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Self-assembled micelles of well-defined pentaerythritol-centered amphiphilic A_4B_8 star-block copolymers based on PCL and PEG for hydrophobic drug delivery

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ABSTRACT

Biodegradable star-shaped poly(ε-caprolactone) (PCL) with four arms were synthesized by ring-opening polymerization (ROP) from a symmetric pentaerythritol core via the "core-first" strategy. Subsequently, two samples of the amphiphilic A_4B_8 star-block copolymers with symmetrical topologies [4s(PCL-b-2sPEG)] were synthesized by a macromolecular coupling reaction between carboxyl-terminated poly (ethylene glycol) (PEG) and 4-arm star-shaped PCL macromers with eight –OH end groups. The latter was prepared by attaching 3-hydroxy-2-(hydroxymethyl)-2-methylpropanoic acid (HHMPA) to 4sPCL using a simple two-step reaction sequence. The in vitro cytotoxicity test indicated no apparent cytotoxicity. The amphiphilic star-block copolymers are capable of self-assembling into spherical micelles in water at room temperature, and they possess low critical micelle concentrations (CMCs) of 2 ~ 8 mg/L in aqueous solution which was determined by fluorescence spectroscopy using pyrene as a probe. Transmission electron microscopy (TEM) measurement demonstrated that the micelles exhibit a spherical shape with a size range of 30 ~ 50 nm in diameter. In addition, the hydrophobic and anticancer drug, quercetin, is loaded effectively in the polymeric micelles, suggesting that these new materials are appropriate candidates as hydrophobic drug nanocarriers.

1. Introduction

Over the past decades, polymeric micelles self-assembled from amphiphilic block copolymers have received significant attention as drug and gene nanocarriers due to their unique characteristics such as core-shell structure, mesoscopic size range and prolonged blood circulation [1–4]. Lipophilic molecules can be incorporated into the hydrophobic core of polymeric micelles by physical entrapment, while the hydrophilic shell composed of flexible polymers provides steric protection and helps these nanoparticles to escape from the reticuloendothelial system (RES) uptake after intravenous administration [5,6]. The PCL is a U.S. Food and Drug Administration (FDA) approved semi-crystalline, hydrophobic and biodegradable polymer with an ester group and five methylene groups in its repeating unit that might be used as a synthetic biomaterial or a controlled drug release matrix due to its good drug permeability, biocompatibility and nontoxicity. The high olefin content imparts polyolefin-like properties to PCL, while hydrolytically labile ester groups make PCL biodegradable under physiological conditions [7]. The PEG is also a FDA approved hydrophilic and nonionic polymer that has been widely used as a hydrophilic polymer in conjunction with hydrophobic block [8] to inhibit the uptake and clearance of colloidal particles by RES and increases the blood circulation time of these carriers. This is due to steric hindrance posed by PEG chains to complement activating system toward the hydrophobic part of the delivery system. Poly(ethylene glycol) is a nonbiodegradable polymer and longer chain length could eventually lead to increased body load of the polymer. Therefore, in place of increasing PEG chain length for increasing the surface coverage, the use of short-chain PEG at higher density on nanomicelles is a preferable strategy. For this objective, PEG is coated physically on the surface of the nanoparticles, or multiple PEG chains are covalently linked to the hydrophobic block of the polymer. The last strategy is apparently more effective whereas physical coating may fail to serve the objective because of the good aqueous solubility of PEG.

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Although micelles self-assembled from PEG and PCL based copolymers have been widely investigated as drug carriers due to their biocompatibility, biodegradability and nonimmunogenicity, most of works were focused on utilizing linear block copolymers [9–18]. Recently several studies have revealed that the architecture of amphiphilic block copolymers has a great influence on the morphology, stability and dimensions of the micelles [19–22] which are very important parameters to determine the drug loading efficiency, releasing behavior, biodistribution and circulation in vivo [23–38].

Star-shaped block polymers are the simplest examples of branched macromolecules with all branches (arms) extending from a single point. Compared to linear polymers, the special unique properties of amphiphilic star-block copolymers originating from their unique shape have been observed in both bulk state and in solution. It has been proved that star-shaped polymers exhibit a smaller hydrodynamic radius, lower solution viscosity and improvement of micelle stability when compared to linear block polymers of the same molecular weight and composition. Moreover, several recent reports also demonstrated that star-shaped polymers have some advantages in drug delivery compared to linear polymers [39–43]. From these reports it is expected that developing new types of star-shaped block copolymers based on PCL and PEG with unique architectures may provide novel insights for fabricating superior drug carriers for clinical applications in drug delivery systems (DDS).

In this study, according to the "arm-first" and "core-first" synthetic methods, a versatile strategy to prepare star-shaped *n* [Poly(ε-caprolactone)-*b*-*m*Poly(ethylene glycol)] ($n = 4$ and $m = 2$) copolymers is successfully introduced, as shown in Scheme 1. In the core-first method, the star-PCL polymer with four arms having terminal primary hydroxyl groups were synthesized by ring-opening polymerization (ROP) from a symmetric pentaerythritol core as a initiating agent. In the arm-first strategy, two samples of the amphiphilic star-block copolymers with symmetrical topologies [4s(PCL-*b*-2sPEG)] and different PCL chain length were synthesized by a macromolecular coupling reaction between carboxyl-terminated PEG and 4-arm star-shaped PCL macromers with eight −OH end groups. The latter was prepared by attaching 2,2-bis(hydroxymethyl)propionic acid (BHPA) (this linker was used to increase PEG density on PCL chain) to 4sPCL using a simple two-step reaction sequence. The star structure of the block copolymers was confirmed by several physicochemical methods. To establish this system as a suitable drug carrier the micellar properties of the star amphiphilic polymer in aqueous media were studied by fluorescence techniques and dynamic light scattering. The anticancer drug quercetin was then chosen as a model lipophilic drug to investigate the drug entrapment efficiency and in vitro drug release profile of drug-loaded 4s(PCL-*b*-2sPEG) micelles.

Quercetin is an anticancer drug and is abundantly found in citrus fruits, vegetables, herbs and related products. However, its administration has been heavily hindered by its extremely poor water-solubility. Many serious problems are associated with the therapeutic use of poorly water-soluble drugs. This includes poor absorption and bioavailability upon oral administration, embolization of blood vessels from intravenous injection of the water-insoluble drug because of drug precipitation, and local tissue toxicity and low systemic drug bioavailability [44].

2. Experimental

2.1. Materials

Monomethoxy poly (ethylene glycol) (MPEG, $M_n = 2000$ g/mol) purchased from Fluka and was dried by azeotropic distillation using anhydrous toluene. Maleic anhydride (MAh) (Aldrich, 98%) was recrystallized from toluene and then dried under vacuum (0.1 mmHg) at room temperature for 24 h ε-Caprolactone (CL) was purchased from Sigma and purified with CaH_2 by vacuum distillation. Pentaerythritol, 3-hydroxy-2-(hydroxymethyl)-2-methylpropanoic acid (HHMPA), diethyl ether, methanol, and dichloromethane were purchased from Merck Chemical Co. Stannous octoate ($Sn(Oct)_2$), 4-(dimethylamino) pyridine (DMAP) and dicyclohexylcarbodiimide (DCC) were purchased from Aldrich and used as received. Quercetin dihydride was obtained from Fluka Chemical Co. All other chemicals were of analytical grade and were used as received. For in vitro cytotoxicity test, amniotic epithelial cells were obtained from elective Cesarean. Dulbecco's Modified Eagle's Medium (DMEM)/F12 and fetal calf serum (FCS) were obtained from GIBCO Invitrogen Corporation. 3-(4,5 Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) and epidermal growth factor (EGF) were from Sigma.

2.2. Synthesis of amphiphilic star-shaped 4s(PCL-b-2sPEG) copolymer

The total synthetic scheme for the star-shaped block polymer is shown in Scheme 1, and the 4s(PCL-*b*-2sPEG) star copolymer was prepared through five steps, which are detailed as following.

2.2.1. Synthesis of 4-arm star-Shaped PCL (4sPCL)

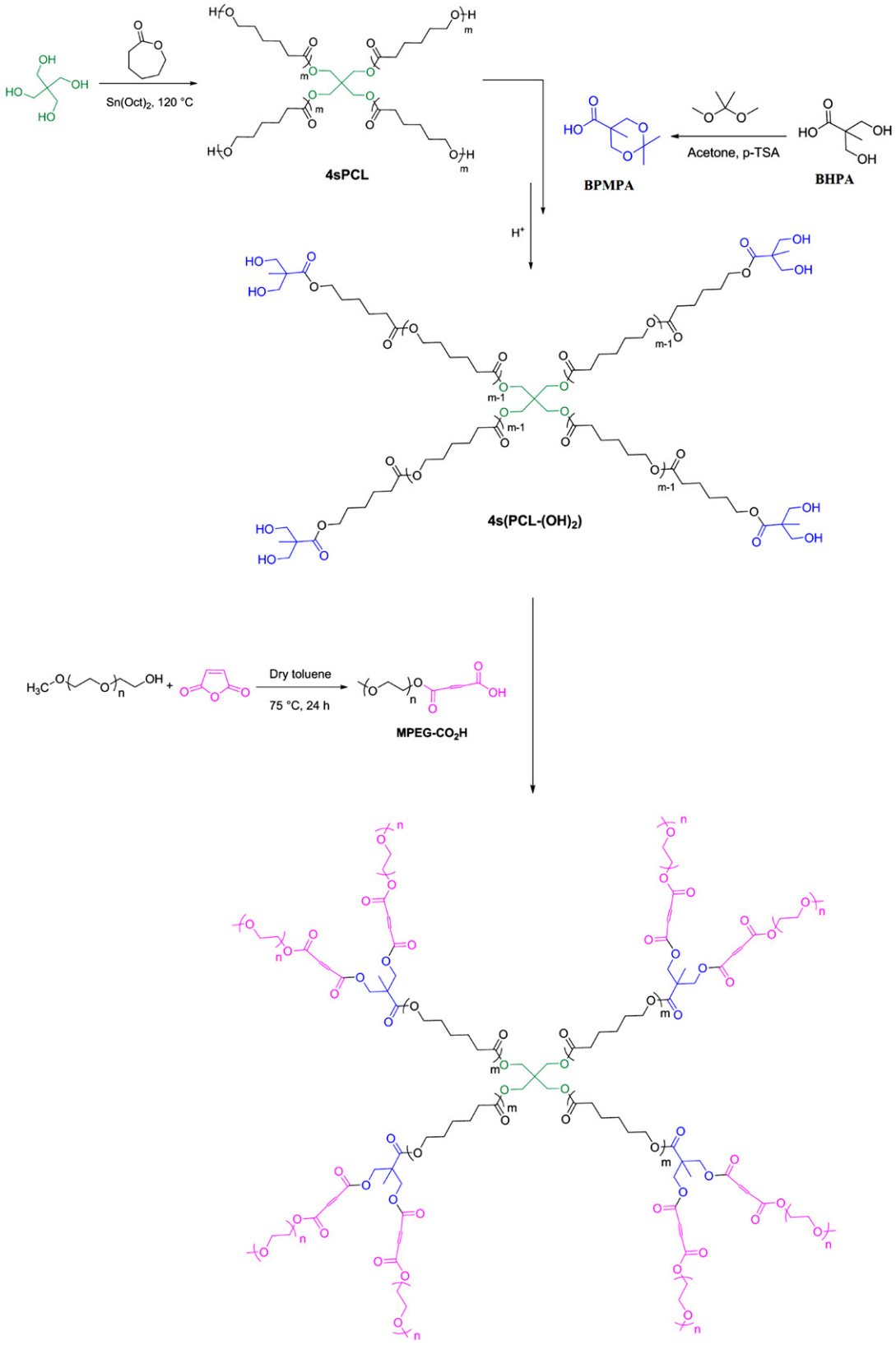
The 4-arm star-Shaped PCL was synthesized by $Sn(Oct)_2$-catalyzed ring-opening polymerization of CL at different monomer/initiator feed ratios. Typical polymerization procedure was as following: A certain amount of pentaerythritol (0.13 g, 1 mmol), $Sn(Oct)_2$ (0.1 wt.% of ε-caprolactone) and ε-caprolactone (9.12 g, 80 mmol) were placed in a three-neck round-bottom flask equipped with a reflux condenser under a nitrogen atmosphere. Then, the reaction vessel was put in oil bath at 120 °C, for 24 h with stirring. After the reaction flask was cooled to room temperature, the resulting product was dissolved in methylene chloride and then poured into excess methanol to precipitate the polymerized product. The 4-arm star-shaped PCL with a hydroxyl group at each chain end was obtained after filtering and drying in a vacuum at room temperature for 48 h. Yield: 8.38 g (90%). Here as shown in Table 1, two products of 4sPCL with different PCL lengths are labeled as 4sPCL-a (monomer to initiator ratio; 80/1) and 4sPCL-b (monomer to initiator ratio; 132/1).

2.2.2. Synthesis of 2,2-Bis[(2,2-propyl)dioxymethyl]propionic acid (BPMPA)

2,2-Bis[(2,2-propyl)dioxymethyl]propionic acid was synthesized through the reaction of HHMPA, 2,2-dimethoxypropane, and p-TSA in acetone [45].

2.2.3. Synthesis of 4-arm star-shaped PCL macromers with eight −OH end groups [4s(PCL-(OH)2)]

In a 50 ml flask with a magnetic stirring bar, 4sPCL ($M_n = 9500$ g/mol, 3.8 g, 0.4 mmol) was dissolved in 10 ml of dry methylene chloride. 4-dimethylaminopyridine (DMAP, 0.29 g, 2.4 mmol) and BPMPA (0.348 g, 2 mmol) were added subsequently. After the flask was cooled to 0 °C, a diluted solution of dicyclohexyl carbodiimide (DCC, 0.41 g, 2 mmol) was added dropwise over 2 h. The reaction mixture was warmed to room temperature and stirred for 24 h. After filtration, the crude product was precipitated into excess methanol and collected by vacuum filtration. After redissolving the solid in CH_2Cl_2, the precipitation cycle was repeated twice. The solid product was dried in a vacuum oven at room temperature for 24 h. Yield: 3.6 g (90%). Then, functionalized 4-arm star-shaped PCL (1 g, 0.1 mmol) was



Scheme 1. Synthesis of pentaerythritol-centered amphiphilic A_4B_8 star-block copolymers based on PCL and PEG.

Table 1
Molecular weight and molecular weight distribution of carboxylated PEG, 4sPCL-a, 4sPCL-b, 4s(PCL-b-2sPEG)-a and 4s(PCL-b-2sPEG)-b

Sample	M_n (g mol^{-1})[a]			M_n (g mol^{-1})[b]	M_w/M_n[b]
	PEG block	PCL block	Total		
Carboxylated PEG	2.2×10^3	–	2.2×10^3	1.6×10^3	1.13
4sPCL-a	–	9.5×10^3	9.5×10^3	$3.8 \times 1\,0^3$	1.15
4sPCL-b	–	15.4×10^3	15.4×10^3	5.5×10^3	1.14
4s(PCL-b-2sPEG)-a	16.7×10^3	9.5×10^3	26.2×10^3	13.9×10^3	1.16
4s(PCL-b-2sPEG)-b	16.7×10^3	15.4×10^3	32.1×10^3	16.3×10^3	1.15

[a] Determined by ^{1}H NMR.
[b] Determined by GPC.

dissolved in a mixture of 25 mL of THF and 10 mL of 0.1 M HCl (aqueous). The reaction mixture was stirred at room temperature for 4 h. Subsequently, the solvent was removed under reduced pressure and the resulting bifunctional 4-arm star-shaped PCL was dissolved in methylene chloride and then poured into excess methanol to precipitate the deprotected product. The 4-arm star-shaped PCL macromer with eight –OH end groups was obtained after filtering and drying in a vacuum at room temperature after 48 h. Yield: 0.96 g (96%).

2.2.4. Synthesis of carboxyl-terminated monomethoxy poly(ethylene glycol) (MPEG-CO_2H)

Carboxyl-terminated MPEG was synthesized as follows: MPEG (5.0 g, $M_n = 2000$ g/mol, 2.5 mmol) was dissolved in dry toluene (25 mL). In order to removing trace water in MPEG azeotropic distillation was carried out at 125 °C. Then, maleic anhydride (2.45 g, 25 mmol) was added. The mixture was stirred at 75 °C for 24 h, and then added into diethyl ether at room temperature to precipitate the reaction product. The precipitate was filtered, and dried at 30 °C in a vacuum oven for 24 h. The carboxyl-terminated poly(ethylene glycol) (MPEG-CO_2H) (4.8 g) was obtained in 96% yield.

2.2.5. The coupling reaction of 4s(PCL-$(OH)_2$) and MPEG-CO_2H

The amphiphilic star-shaped 4s(PCL-b-2sPEG) copolymers were synthesized using a molar feed ratio [4s(PCL-$(OH)_2$) (4.75 g, $M_n = 9500$ g/mol)]/[MPEG-CO_2H (10.56 g, $M_n = 2200$ g/mol)]/[DCC (1.64 g)]/[DMAP (0.97 g)] of 1:8.8:16:16. The calculated amounts of reactants were added into a three-necked round-bottom flask. Then, anhydrous dichloromethane was poured into the mixture under a nitrogen atmosphere. The reaction was performed at room temperature for 24 h under vigorous stirring [33]. After that the mixture was evaporated to dryness, the obtained product was dissolved in distilled water. The solution was put into a dialysis bag (MWCO = 3500) and dialyzed against 2000 mL distilled water, which was renewed every 12 h at room temperature for 5 days before freeze-drying for 48 h.

2.3. Characterization of copolymers and micelles

FT-IR spectra were recorded on a 102 MB BOMEM apparatus. The samples of freeze-dried micelles were pressed into potassium bromide (KBr) pellets. Additionally, other samples for FT-IR measurements were prepared by dissolving them in 1.5 wt% chloroform solution on the surface of a silicon wafer and the solvent was completely evaporated before measurements. ^{1}H NMR spectra were recorded on a BRUKER DRX-300 AVANCE spectrometer at 300.13 MHz using $CDCl_3$ as the solvent. Crystallographic assay measurement was performed on lyophilized micelles using an X-ray powder Diffractometer (XD-3A) with Cu Kα radiation. The gel permeation chromatographic (GPC) system was used to determine the number-average molecular weights (M_n) of 4sPCL and 4s(PCL-*b*-2sPEG) copolymers. The GPC system was equipped with Waters 2690D separations module, Waters 2410 refractive index detector. THF was introduced as the eluent at a flow rate of 0.3 ml/min. Waters millennium module software was used to calculate molecular weight on the basis of a universal calibration curve generated by polystyrene standard with narrow molecular weight distribution. The morphology of the self-assembled micelles was performed on a ZEISS EM-900 transmission electron microscopy operating at an acceleration voltage of 80 kV. TEM sample was prepared by dipping a copper grid with Formvar film into the freshly prepared micelles solution. A few minutes after the deposition, the aqueous solution was blotted away with a strip of filter paper and stained with phosphotungstic acid aqueous solution, then dried in the air. The mean particle size in aqueous solution at room temperature and size distribution of self-assembled micelles were determined by Nano-ZS ZEN3600. Each sample was diluted to appropriate concentration with distilled water. The polymeric solutions (mg/L) were passed through a 0.2 μm pore size filter before measurement. The thermal transitions of the polymers were determined using a Perkin–Elmer DSC7 differential scanning calorimeter under nitrogen purge. The samples were heated at a rate of 10 °C/min.

2.4. Micelle formation

We used dialysis bag method to fabricate the micelles. Briefly, 4s(PCL-*b*-2sPEG) copolymer (10 mg) was dissolved in 3 ml DMF. The solution was put into a dialysis tube and subjected to dialysis against 1000 ml distilled water, which was renewed every 3 h, then lyophilized before further examinations.

2.5. Fluorescence measurements

Fluorescence spectra were recorded on a Varian Cary Eclipse spectrofluorophotometer. Pyrene was used as a hydrophobic fluorescent probe to determine the critical micelle concentration (CMC). Aliquots of pyrene solutions (6×10^{-6} M in acetone, 1 ml) were added to containers, and acetone was allowed to evaporate at room temperature. 10 ml aqueous copolymer solutions at different concentrations were then added to the containers containing the pyrene residue. The solutions were kept at room temperature for 24 h to reach the solubilization equilibrium of pyrene in the aqueous phase. Excitation was carried out at 340 nm, and emission spectra were recorded ranging from 350 to 470 nm. The excitation and emission bandwidths were 10 nm and 5 nm, respectively. A CMC value was determined from the ratios of pyrene intensities at 381 (I_3) and 373 (I_1) nm and calculated from the intersection of two tangent plots of I_3/I_1 versus log concentrations of polymers.

2.6. Cell cultures

Human amniotic membrane was prepared using fresh human placenta as described previously [46]. Briefly, the amnion was mechanically peeled of the chorion and washed several times with PBS. To release amniotic epithelial cells, the amniotic membrane was incubated at 37 °C with 0.15% trypsin-EDTA. Trypsin was inactivated with FCS and the solution centrifuged at 2500 rpm for 12 min. Cells were washed with PBS and cultured in DMED/F12 containing 10% FCS, 10 mg/ml EGF, 2 mM L-glutamine, 1% nonessential amino acid, 55 μM 2-mercaptoethanol, 1 mM sodium pyruvate and 100 U/ml penicillin/streptomycin solution.

2.7. In vitro cytotoxicity studies

Amniotic epithelial cells (AEC) were seeded at density of 7.5×10^4 per well in gelatin-coated 24-well plate and incubated overnight in incubator (37 °C, 5% CO_2). The next day, AEC was treated with 4s(PCL-*b*-2sPEG)-a copolymer with concentration 1 mg/ml and incubated at 37 °C for 24 h. The cells without treatment were used as control group. For cytotoxicity assay, 250 μL of MTT solution (5 mg/mL) was added to each cell and the plates were incubated for 4 h. The MTT formazan crystals were then dissolved with 1 ml DMSO at room temperature. The optical density (OD) was measured at 570 nm with a spectrophotometer (CE7500, Cecil, UK). The blank well containing only medium material was used for zero adjustment. The viable rate was calculated by the following equation:

$$\text{Viable rate} = (\text{OD}_{\text{treated}}/\text{OD}_{\text{control}}) \times 100\%$$

Where $\text{OD}_{\text{control}}$ was obtained in the absence of polymers and $\text{OD}_{\text{treated}}$ was obtained in the presence of polymers.

2.8. General procedure for the preparation of drug-loaded micelles

4s(PCL-*b*-2sPEG)-a (10 mg) and quercetin (0.5 mg) were dissolved in 3 mL DMF, then the solution was put into a dialysis tube and subjected to dialysis against 1000 ml distilled water. The whole dialysis process lasted for 24 h and the water was renewed every 2 h during the initial 12 h to remove the unloaded drug. To determine the entrapment efficiency (EE), the drug-loaded micelle solution was lyophilized, and then dissolved in DMF and analyzed by UV absorbance at 373 nm, using a standard calibration curve experimentally obtained with quercetin/DMF solutions. The EE was calculated based on the following formula:

$$\text{EE(wt.\%)} = (\text{Weight of the drug in micelles}/\text{Weight of the feeding drugs}) \times 100$$

2.9. In vitro drug release

The drug-loaded micelle solution (2 mL, [Quercetin] = 0.008–0.011 g/L) was placed in a dialysis tube (MWCO = 12 kDa). The dialysis tube was sealed and immersed in 30 mL of phosphate-buffered saline (PBS) (pH 7.4). In vitro drug release study of drug-loaded micelle was carried out in a shaking water bath at 25 °C. A 3 mL aliquot of solution was taken out and the same volume of PBS solution was added after each sampling at predetermined time intervals. Also, as a control experiment, quercetin solution was used with 0.01 g/L in a solvent mixture of ethanol-water-PEG400 (4:3:3 v/v). The concentration of the drug in the release samples was determined by UV spectra.

3. Results and discussion

3.1. Synthesis and characterization of the star-shaped poly (ε-caprolactone)

The star-shaped poly(ε-caprolactone) homopolymer was synthesized by ring-opening polymerization of CL at 120 °C. The pentaerythritol with four hydroxyl groups was chose as the initiator in order to produce the hydroxyl-terminated star-shaped PCL. By changing the feed ratio of monomer to initiator, two star-shaped polymers of 4sPCL-a and 4sPCL-b with different lengths of PCL chain were easily obtained.The IR spectrum of the 4sPCL is shown in Fig. 1A. The IR spectrum of the star-shaped PCL had a band characteristic for the ester carbonyl at 1727 cm^{-1} and two bands for hydroxyl at 3540 and 3439 cm^{-1}. These bands could be associated to different hydrogen bonding interactions. There is only one hydroxyl group per PCL chain so the intensity of the hydroxyl band was low; reflecting the low content of –OH in the sample. ^{1}H NMR spectrum of the star-shaped PCL is shown in Fig. 2A. The typical signal of the methylene (a) protons of the initiator pentaerythritol was clearly detected at 4.37 ppm. The major resonance peaks (b–e) were attributed to PCL. The peak of the methylene (e) protons was detected at 4.05 ppm when the peak of the protons of the terminal methylene (e') was observed at 3.65 ppm indicating that PCL is terminated by hydroxyl groups. The average degrees of polymerization for the PCL arms were calculated from the integration ratio between the methylene protons in the repeat units (e) and those in the terminal unit (e') based on ^{1}H NMR spectrum. The molecular weights of 4sPCL-a and 4sPCL-b was determined by ^{1}H NMR spectrum and GPC analysis were listed in Table 1.

3.2. Synthesis and characterization of the 4-arm star-shaped PCL macromers with eight –OH end groups

The star-shaped 4s(PCL-$(OH)_2$) macromer was obtained by the reaction of 4sPCL with the BPMPA. The molecular weight of 4s(PCL-$(OH)_2$) increased slightly and the molecular weight distribution was similar to that of 4sPCL. The IR spectrum of the star-shaped 4s(PCL-$(OH)_2$) macromer (Fig. 1B) had a band characteristic for the ester carbonyl at 1728 cm^{-1} and two bands for hydroxyl at 3544 and 3441 cm^{-1} ^{1}H NMR spectrum was shown in Fig. 2B. The peak assigned to the methylene protons of the PCL block at 3.65 ppm disappeared, while novel signals corresponding to methyl (g) protons and ester methylene (f) protons of terminal BHPAs appeared at 1.16 and 3.72 ppm, indicating complete reaction of all the terminal hydroxyl groups.

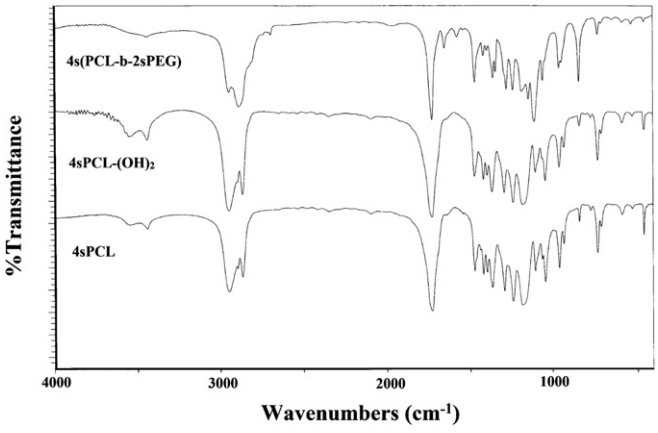


Fig. 1. FT-IR spectra of 4sPCL, 4s(PCL-$(OH)_2$), and 4s(PCL-b-2sPEG).

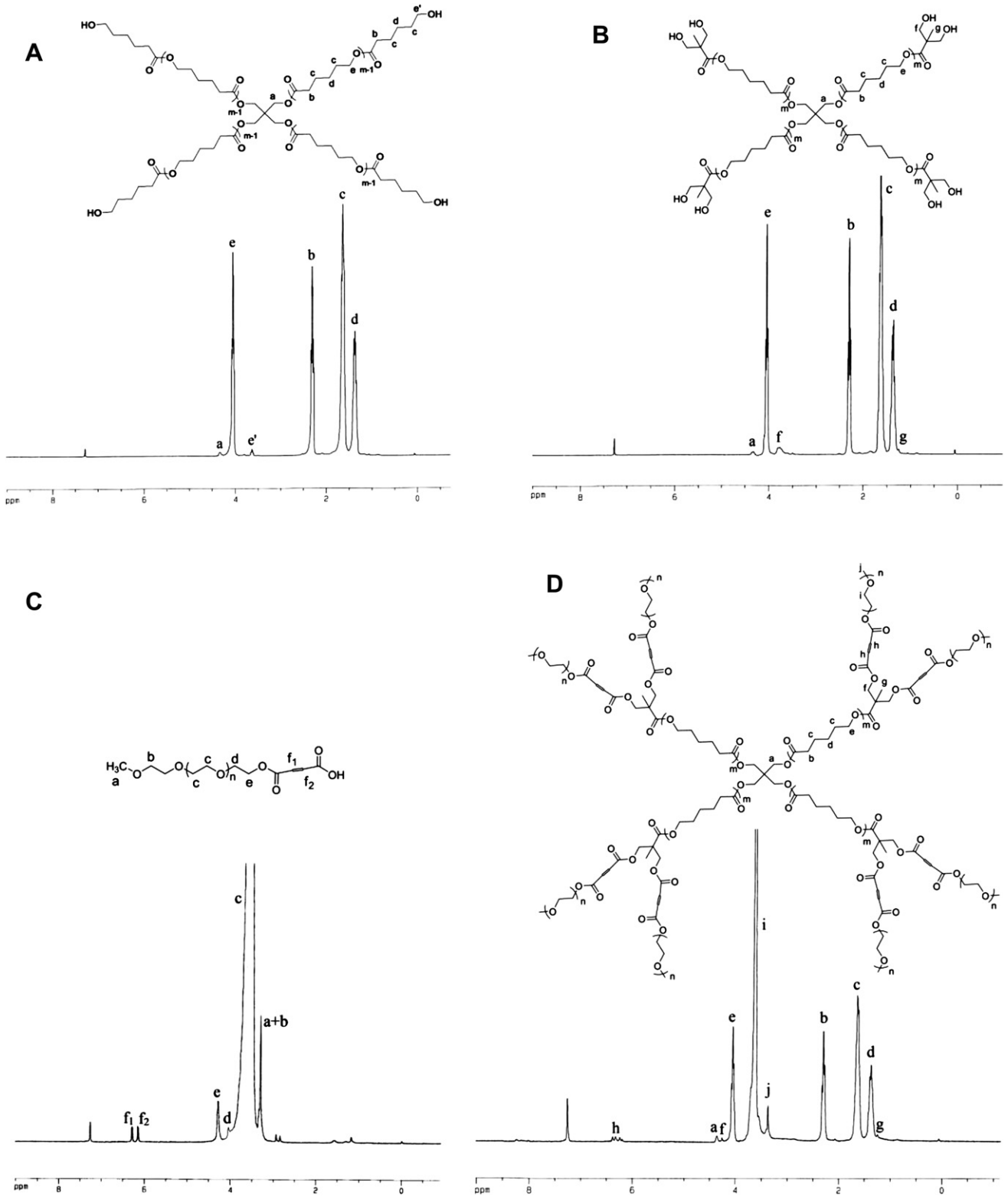


Fig. 2. Chemical structures and ^{1}H NMR spectra of copolymers: (A) 4sPCL, 4s(PCL-$(OH)_2$) (B), carboxylated PEG (C), 4s(PCL-b-2sPEG) (D).

3.3. Synthesis of carboxyl-terminated PEG

Carboxyl-terminated monomethoxy poly(ethylene glycol) (MPEG-CO_2H) was prepared from the reaction of MPEG-OH with maleic anhydride. Fig. 2C exhibits the ^{1}H NMR spectrum of MPEG-CO_2H. Two signals at 6.18 (f_1) and 6.37 ppm (f_2) are assigned to vinyl protons in the terminal group; the signals at 3.65 (c) and 4.30 ppm (e) belong to ether and ester methylene protons, respectively; terminal methyl and methylene (CH_3OCH_2) peaks (a + b) appear at 3.32–3.40 ppm. The reaction extent is estimated by the integration

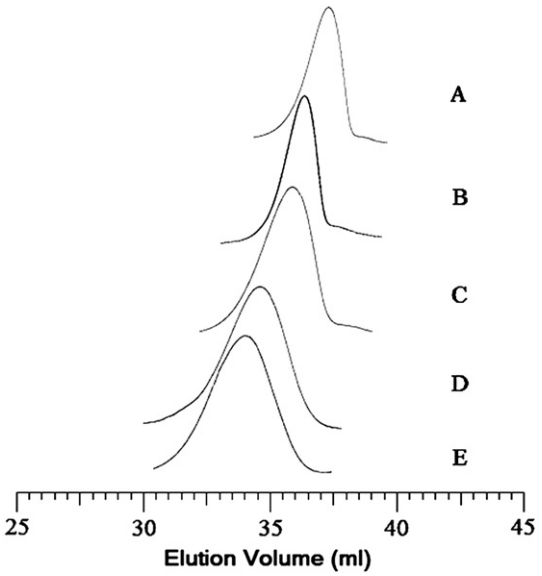


Fig. 3. GPC traces of polymers: (A) carboxylated PEG, (B) 4sPCL-a, (C) 4sPCL-b, (D) 4s(PCL-b-2sPEG)-a, (E) 4s(PCL-b-2sPEG)-b.

ratio of the peaks f_1 and f_2 to the peak a + b, the value is close to 2:5, indicating almost quantitative reaction of hydroxyl group of MPEG with MAh. The MAh-terminated MPEG was successfully prepared.

3.4. Synthesis and characterization of the star-shaped 4s(PCL-b-2sPEG) copolymers

Finally, the pre-synthesized 4-arm star-shaped PCL macromer [4s(PCL-$(OH)_2$] was coupled with the carboxylated PEG to yield star-shaped block copolymers of 4s(PCL-b-2sPEG)-a and 4s(PCL-b-2sPEG)-b with different lengths of PCL chain. In the IR spectrum of star 4s(PCL-b-2sPEG) copolymer (Fig. 1C), the band belonging to the hydroxyl end groups of the 4-arm star-shaped PCL macromer [4s(PCL-$(OH)_2$] at 3544 to 3441 cm^{-1} disappeared, confirming the conjugation of 4-arm star-shaped PCL macromer [4s(PCL-$(OH)_2$] and PEG blocks. A stronger band at 1112 cm^{-1} for C—O—C appeared, consistent with the addition of PEG ether units. The ^{1}H NMR spectrum of 4s(PCL-b-2sPEG) copolymer is shown in Fig. 2D. In ^{1}H NMR, peaks at 1.37, 1.62, 2.28, and 4.03 ppm are assigned to methylene (b-e) protons of $-(CH_2)_3-$, $-CH_2COO-$, and $-OCCH_2-$ in PCL units respectively. The sharp peak at 3.65 ppm is attributed to methylene (i) protons of $-CH_2CH_2O-$ in MPEG units in block copolymer. The very weak peaks at 1.18 and 4.17 are assigned to the methyl (g) and ester methylene (f) protons in BHPA units. The peak at 3.38 is attributed to the methyl (j) protons of $-CH_3$ in MPEG units. Two signals at 6.2 and 6.4 ppm are assigned to vinyl (h) protons in the maleic anhydride units and the signal at 4.37 ppm belongs to ester methylene (a) protons in pentaerythritol core. All of these peaks indicate that the 4s(PCL-b-2sPEG) copolymer was successfully synthesized.

Fig. 2D shows that the proton resonance of vinyl group between the two ester groups is clearly observed at 6.2 and 6.4 ppm, indicating that carboxylated PEGs are successfully coupled with the 4-arm star-shaped PCL. Since the proton resonance of the pentaerythritol moiety is observed at 4.37 ppm, the degree of coupling between carboxylated PEG and 4-arm PCL can be estimated from the ratio of the peak area at 6.2 and 6.4 ppm to the peak area at 4.37 ppm; the degree of coupling for our system is estimated to be 95%. All the GPC traces of carboxylated PEG, 4sPCL and 4s(PCL-b-2sPEG) copolymer exhibit monomodal molecular weight distributions, as shown in Fig. 3. Therefore, both the results of ^{1}H NMR and GPC confirm the successful synthesis of the star-shaped copolymer. The molecular weights and molecular weight distributions of carboxylated PEG, 4-arm star-shaped PCL and star-shaped copolymers are listed in Table 1. It has been demonstrated that star-shaped polymers exhibit a smaller hydrodynamic radius when compared to linear polymers of the same molecular weight and composition. So, these results (Table 1) are predictable: the star copolymers compared to linear polymers of the same molecular weight can be detected in higher elution time. This lead to smaller M_n compared to actual M_n or M_n (^{1}H NMR).

3.5. Self-assembly amphiphilic star-shaped copolymers [4s(PCL-b-2sPEG)]

The amphiphilic star-block copolymer self-assembled into micelles in aqueous solution using the dialysis method. The critical micelle concentrations (CMC) of these star-shaped block copolymers were analyzed by fluorescence spectra using pyrene as a hydrophobic probe. Pyrene, initially dissolved in water, undergoes partition in the hydrophobic core of forming micelles, where it is more soluble. The variation of microenvironmental polarity results in a red shift of pyrene excitation maximum. The excitation spectra of pyrene at different copolymer concentrations in water were acquired and the intensities of absorptions at 381 (I_3) and 373 (I_1) nm are plotted as I_3/I_1 ratio versus polymer concentration in solution (Fig. 4). It was found that the ratio is almost constant at relatively low concentrations. After the concentration of the copolymer got to a critical value, ratio starts to enhance dramatically with the increasing concentration, then the creation of micelles as well as

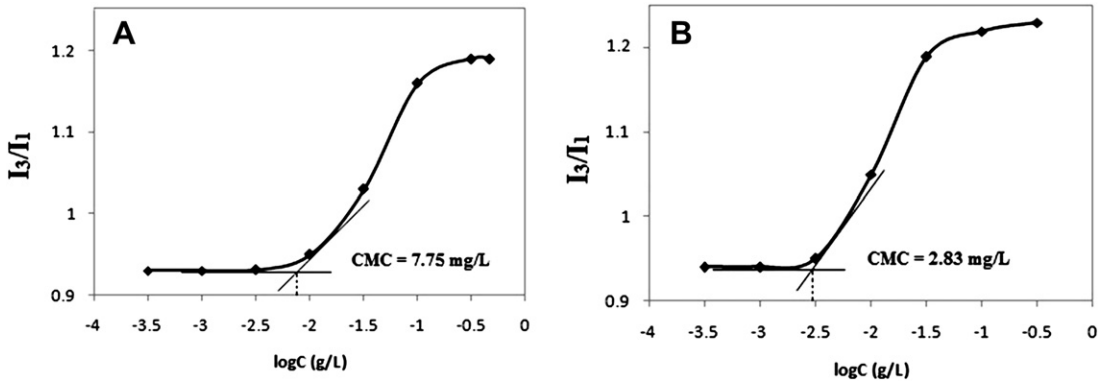


Fig. 4. I_3/I_1 plotted as a function of polymer concentrations of 4s(PCL-b-2sPEG)-a (A) and 4s(PCL-b-2sPEG)-b (B).

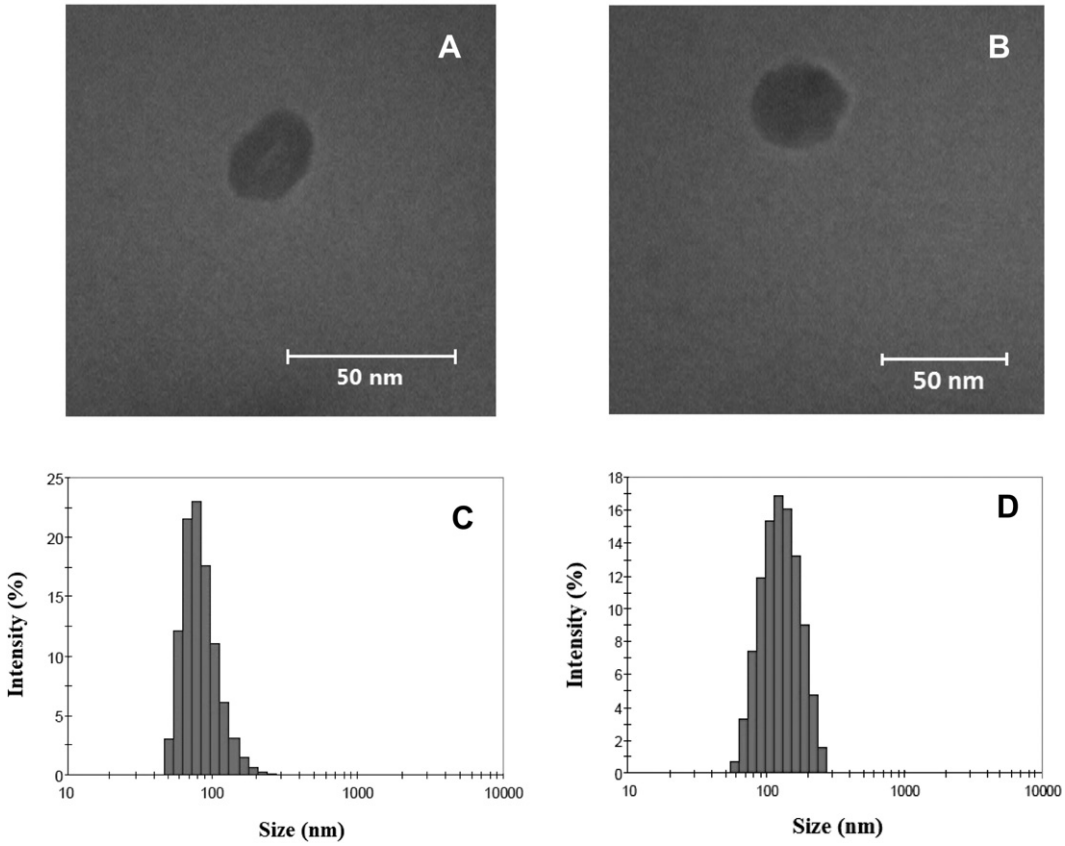


Fig. 5. TEM micrography of micelles from 4s(PCL-b-2sPEG)-a (A), 4s(PCL-b-2sPEG)-b (B) and size distribution detected by DLS of the micelles of 4s(PCL-b-2sPEG)-a (C), 4s(PCL-b-2sPEG)-b (D) in distilled water.

the simultaneous transfer of pyrene into the hydrophobic micellar core are initiated. Thus, the transition concentration was defined as the CMC of 7.75 and 2.83 mg/L for 4s(PCL-b-2sPEG)-a and 4s(PCL-b-2sPEG)-b, respectively. The difference of the CMC values is probably due to the relatively stronger hydrophobic interactions or decreased solvency resulting from the more hydrophobic core in the presence of longer PCL chains. Thus the CMC value exhibits a decreasing tendency with the increased length of PCL chain and might be tuned accordingly to a certain extent. Micelles with small CMC values are expected to be more stable against dilution after the IV injections [47].

To understand the micellization behavior of the obtained star-block copolymers, the HLB values were estimated based on composition of the copolymers according to equation [48,49]:

$$\mathrm{HLB} = [W_H/(W_H + W_L)] \times 20$$

Here, W_H and W_L are the weight fraction of the hydrophilic (PEG) and hydrophobic (PCL) segment, respectively. The HLB values of the copolymers are 12.7 and 10.4 for 4s(PCL-b-2sPEG)-a and 4s(PCL-b-2sPEG)-b, respectively. These results demonstrate that the HLB values decrease as the length of PCL chain increases, with similar trends observed for the CMC values and this partly sustains the conclusions concerning the CMC result.

To further evaluate the properties of micelles, both the morphology and the mean size of these 4s(PCL-b-2sPEG) micelles were studied by the TEM measurement and dynamic light scattering (DLS). It is seen from TEM pictures (Fig. 5A and B) that the self-assembled micelles are well dispersed as individual nanoparticles with regularly spherical shapes confirming the micellization. Furthermore, the diameters of the nanomicelles are around 30–50 nm. The information is more likely to support the opinion that the micellization occur as a result of molecular association rather than aggregation of smaller micelles. The PDI of corresponding micelles, very low about 0.135 and 0.092 determined by the DLS, also reinforced this opinion though the micelles exhibit larger average diameter of 78 nm and 122 nm, respectively, determined by the DLS (Fig. 5C and D). This difference in micelles size measured by TEM and DLS should be attributed to that the latter is the hydrodynamic diameter of micelles in water, whereas the former reveals the morphology size of the micelles in solid state.

3.6. DSC characterization

The thermal behavior of the synthesized star-block copolymers as well as 4sPCL-b and PEG-COOH samples was investigated using differential scanning calorimetry (DSC). Results are summarized in Table 2. Peak attribution was made taking into account the T_m of precursor PEG and PCL blocks. The melting enthalpy of PCL blocks

Table 2
DSC result of polymers.

Samples	T_m (°C)		ΔH_m (J/g)	
	PEG	PCL	PEG	PCL
PEG-COOH	57	–	164	–
4sPCL-b	–	55.7	–	80
4s(PCL-b-2sPEG)-a	55.5	62	99	81
4s(PCL-b-2sPEG)-b	55	61	94	80

Table 3
Properties of blank and quercetin-loaded polymeric micelles.

Samples	CMC[a] (mg/L)	DL[b] (%)	EE[c] (%)	Quercetin St[d] (μg/ml)
4s(PCL-b-2sPEG)-a	7.75	3.75	75	750
4s(PCL-b-2sPEG)-b	2.83	5	80	800

[a] Critical micelle concentration of the micelles in water.
[b] Percent drug loading = weight of quercetin in micelles × 100/weight of micelles tested.
[c] Percent entrapment efficiency = weight of quercetin in micelles × 100/weight of quercetin used in micelles preparation.
[d] Quercetin solubility in water.

in copolymers was almost equal to that of parent macromers, thus suggesting that PCL crystallization is difficulty influenced by the presence of PEG blocks. A decrease in ΔH_m for the PEG component in star copolymers was observed compared with PEG-COOH. This result is due to the crystallization of PCL blocks which is related to the higher molecular weight of PCL blocks ($M_n = 2.3-3.8$ kDa) and therefore disturbing the organization of PEG blocks ($M_n = 2.0$ kDa). As micelle stability is related also to the physical form of the core-forming blocks, development of PCL crystallinity in the micelle core contributes to improve their kinetic stability.

3.7. Drug solubilization in aqueous solutions

One of the main aims of encapsulating quercetin into 4s(PCL-b-2sPEG) micelles was to increase its aqueous solubility. Quercetin is a lipophilic molecule with very low aqueous solubility, <10 μg/mL [50]. Low bioavailability, low drug efficacy and limited treatment options of poorly soluble drugs are common problems in drug development and can have negative consequences for patients [51,52]. Table 3 presents quercetin aqueous solubility up to 800 μg/mL observed for 4s(PCL-b-2sPEG) micelles at polymer concentration of 10 g/L; this corresponds to ~80 folds increase in quercetin

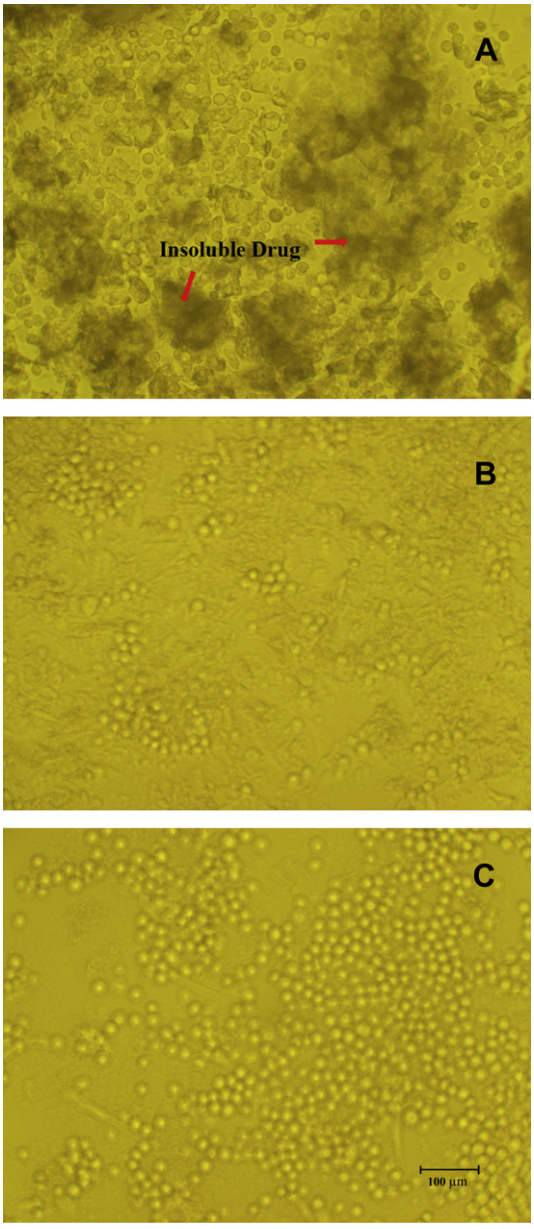


Fig. 6. Light microscopic images of quercetin (A), empty micelles (B) and quercetin-loaded micelles (C) added into cell culture medium.

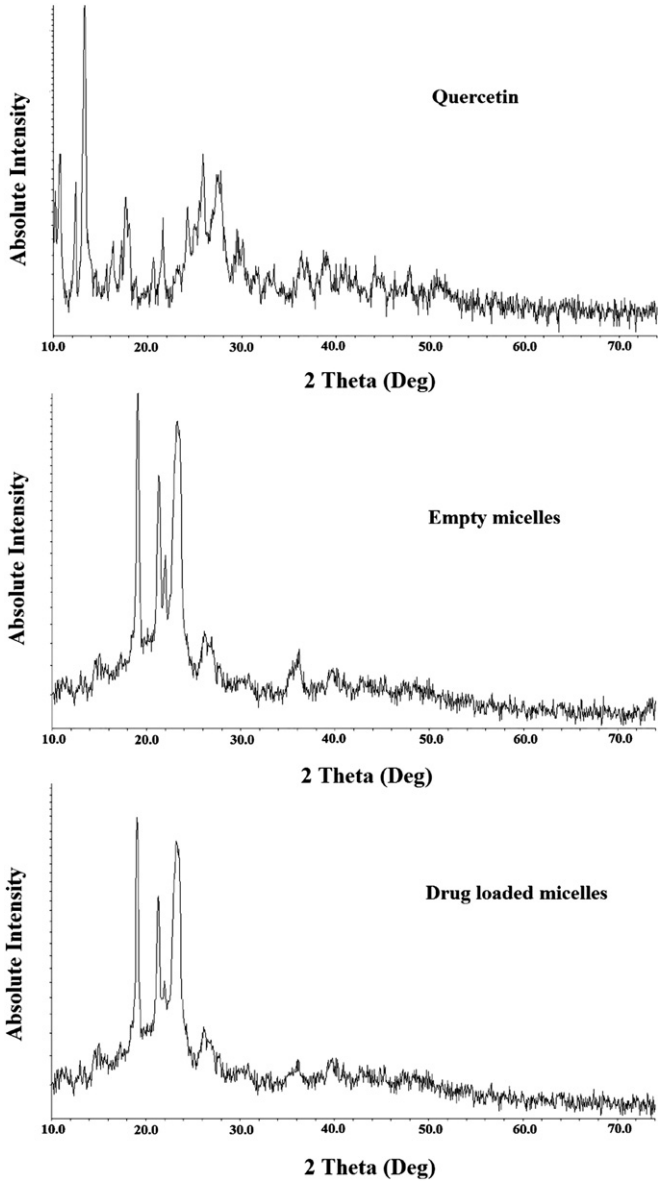


Fig. 7. X-ray diffraction spectra of quercetin crystal, empty micelles and drug-loaded micelles.

aqueous solubility. Also, we provide light microscopic images of quercetin, empty micelles, and quercetin-loaded micelles added to cell culture medium. As shown in Fig. 6A–C, the culture mediums contain empty micelles and drug-loaded micelles which are quite soluble whereas, quercetin in culture medium is partially insoluble; these are in good agreement with above mentioned results.

3.8. Drug loading and encapsulation efficiency

The anticancer drug, quercetin-loaded micelles were prepared by the dialysis method. The mood of the entrapped drug in the nanomicelles matrix is one of the main factors in the release profile of drug delivery systems. Fig. 7 shows the X-ray diffraction scans of the pure quercetin, drug free micelles and drug-loaded core-shell type micelles. It is observed that there is no diffraction peak of the pure quercetin in the drug-loaded micelles and the diffraction peaks of quercetin-loaded micelles are similar to those of quercetin-free micelles. This means that no drug crystal is visualized even on the surface of the nanoparticles demonstrating that the quercetin is dispersed molecularly in the core of nanoparticles. Indeed, similar results were reported for other drug-loaded polymeric micelles [53–55].

The drug loading contents of 4s(PCL-b-2sPEG)-a and 4s(PCL-b-2sPEG)-b were 3.75 and 5%, respectively, indicate that the quercetin is effectively loaded into the micelles. Quercetin loading capacity and encapsulation efficiency increase from 3.75 to 5.0 wt% and from 75.0 to 80.0 wt%, respectively by increasing the PCL molecular weight from 2.3 to 3.8 kDa. These results confirm that quercetin loading capacity and encapsulation efficiency of the micelles depend on the block length of the PCL arm. Thereby, hydrophobic interactions of quercetin with PCL arm are an important factor for the drug loading capacity and encapsulation efficiency of 4s(PCL-b-2sPEG) micelles (Table 3) [56].

The in vitro release behavior of quercetin-loaded 4s(PCL-b-2sPEG) micelles and free quercetin in PBS (pH 7.4) at 25 °C was studied and the results are shown in Fig. 8. Quercetin solubility in the PBS medium was 150 μg/mL confirming the maintenance of sink conditions during the release experiments given the release volume (30 mL) and quercetin amounts in the micelles (260–350 μg). Free quercetin quickly diffused out of the dialysis tube when dissolved in the dialysis medium. Almost complete release was achieved after 15 h. It was observed that the release pattern of quercetin-loaded 4s(PCL-b-2sPEG)-a and 4s(PCL-b-2sPEG)-b micelles were a typical two phase release pattern which means that the release rate is very fast at the first stage. In the second stage, the release rate is much slower and the process may persist for several days. For the quercetin-loaded 4s(PCL-b-2sPEG)-a and 4s(PCL-b-2sPEG)-b micelles, there were both a similar burst release for initial 15 h, followed by a gradual release up to 160 h. In contrast, the release rate of quercetin from micelles is much slower than that of the free quercetin. After 160 h, 4s(PCL-b-2sPEG)-a and 4s(PCL-b-2sPEG)-b micelles released ~94 and 86% of their quercetin contents, respectively. These results demonstrate that a strong interaction between the drug and core-forming block in nanoparticles leads to a decreased rate of drug release from micelles.

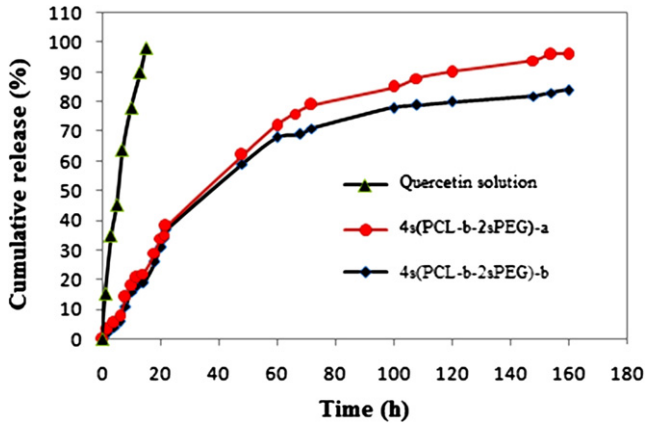


Fig. 8. Cumulative drug release profiles of the 4s(PCL-b-2sPEG)-a and 4s(PCL-b-2sPEG)-b in PBS pH 7.4 at 25 C°.

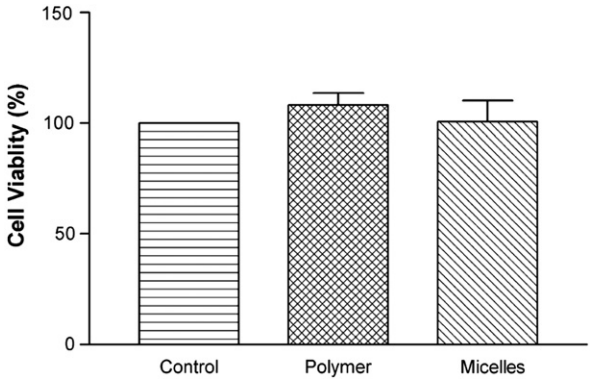


Fig. 9. In vitro cytotoxicity of the 4s(PCL-b-2sPEG)-a copolymer in two form of nanoparticles (micelle) and synthesized polymer with concentration 1 mg/ml.

3.9. In vitro cytotoxicity studies

Biocompatibility is a great concern for biomaterials and in this study we performed preliminary evaluation on the cytotoxicity of obtained materials by the MTT assay. The highest possible concentration (1 mg/ml) of the copolymer was used to evaluate the cytotoxicity. The results in Fig. 9 demonstrate that the resulted copolymer does not exhibit apparent cytotoxicity.

4. Conclusions

Well-defined pentaerythritol-centered amphiphilic A_4B_8 star copolymers [4s(PCL-b-2sPEG)] have been successfully designed and synthesized. These amphiphilic star-block copolymers self-assemble into micelles in which hydrophobic arms of the copolymers form a core while the hydrophilic arms form a corona. The results demonstrate that such micelles provide an excellent nanocarrier for hydrophobic drugs such as quercetin, in which aqueous solubility of the drug is dramatically enhanced. Moreover, the micelles can effectively load hydrophobic quercetin and encapsulation efficiency of up to 80 wt% is achieved. The release behavior of quercetin from loaded micelles is shown a sustained manner which protects drug from precipitation in physiological medium. The star copolymers exhibit no apparent cytotoxicity. With these enhanced properties the star-block copolymer micelles composed of PEG and PCL have potential applications as a versatile nanocarrier of various liposolubility drugs.

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