confirmed by the Wet-STEM micrograph. Besides, thanks to backscattered electron, the Wet-STEM technique informed us about the surface characteristics of the microparticles, which were found to be smooth (Figure 5b). Also, the size values obtained by laser diffraction technique correlated well with the TEM and Wet-STEM results (Figure 5c). No drug crystals were visible in the micrographs obtained in both techniques.

Confocal laser scanning microscopy

It is so far important to investigate the distribution profile of the drug inside the obtained microparticles and to check out if the drug is homogenously encapsulated inside or/and adsorbed at the microparticle surfaces. For this purpose a hydrophilic fluorescent molecule model, FD40, has been used. The main advantage of confocal laser scanning microscopy is its ability to provide visualization of images parallel to the sample surface at both internal and external levels, at multiple depths, without any mechanical sectioning. Furthermore, this technique has been already applied to determine the internal structure of microparticles[26,42–44] and thereby we applied it in our study to assess the success of microparticle preparation using our synthesized copolymers and our double emulsion–solvent evaporation method.

Figure 6 shows that microparticles are composed of homogenously distributed fluorescent aqueous vesicles in the polymer matrix where the hydrophilic molecule is dissolved in their internal aqueous medium.

According to confocal laser scanning microscopy images we suppose that Ara-C would be homogenously distributed in the internal aqueous vesicles in the microparticles, thus demonstrating the ability of the W/O/W technique to provide an adequate entrapment of hydrophilic drugs in PCL/mPEG–PCL microparticles.

Residual solvent measurement by gas chromatography

Solvents commonly used in microencapsulation, such as DCM, may be retained in microparticles as residual

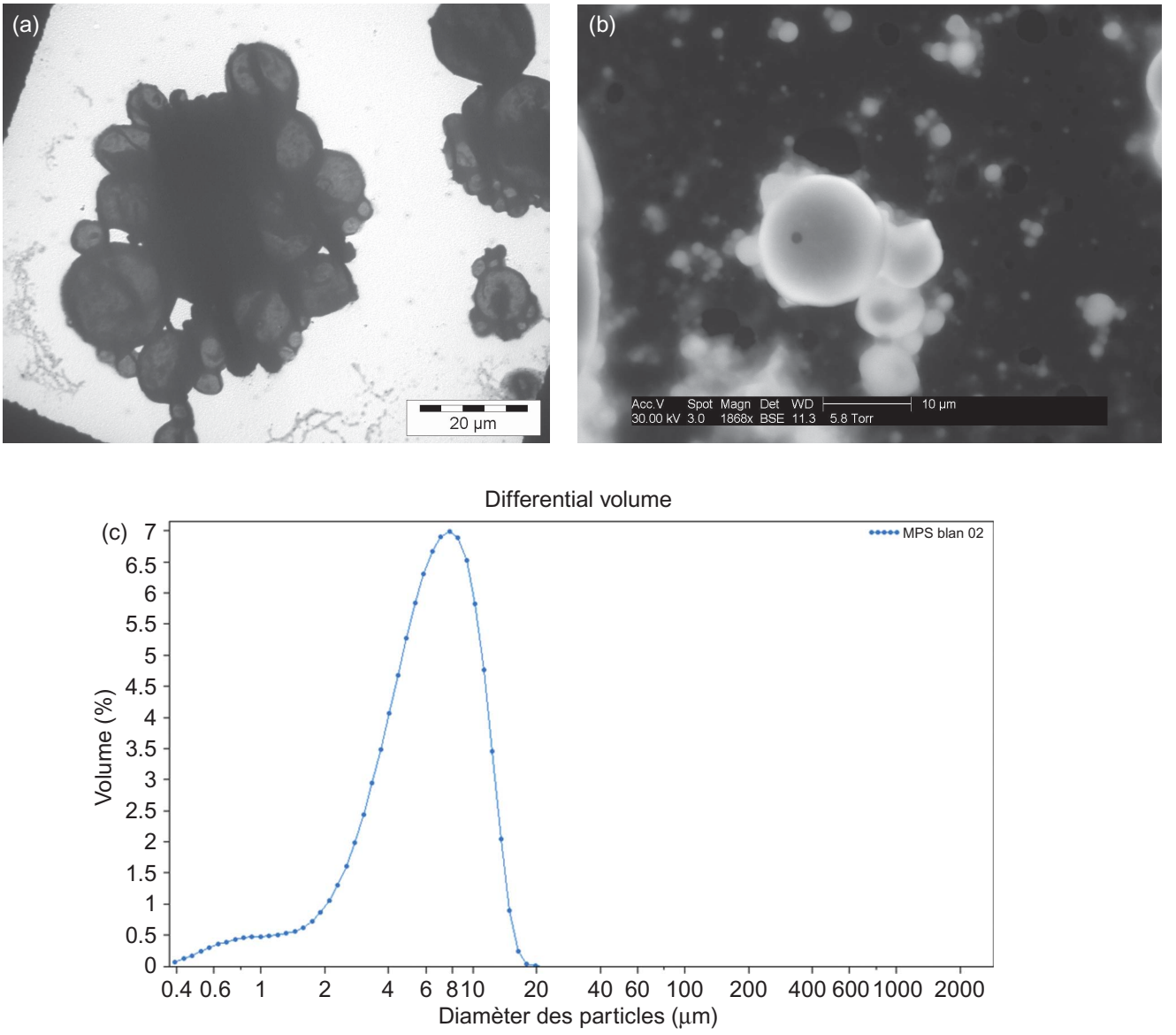

Figure 5. Morphological characteristics of the MP5 microparticles (Ara-C MPs prepared using CP5 as stabilizer), as observed by TEM imaging (a), by Wet-STEM technique (b), and the distribution of microparticle sizes (c) as determined by laser diffraction technique.

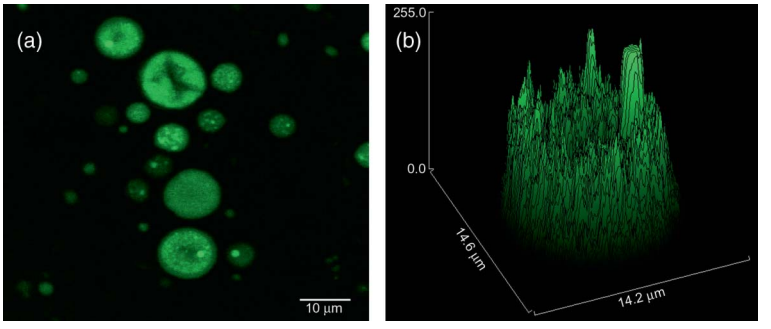

Figure 6. Distribution of FD40 in MP5 microparticles: (a) overview, (b) FD40 distribution with surface intensity plot.

organic volatile impurities. Because of the toxicological risks associated with such substances, the USP XXIII has outlined limits for these residual impurities, which is of 500 ppm for DCM.

The content of residual DCM inside the microparticles can be analyzed by GC[45–49]. It was found that the content of residual DCM in microparticles (MP5) was less than 5 ppm and well satisfied the USP XXIII regulation recommendations. This result indicates that solvent evaporation and microparticles drying can be efficiently carried out as shown in our study (under vacuum at room temperature).

Differential scanning calorimetry

The DSC technique has been widely carried out to check for the possibility of any interactions between the polymer and the loaded drugs in polymeric micro- and nanoparticulate delivery systems[50–53]. DSC analysis informs about the physical status of drugs within the polymeric matrix, which can emerge from crystalline to amorphous form[51] or molecularly dispersed and dissolved in the polymer,[54,55] influencing by consequence the relevant in vitro release properties[49,51].

The physical status of pure microparticle components and their physical mixture was determined before the microparticle preparation. Then blank microparticles and the optimized Ara-C-MPs (MP5) were also characterized with DSC (Figure 7). The pure component thermograms showed a sharp endothermic peak at 220°C and a melting peak (T_m) at 67°C, belonging to Ara-C and polymeric components (PCL and mPEG–PCL), respectively.

The DSC thermograms of the blank and drug loaded-microparticles were identical, with an endothermic peak at 58°C corresponding to the PCL T_m. Obviously, the melting point values of PCL in blank and drug-loaded microparticles were shifted to lower values after microparticle preparation. The thermal behavior of the polymer could be altered after microparticle preparation by emulsification/solvent evaporation process, as previously reported[55]. Changes of the polymer status occurred, probably because the emulsion/evaporation method causes the polymer precipitation from the previously dissolved status in DCM during microparticle formation.

Furthermore, on the drug-loaded microparticle thermogram, the endothermic peak of Ara-C has disappeared, suggesting the presence of the drug in an amorphous form.

To investigate the sensitivity of this technique to detect the drug in the concentration that was used in formula MP5, a thermogram for a physical mixture of PCL, mPEG–PCL, and Ara-C (at a ratio of 2.5/5/1, w/w, respectively) was recorded. It was found that the T_m values of the physical mixture were similar to those of the

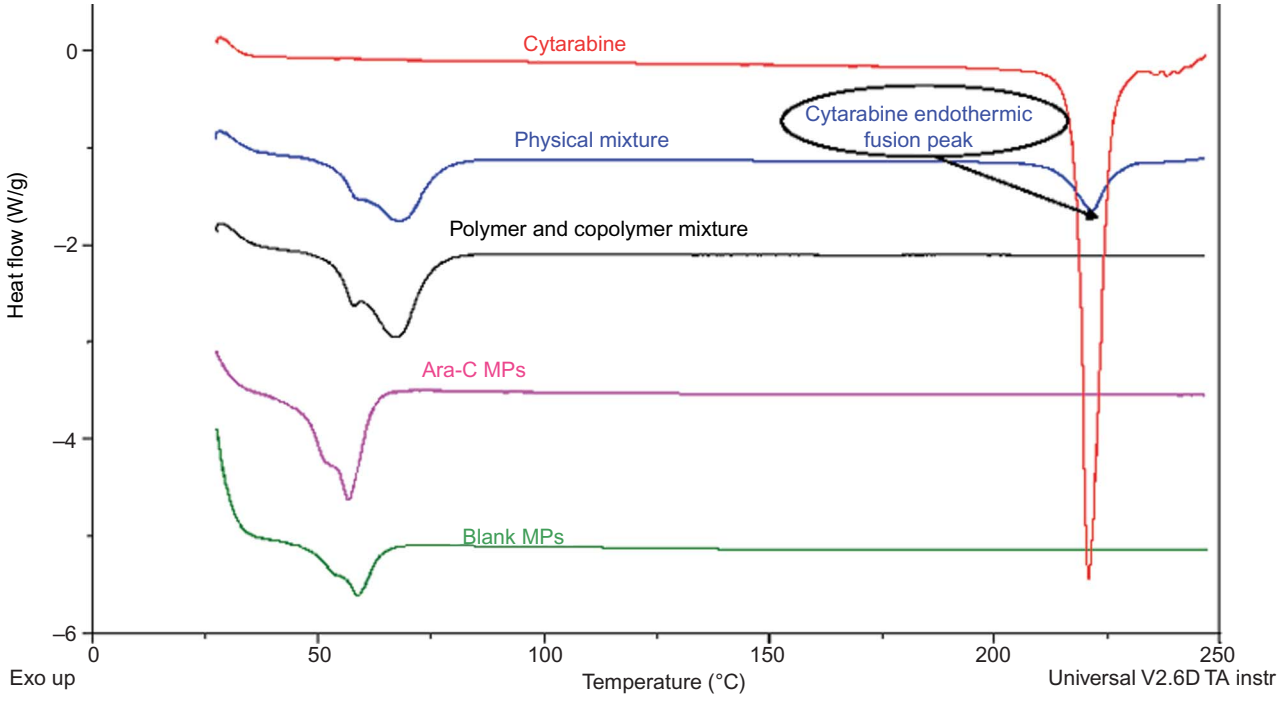

Figure 7. DSC thermograms for (from top to bottom): pure cytarabine, physical mixture of polymeric components with cytarabine, microparticle polymeric components, blank microparticles, and cytarabine-loaded microparticles (MP5).

raw materials, with a clearly observed peak for Ara-C, indicating that the Ara-C is detectable by DSC at the concentration used in microparticle preparation and the absence of its endothermic peak is actually related to the encapsulation in the polymeric matrix.

In vitro drug release study

Figure 8 shows the in vitro release profile of Ara-C MPs (MP5 formulation, with the higher EE), in pH 7.4 phosphate buffer, by representing the percentage of released Ara-C with respect to the amount of encapsulated Ara-C. The saturation concentration of Ara-C in pH 7.4 phosphate buffer is 150 mg/mL,[56] which definitely demonstrates that the sink conditions (defined as 30% of the concentration saturation) were well maintained during the whole dissolution experiment.

The release kinetics of the drug from MP5 microparticles showed a hyperbolic profile (Figure 8). A burst effect was observed in the first hour when 45% of the drug was released. We assume that this portion of drug was deposited at the regions near the PEG shell and could get access to aqueous medium without the need of long-time diffusion. Besides, the hydrophilicity of the PEG copolymers provides water uptake inside the particles[57,58] and facilitates the diffusion of the drug close to the particle surface to the release medium. The maximum drug release had taken place after 6 hours, when 81% of Ara-C was released. Afterwards, the release rate of Ara-C became steady.

Different formulations have been designed to control the release of hydrophilic molecules. Irrespective of the formulation an initial burst release profile was observed followed by a low-rate release profile. The main factors that generally influence the drug release are the polymer concentration[26], the hydrophilicity of the polymeric matrix[59], the cross-linking density of hydrophilic polymers[60], particle size, surface porosity,[61] and the preparation method[44,62].

Conclusion

In this work a series of mPEG–PCL diblock copolymers with different PCL chain lengths have been successfully synthesized to be used as surfactant agents for the Ara-C microencapsulation. This was confirmed by Fourier transform infrared spectroscopy and ^{1}H NMR data. The diblock copolymer molecular weights have been calculated by the integration of the characteristic resonance peaks in the ^{1}H NMR spectra of the synthesized copolymers. And then the calculated molecular weights of CP5

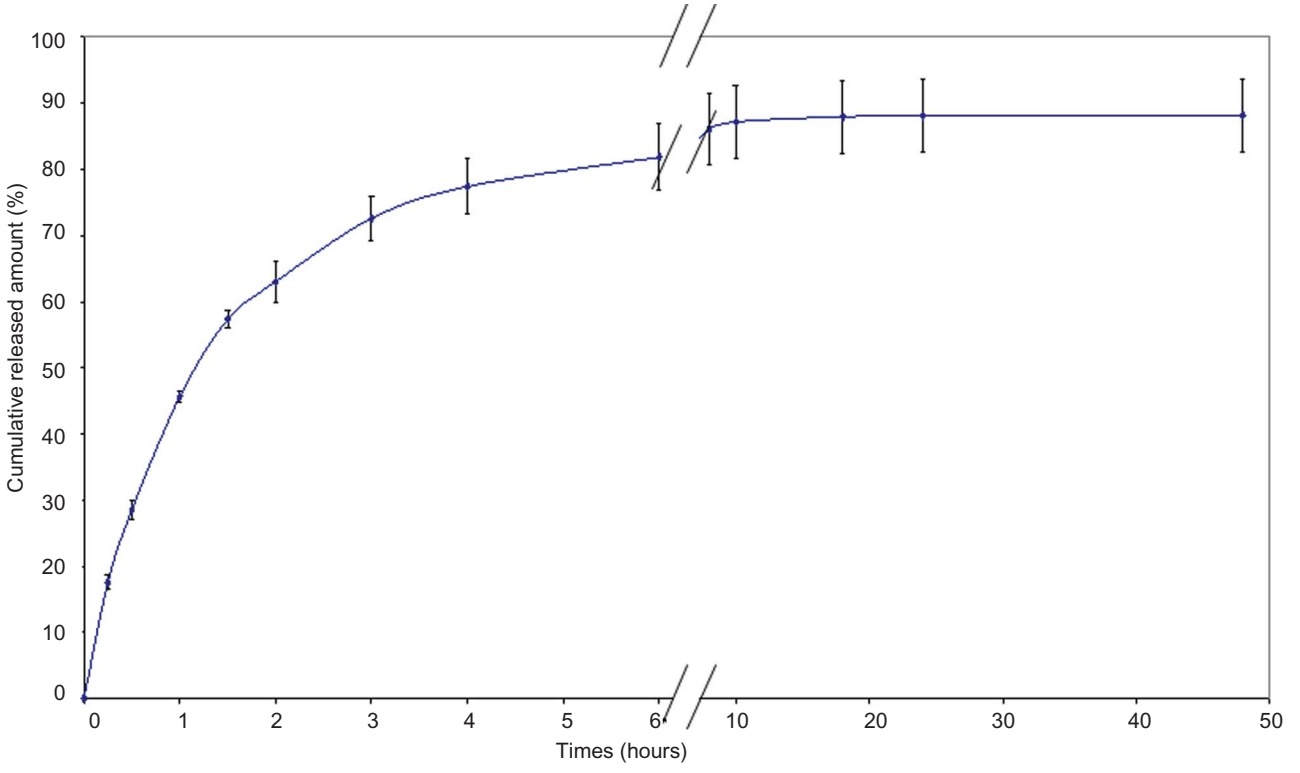

Figure 8. Cumulative percentage amount of released cytarabine from MP5 formulation in PBS (pH 7.4) at 37°C. The graph represents the mean ± SD, and each group was composed of three sets ($n = 3$).

and CP6 were verified using the SEC–MALLS/RI technique being applicable only to the polymers of relatively high molecular weight. The synthesized diblock copolymers showed narrow molecular weight distributions according to the SEC analysis.

It is found that the composition of the amphiphilic diblock copolymers, precisely the PCL block length, is influential on the particle size, Ara-C encapsulation efficiencies as well as zeta potential values of the obtained microparticles. An increase in microparticle size was observed for all the formulations prepared using the synthesized copolymers when compared to PCL microparticle size. Besides, the microparticle size increased with the increase of PCL chain length of the used copolymer. These results confirm our hypothesis about the contribution of copolymers to the microparticle polymer matrix formation. Also, an increase in the Ara-C EE was noticed, with the increase of the PCL chain length of the copolymer used in the formulation. For instance, the use of CP5 yielded the highest EE of Ara-C, increasing the latter to about 10-fold compared to PCL microparticles prepared without the copolymer.

The microparticles prepared using the CP5 showed a hyperbolic release profile of Ara-C with an initial burst release that could be attributed to the drug deposited at the region near the PEG shell. Therefore, we conclude that the use of amphiphilic diblock copolymers as surfactant agents in the encapsulation of hydrophilic molecules can largely improve the EE while keeping the known biphasic drug release profile.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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