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Preparation, pharmacokinetics and tissue distribution of micelles made of reverse thermo-responsive polymers

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ABSTRACT

New reverse thermo-responsive polymers, poly(ethylene oxide)–poly(propylene oxide) multi-block copolymers (poly(ether-carbonate)s) were synthesized. The micelles made of new reverse thermo-responsive polymers were also prepared loaded with the poorly soluble anticancer drug, hydroxycamptothecin (HCPT). The structure characterization of poly(ether-carbonate)s was determined by ^{1}H NMR and FT-IR analysis. The critical micelle concentration (CMC), critical micelle temperature (CMT), size distribution and drug release in vitro were determined. The pharmacokinetics and tissue distribution in vivo for novel copolymer micelles were studied. The experimental results showed that the micelles was spherical in appearance and dispersed well. The process of HCPT release from micelles in vitro was composed of two steps, abrupt release and sustained release. After i.v. administration (2 h), the drug concentration of poly(ether-carbonate) micelles group in liver in mice was 3.46 μg/g, while that of HCPT injection group was 0.401 μg/g. Compared with HCPT injection, the elimination half-life of poly(ether-carbonate) micelles group was prolonged remarkably from 1.3 to 12.5 h. The poly(ether-carbonate) micelles showed a combination of liver targeting and sustained drug release in experiments on animals.

1. Introduction

The reverse thermo-responsive phenomenon is usually known as reverse thermal gelation (RTG) and it provides one of the most promising strategies for the development of injectable systems for biomedical applications. The basic feature shared by these different polymeric systems is that water solutions of these materials display low viscosity at ambient temperature and exhibit a sharp viscosity increase following a small temperature rise, producing a semi-solid gel at body temperature (Sosnik and Cohn, 2005). A number of reverse thermo-responsive materials have been investigated for drug solubilization and controlled release (Kabanov et al., 1992; Yokoyama, 1992), for the prevention of post-surgical tissue adhesion (Steinleitner et al., 1991).

There are several RTG-displaying polymers. One of the most important RTG-displaying materials is the family of poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide) (EO–PO–EO) triblocks, commercially available as Poloxamers or Pluronics. A prominent feature of Pluronic copolymer is the ability of individual block copolymer molecules to self-assemble into polymeric micelles in aqueous solutions, which has a hydrophobic core formed by PO chains and a hydrophilic corona formed by EO chains. The PO core can serve as a 'pool' and the hydrophobic drug can be incorporated into the hydrophobic PO core, while the hydrophilic corona maintains the dispersion stability of Pluronic micelles. Incorporation into micelles leads to increased solubility, metabolic stability and circulation time of the drug (Kabanov et al., 2002). Unfortunately, the viscosity increase achieved by Pluronic triblocks is not large enough, resulting in systems displaying limited stability, poor mechanical properties, short residence times and unacceptably high permeabilities. These drawbacks render these systems unsuitable for most biomedical applications (Sosnik and Cohn, 2005).

Hydroxycamptothecin (HCPT), a plant alkaloid from Camptotheca acuminate demonstrated strong antitumor activity against lung, ovarian, breast, pancreas, and stomach cancers. However, HCPT is insoluble in water and exists in two forms depending on the pH value: the active lactone forms at pH below 5 and the inactive open ring-carboxylated forms, which presents at neutral pH values (Ling and Xu, 1993). Although the lactone form of HCPT is crucial for its anticancer activity, at physiological pH values most HCPT molecules exist in the inactive carboxylated form. Thus, sparing solubility and labile lactone ring hinder the clinical application of HCPT.

The present study focused on the synthesis and analysis of PEO/PPO-containing block copolymers, poly(ether-carbonate)s, by covalent binding of poly(ethylene glycol) and poly(propylene gly-

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col) chains using phosgene as the coupling unit. The micelles made of new reverse thermo-responsive polymers were also prepared. HCPT was used as a model drug and incorporated into micelles. The pharmacokinetics and tissue distribution in vivo for novel poly(ether-carbonate) micelles were studied.

2. Materials and methods

2.1. Materials

The solvents used were of analytical grade. Poly(ethylene glycol) (6000 and 2000 Da) (PEG6000 and PEG2000) and poly(propylene glycol) (6000 and 2000 Da) (PPG 6000 and PPG 2000) were purchased from Sigma–Aldrich (St. Louis, MO, USA) and dried before use, at 120 °C under vacuum for 1 h. The phosgene chloroformic solution was prepared from 1,3,5-trioxane (Jiangsu Changde Company, China) and carbon tetrachloride, using aluminum chloride as catalyst, following a technique previously published (Kheyfets et al., 1968). Pyridine was purchased from Tianjin Reagent Company. Hydroxycamptothecin (HCPT) was purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Synthesis of poly(ether-carbonate)s

Poly(ether-carbonate)s were synthesized by polymerizing poly(ethylene glycol) and poly(propylene glycol) segments utilizing phosgene as the coupling molecule. The different reactivity of phosgene's two functionalities allowed to bind the two constituents. The random poly(ether-carbonate)s were synthesized by a one-pot reaction, as described in detail (Cohn et al., 2003).

2.3. Gel permeation chromatography (GPC)

The average molecular weights and polydispersity were determined by gel permeation chromatography (Differential Separation Module Waters 2690 with refractometer detector waters 410 and Millenium Chromatography), using polystyrene standards between 472 and 360,000 Da.

2.4. Nuclear magnetic resonance spectroscopy (NMR)

The PEO/PPO ratio was determined by ^{1}H NMR spectroscopy (Bruker 300 MHz AMX 300), using the integral intensity of the peaks based on blends of known PEG6000/PPG2000 weight ratios. All spectra were obtained at room temperature from 15% (w/v) $CDCl_3$ solutions.

2.5. FT-IR analysis

The characterization of the functional groups was carried out by FT-IR analysis using a FT-IR spectroscopy (Thermo Electron Scientific Instruments Corp.). The samples were prepared by compressing poly(ether-carbonate)s into pellets with potassium bromide.

2.6. Critical micelle concentration (CMC) and critical micelle temperature (CMT) determination

The CMC values were obtained by determination of surface tension of water solutions with different concentrations of poly(ether-carbonate) using Krüss K12 tension apparatus (Krüss Company, Switzerland).

The CMT values of the micelles were measured by a fluorescence probe technique using pyrene as a hydrophobic probe, as described previously (Bae et al., 2006). Pyrene dissolved in acetone was diluted in deionized water to adjust at a concentration of 6.0×10^{-7} M, and then the acetone was evaporated. Pyrene solution (1.8 ml) and 200 μL of micelle solution were mixed and incubated for 2 h at each temperature in the dark. Excitation spectra of pyrene were monitored at an emission wavelength of 390 nm.

2.7. HCPT-loaded micelle preparation

20 mg poly(ether-carbonate) (PEG:PPG molar ratio is 0.7:1) was dissolved into 10 ml DMF. 20 mg HCPT dissolved in chloroform and adjusted with acetic acid was added to poly(ether-carbonate) solution in DMF. The organic solvent was subsequently removed by rotary vacuum evaporation. The film formed was additionally freeze-dried in vacuum, hydrated with a suitable amount of 5 mM HEPES-buffered saline (HBS), incubated and sonicated for a few minutes. The resulting mixture was filtered through a 0.2 μm nylon filter.

2.8. Appearance and size distribution measurement

The surface morphology of the micelles was observed by transmission electron microscope (TEM). Micelle size and size distribution were measured by the laser dynamic light scattering (DLLS) method using a Dawn Heleos, Wyatt QELS, and Optilab DSP instrument (Wyatt Technology Co., Santa Barbara, CA, USA).

2.9. Determination of HCPT into poly(ether-carbonate) micelles

The amount of HCPT in the micellar phase was determined by the reversed phase-HPLC. The LC-10ATVP-ODS HPLC system equipped with a diode array and fluorescence detector (Shimadzu, Japan) and Spherisorb ODS2 column, 4.6 mm × 250 mm (Analytical Cartridge Waters, Ireland) was used. After filtration of HCPT-containing micelle preparation through a 0.2 μm nylon filter, the micelles were diluted by 200-fold with acetonitrile (to destroy micelles and release HCPT), and an aliquot of the diluted solution was injected into HPLC system. The determination of the micellar HCPT was performed using the calibration curve of pure HCPT. The mobile phase composed of 66% (v/v) acetonitrile (HPLC grade) in water (pH 5.0 adjusted with acetic acid) was used with a flow rate of 1.1 ml/min. HCPT was detected by a fluorescence spectrometer set at λ_{ex} = 360 nm and λ_{em} = 430 nm. The drug loading and drug encapsulating efficiency were calculated using the following formulae:

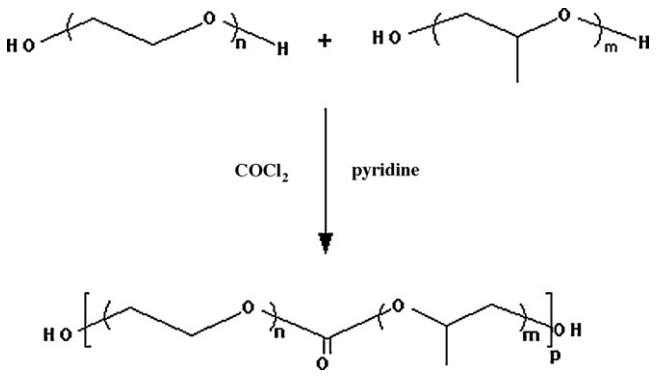
$$\text{drug loading}\,(\%) = \frac{D_{\text{HCPT}}}{Q_{\text{sys}}} \times 100$$

$$\text{drug encapsulating efficiency} = \frac{D_{\text{HCPT}}}{D_{\text{add}}}$$

where D_{HCPT} is the amount of HCPT in each composite, Q_{sys} is the amount of HCPT-loaded micelles and D_{add} is the amount of HCPT added in the experimental progress.

2.10. HCPT release from micelle formulation

The in vitro HCPT release from micelles was investigated with a hydrotropic agent, sodium salicylate, to create pseudo-sink conditions according to the reported procedure with modifications (Mu et al., 2005). Briefly, 1 ml of HCPT-loaded micelles was introduced into a dialysis membrane bag (MWCO = 1000 Da) and the end-sealed dialysis bag was incubated in 100 ml 1 M sodium salicylate at 37 °C. The dialysis solution was shaken at a speed of 100 rpm. At predetermined time intervals, 2 ml aliquots of the sodium salicylate solution were withdrawn and replaced with an equal volume of the fresh 1 M sodium salicylate. The concentration



Scheme 1. Synthesis and structure of the random poly(ether-carbonate)s.

of HCPT in a sample was measured by HPLC method with the correction for the volume replacement. The mobile phase composed of 66% (v/v) acetonitrile (HPLC grade) in water (pH 5.0 adjusted with acetic acid) was used with a flow rate of 1.0 ml/min. HCPT was detected by a fluorescence spectrometer set at λ_{ex} = 360 nm and λ_{em} = 430 nm.

2.11. In vivo pharmacokinetics of micelles

The healthy rabbits (half males and half females, weight 2.5 ± 0.5 kg) were randomly divided into three groups with six for each group. One group was administered HCPT injection via ear marginal vein at a dose of 0.15 mg/kg, while other groups were administered HCPT-loaded poly(ether-carbonate) micellar solution and HCPT-loaded Pluronic micellar solution, respectively, at a dose of 0.15 mg/kg. All rabbits were kept on starvation for 12 h before injection (drinking freely). For HCPT injection group blood samples were taken from ear marginal vein at 0 min, 15 min, 30 min, 1.0 h, 2.0 h, 4.0 h, 8.0 h; for HCPT-loaded micellar solution groups blood samples were taken at 0 min, 15 min, 30 min, 1.0 h, 2.0 h, 4.0 h, 8.0 h, 12.0 h, 24.0 h and 48.0 h. The concentration of HCPT in a sample was measured by HPLC method.

Table 1
Physical parameters of random poly(ether-carbonate)s.

Sample	Mol ratio of PEG/PPG	Mw	Polydispersity	Productivity/%
a	0.5:1	10,416	1.41	83.56
b	0.6:1	10,813	1.40	86.38
c	0.7:1	13,487	1.33	89.26
d	0.8:1	14,263	1.41	80.46
e	0.9:1	15,238	1.38	88.39

2.12. Tissue distribution of polymeric micelles

Healthy mice (half males and half females, weight 18–22 g) were randomly divided into two groups. HCPT injection and HCPT-loaded micellar solution were, respectively, injected via tail vein at a dose of 1 mg/kg. For HCPT injection group three mice were killed at 30 min, 1.0 h, 2.0 h, 4.0 h, 8.0 h, 12.0 h after administration. For HCPT-loaded micellar solution group three mice were killed at 30 min, 1.0 h, 2.0 h, 4.0 h, 8.0 h, 12.0 h, 24.0 h, 48.0 h. Heart, liver, spleen, lung and kidney were taken out immediately and washed with distilled water, and then 1 g of tissue sample was collected, respectively, after surface water was dried. The drug concentration in tissues was determined by HPLC method.

3. Results and discussion

3.1. The synthesis of poly(ether-carbonate)s

These poly(ether-carbonate)s synthesized have the following general structure: [–PEO–E–PPO–]$_n$, where E is phosgene, performing as a bifunctional coupling molecule and *n* designates the degree of polymerization. The general synthesis of the poly(ether-carbonate)s is presented in Scheme 1. The physical parameters of poly(ether-carbonate)s are listed in Table 1.

3.2. ^{1}H NMR

As already stated in Section 2, the PEO/PPO ratio was determined by ^{1}H NMR spectroscopy (Bruker 300 MHz AMX 300), using the integral intensity of the peaks based on blends of known

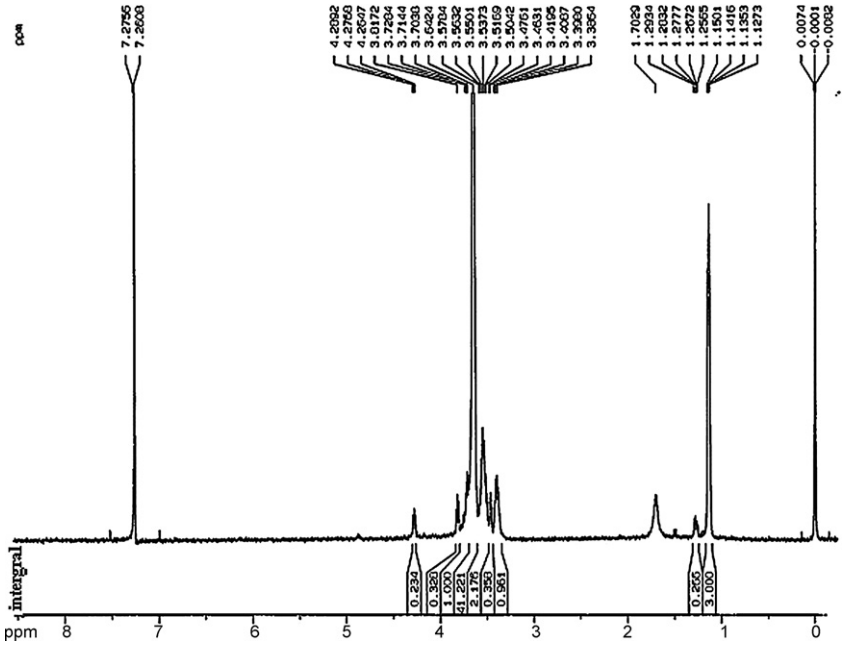


Fig. 1. ^{1}H NMR spectrum of a random poly(ether-carbonate)s.

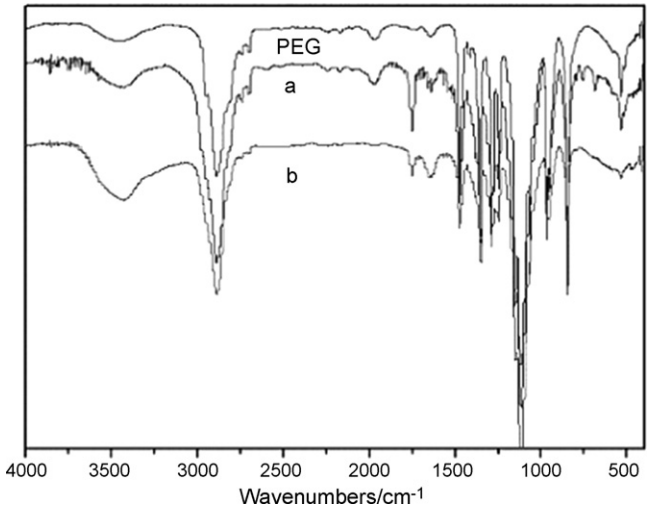


Fig. 2. FT-IR spectrum of PEG and two random poly(ether-carbonate)s. (a) Mol ratio (PEG:PPG) 0.7:1 and (b) mol ratio (PEG:PPG) 0.9:1.

PEG6000/PPG2000 weight ratios. The peaks used to determine the composition of copolymer were the one assigned to PEOs methylene protons, at 3.64 ppm (triplet), and the peak at 1.13 ppm (singlet), due to the protons of PPOs methyl pendant groups (see Fig. 1).

3.3. FT-IR

The functional groups of copolymers were analyzed by FT-IR spectroscopy (see Fig. 2). The peak at 1743 cm^{-1} was assigned to the characteristic of the carbonate group and the peak at 3425 cm^{-1} was assigned to the OH end groups. The peak at 2868 cm^{-1} was assigned to the characteristic of PEOs methylene groups.

3.4. Characteristics of drug-loaded micelles

Figs. 3 and 4 show representative size distributions and TEM micrographs of the poly(ether-carbonate) micelles. It was found that the mean particle size of the poly(ether-carbonate) micelles obtained by dynamic light scattering was 65.5 nm. Size distribution may play important roles in determining the fate of micelles after administration. It was reported that drug vehicles with a diameter larger than 200 nm are readily scavenged non-specifically by monocytes and RES. In contrast, smaller particles tended to accumulate

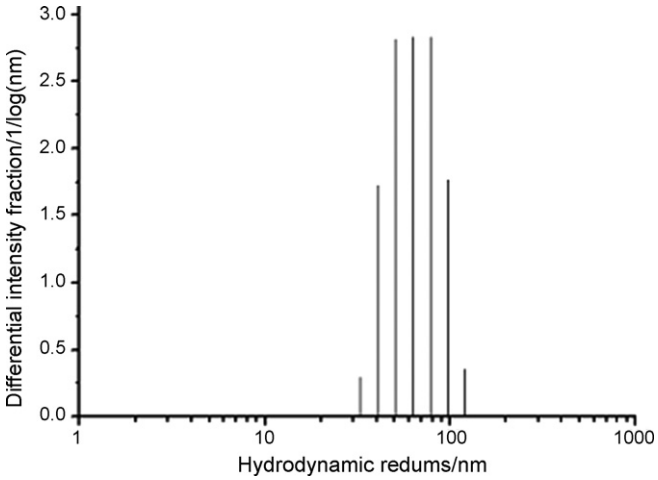


Fig. 3. Size distributions of HCPT-loading micelles.

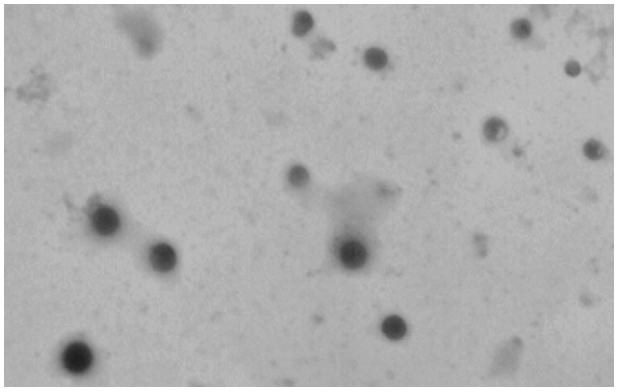


Fig. 4. TEM micrograph of HCPT-loading micelles.

in the tumor sites due to the enhanced permeability and retention (EPR) effect and a greater internalization was also observed.

The results obtained by TEM showed that the morphology of the HCPT-loaded copolymeric micelles was spherical in shape with a smooth surface.

The average drug loading and drug encapsulating efficiencies were 0.0363 ± 0.0011% and 43.6 ± 0.21% (w/w), respectively.

3.5. Critical micelle concentration (CMC) and critical micelle temperature (CMT) determination

The CMC values were obtained by the determination of surface tension of polymer aqueous solutions with different concentrations (see Fig. 5). Surface tension decreased with the increase of polymer concentration at low concentration and became stable when the polymer concentration was at the CMC value. The CMC values of poly(ether-carbonate)s was about 38.5 mg/L, which was lower than that reported for Pluronic F127 micelles (45.28 mg/L, Kabanov et al., 2002). The CMT of the micelles was measured by a fluorescence probe technique using pyrene as a hydrophobic probe (see Fig. 6). The intensity from pyrene excitation spectra was flat at temperatures lower than 20 °C, but dramatically increased as temperature increased, which demonstrated that the

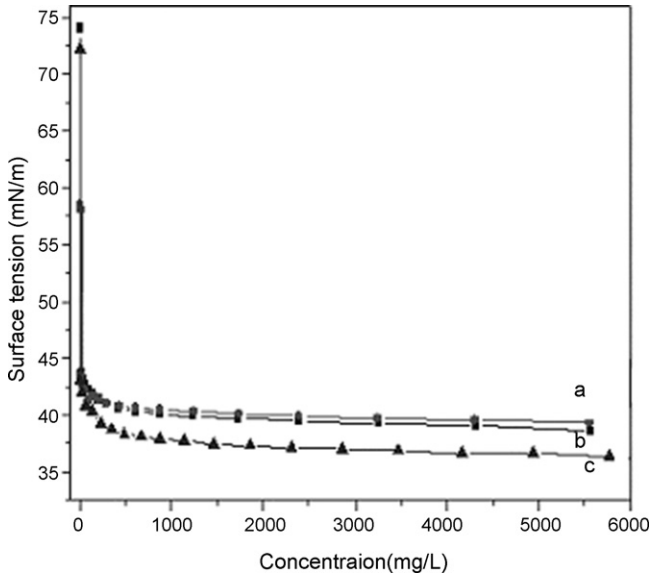


Fig. 5. Surface tension isotherms of three different random poly(ether-carbonate)s at different concentrations in aqueous solution at 25 °C. (a) Mol ratio (PEG:PPG) 0.8:1, (b) mol ratio (PEG:PPG) 0.6:1 and (c) mol ratio (PEG:PPG) 0.7:1.

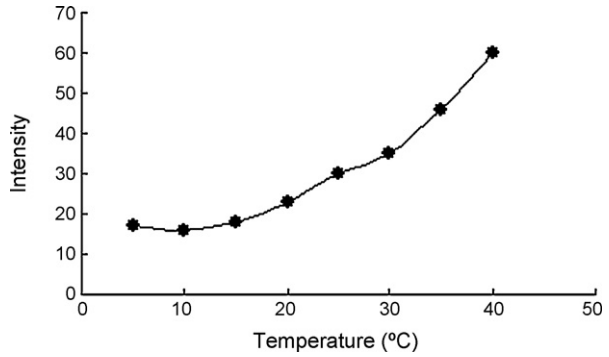


Fig. 6. The intensity of excitation spectra of pyrene as a function of temperature (mol ratio (PEG:PPG) 0.7:1).

CMT of poly(ether-carbonate) micelles was about 20 °C. The sharp increase of the intensity near the CMT was closely related to the formation of micelles composed of a hydrophobic core into which pyrene was preferentially partitioned. Interestingly, the CMT value of poly(ether-carbonate) micelles was much lower than that reported for Pluronic F127 micelles (around 26.5 °C) (Bae et al., 2006). This suggested that the viscosity increase of poly(ether-carbonate) solution following a small temperature rise increased the local concentration of poly(ether-carbonate) copolymers within micelles.

3.6. HCPT release from micelle formulation

In vitro release of HCPT from poly(ether-carbonate) micelles and Pluronic F127 micelles were performed using oscillation in constant temperature (37 °C). Fig. 7 shows HCPT release curve from poly(ether-carbonate) micelles, Pluronic F127 micelles and HCPT injection. The HCPT release curve from micelles presents two phases: the fast release in the first 4 h and sequential slow release. The drug incorporated into the poly(ether-carbonate) micelles showed a very slow release even at sink conditions, 20–30% of the initially incorporated drug was still associated with the micelles after 72 h incubation at 37 °C. In comparison with HCPT-loaded micelle composite, the HCPT injection releases the HCPT very fast. In approximately 4 h, 90% of HCPT has been released, which indicated that the HCPT-loaded poly(ether-carbonate) micelles had a well-sustained release efficacy, which could be explained by the HCPT location within the micelles and viscosity increase of copolymer solution with the increase of temperature (Fig. 7).

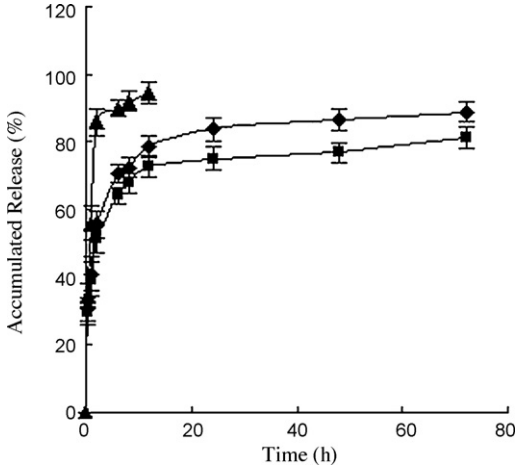


Fig. 7. In vitro release of HCPT-loading micelles in vitro. (♦) HCPT injection; (■) HCPT-loaded micelles (mol ratio (PEG:PPG) 0.7:1); (▲) HCPT-loaded Pluronic F127 micelles.

Table 2
The pharmacokinetic parameters of poly(ether-carbonate) micelles, Pluronic micelles and HCPT injection ($n = 3$).

Parameter	Unit	HCPT injection	HCPT polymeric micelles	HCPT Pluronic micelles
V(c)	L	0.001	0.002	0.002
$t_{1/2(\alpha)}$	h	0.249	0.782	0.689
$t_{1/2(\beta)}$	h	1.325	12.492	10.453
K_{21}	h^{-1}	1.157	0.455	0.501
K_{10}	h^{-1}	1.259	0.108	0.231
K_{12}	h^{-1}	0.893	0.379	0.413
AUC	$ng\,ml^{-1}\,h$	157.400	639.480	601.234
CL(s)	$L\,h^{-1}$	0.001	0.0002	0.0005

It also looks like poly(ether-carbonate) micelles retain the drug even better than "pure" Pluronic F127 micelles indicating that the enhanced stability of poly(ether-carbonate) micelles probably because of viscosity increase of copolymer solution with the increase of temperature (Fig. 7).

3.7. The in vivo pharmacokinetics of poly(ether-carbonate) micelles

The in vivo pharmacokinetics of HCPT-loaded micelles was studied with "practical pharmacokinetic program version 97" and was fitted by one-compartment model, two-compartment model and three-compartment model, respectively. Based on the analysis of the models and parameters, it was concluded that the in vivo pharmacokinetics of HCPT-loaded micelles in blood could be described by two-compartment model with i.v. injection. The pharmacokinetic parameters are reported in Table 2. In comparison with HCPT injection, the half-life after i.v. injection of HCPT-loaded micelles ($t_{1/2(\alpha)} = 0.782$ h, $t_{1/2(\beta)} = 12.492$ h for poly(ether-carbonate) micelles and $t_{1/2(\alpha)} = 0.689$ h, $t_{1/2(\beta)} = 10.453$ h for Pluronic micelles) were prolonged remarkably than those ($t_{1/2(\alpha)} = 0.249$ h, $t_{1/2(\beta)} = 1.325$ h) after i.v. injection of HCPT injection. The results indicated that the HCPT-loaded micelles had a well-sustained release efficacy, which could be explained by the HCPT location within the micelles and viscosity increase of copolymer solution with body temperature increase. In addition, a slight difference in half-life was observed between poly(ether-carbonate) micelles and Pluronic F127 micelles, which could be explained by the enhanced stability of poly(ether-carbonate) micelles caused by viscosity increase of copolymer solution of poly(ether-carbonate) micelles with the increase of temperature.

3.8. Distribution of HCPT in tissues

The drug concentration of tissues was determined by HPLC method (Figs. 8 and 9). The results indicated that the poly(ether-carbonate) micelles could delivery HCPT mainly to liver after i.v.

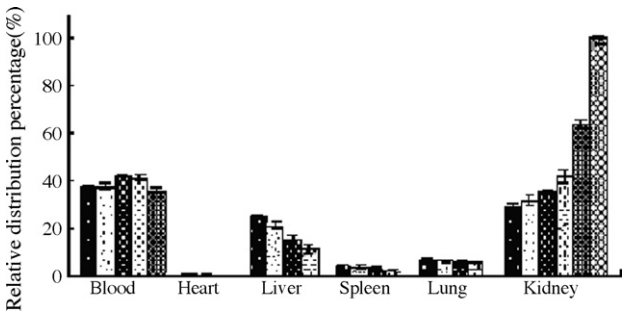


Fig. 8. Distribution in tissue in mice after i.v. administration HCPT injection [(■) 0.05 h; (□) 1 h; (▩) 2 h; (⊡) 4 h; (▩) 8 h; (▨) 12 h].

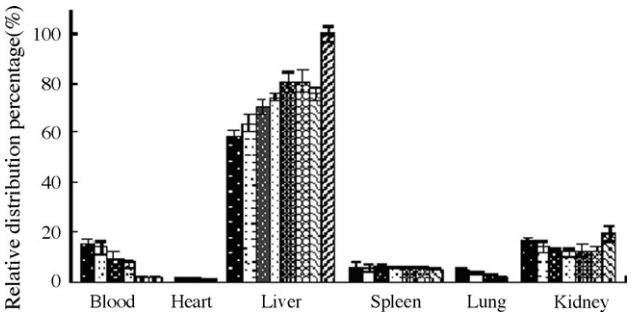


Fig. 9. Distribution in tissue in mice after i.v. administration HCPT micelles [( ) 0.5 h; ( ) 1 h; ( ) 2 h; ( ) 4 h; ( ) 8 h; ( ) 12 h; ( ) 24 h; ( ) 48 h].

injection to mice and the concentration of HCPT in liver (3.46 μg/g, 2 h) was significantly higher than those in other tissues and blood. Compared with HCPT injection, the drug concentration of HCPT in liver after i.v. injection of HCPT-loaded micelles enhanced from 0.401 ± 0.0216 to 3.46 ± 0.0134 μg/g (2 h).

4. Conclusions

Poly(ether-carbonate)s are new reverse thermo-responsive polymers. Water solutions of these materials display low viscosity at ambient temperature, and exhibit a sharp viscosity increase following a small temperature rise at body temperature, which could increase the local concentration of poly(ether-carbonate) copolymers. So the micelles made by poly(ether-carbonate)s can preserve their integrity even upon the dilution in the blood pool. In vitro release of HCPT from poly(ether-carbonate) micelles indicated that the HCPT-loaded poly(ether-carbonate) micelles had a well-sustained release efficacy. The half-life after i.v. injection of HCPT-loaded micelles was prolonged remarkably than those after i.v. injection of HCPT injection. Thus, the micelles made of poly(ether-carbonate) copolymers can be used as drug delivery system.

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