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# Injectable block copolymer hydrogels for sustained release of a PEGylated drug

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**Abstract**

The paper employs the spontaneous physical gelling property of a biodegradable polymer in water to prepare an injectable sustained release carrier for a PEGylated drug. A series of thermogelling PLGA–PEG–PLGA triblock copolymers were synthesized. The PEGylated camptothecin (CPT) was also prepared and employed as the model of a PEGylated drug, and the solubility of this hydrophobic drug was significantly enhanced to over 150 mg/mL. The model drug was completely entrapped into the polymeric hydrogel, and the sustained release lasted for 1 month. The mechanism of the sustained release was diffusion-controlled at the first stage and then was the combination of diffusion and degradation at the late stage. In vivo anti-tumor tests in mice further confirmed the efficacy of the model PEGylated drug released from the hydrogel. This work also revealed the specificity of the PEGylated drug in such a kind of carrier systems by decreasing the critical gelling temperature and increasing the viscosity of the sol. Due to the very convenient drug formulation and highly tunable release rate, an injectable carrier platform for PEGylated drugs is thus set up.


*Keywords:* Injectable hydrogel; Block copolymer; PEGylation; Camptothecin (CPT); Sustained release

## 1. Introduction

Over the past few decades, polymeric drug delivery systems have been an issue of intensive research (Csaba et al., 2006; Hoffman, 2002; Langer, 1998; Park et al., 2006; Sadzuka et al., 2006; Yamagata et al., 2006; Zhang et al., 2005a). Among various sorts of sustained release carriers, in situ-formed polymeric hydrogels have recently been paid much attention as an injectable topical carrier due to the advantages of easy formulation, high loading and free of any organic solvent etc. (Itoh et al., 2006; Jeong et al., 1997; Kang et al., 2006; Kissel et al., 2002; Ricardo et al., 2005; Shim et al., 2007; van de Wetering et al., 2005). Compared to chemically crosslinked hydrogels (van de Wetering et al., 2005; Wang et al., 2006), physical thermogelling of some polymer aqueous solutions is especially attractive due to free of initiator and unreacted agents, and also due to convenience of formulation (Jeong et al., 1997; Kang et al., 2006; Zentner et al., 2001). Such a system makes drugs or bioactive molecules easily entrapped in situ by a simple syringe injection of their aqueous solutions at target sites. It is not hard to understand that as an ideal drug vehicle, the favorite thermogelling material might exhibit lower critical solution temperature (LCST) behaviors, namely, the underlying polymer aqueous solution is in a sol state below room temperature or body temperature while gelling at body temperature. The mixing of drug with polymer at low temperatures is beneficial for protecting drug away from denaturing, aggregation and any undesired chemical reaction.

So far, several temperature responsive polymers have been tried in drug delivery (Coughlan and Corrigan, 2006; Ruel-Gariepy and Leroux, 2004; Wu et al., 2006; Zhang et al., 2005a,b). Poloxamer or pluronic hydrogels composed of poly(ethylene glycol-*b*-propylene glycol-*b*-ethylene glycol) perhaps represent the most extensively researched LCST thermogelling drug delivery system. For example, poloxamer hydrogel displayed a zero-order release profile for urease and interleukin-2 over 8 h (Fults and Johnston, 1989; Johnston et al., 1992). However, poloxamer is not considered an optimal drug delivery system due to its non-biodegradability and relatively fast dissolvability at the injection site. A kind of novel

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thermogelling triblock copolymers has thus been reported as controlled release drug carriers (Chen et al., 2005; Qiao et al., 2005; Tyagi et al., 2004; Zentner et al., 2001). These polymers are composed of hydrophilic poly(ethylene glycol) (PEG) (A) and biodegradable hydrophobic polyester (B), for instance, poly(lactic acid-*co*-glycolic acid) (PLGA), usually in the form of a symmetric triblock copolymer with an architecture of BAB or ABA type, with some appropriate compositions leading to also LCST behaviors (Jeong et al., 1999a; Shim et al., 2002; Yu et al., 2007, 2006). The integrity of the gel remains in rats over 4 weeks and the final degradation products are no toxic and can be obviated (Jeong et al., 2000). The degradation rate, burst release rate, sol–gel transition temperature and permeability of hydrogel matrix can be modified by molecular weight ($M_W$) of polymers, PLGA/PEG ratio, lactide/glycolide (LA/GA) ratio, concentration, and even the end group, etc. (Chen et al., 2005; Qiao et al., 2005; Shim et al., 2002; Yu et al., 2007, 2006).

On the other hand, PEG has been approved by the Food and Drug Administration (FDA) and many authority bureaus for internal consumption and injection in a variety of foods, cosmetics, personal care products, and pharmaceuticals. The attachment of a PEG chain to a protein, an organic drug, or a liposome, so-called PEGylation, has so far been well known to prolong circulating time of many drugs in body. PEGylation can lead to a stealthy liposome (Sadzuka et al., 2006). This stealthy property is beneficial for alleviating protease degradation and immunogenicity. PEGylation has indeed been a fashion in the field of controlled release during the past few decades (Greenwald et al., 2003; Sadzuka and Hirota, 1997; Vyas et al., 2006).

We here suggest the combination of the PEGylation technique of drug and the in situ implant technique of release carriers based upon the above thermogelling material. Such a combination might be with significant progress and striking advantages: the topically formulated drug could be sustained released from the hydrogel for a quite long time, meanwhile the released drug could be circulated for a long time, and thus the efficacy might be greatly enhanced. To our best knowledge, there is so far no report about encapsulation of a PEGylated drug into the thermogelling PLGA–PEG–PLGA block copolymers. Will this physical gel system be valid to encapsulate and deliver a PEGylated drug? Will the gel formation be influenced by loading of a PEGylated drug into the material? These fundamental problems are still open, and thus the methodology studies are highly called for at this stage.

Camptothecin (CPT) is an anti-tumor Chinese medicine which can kill cells by converting DNA topoisomerase I into a DNA-damaging agent. However, both the low water solubility of the drug and the opening of its active lactone ring at physiological pH (and higher pH) limit its clinical application. It has been reported that modifying CPT at the 20th position as a PEG ester not only achieves the soluble transport form of CPT but also stabilizes the active lactone ring under physiological conditions (Greenwald et al., 1996). In the present paper, PEGylated CPT was prepared and it was employed as the model drug to examine the validity of the carrier system of PLGA–PEG–PLGA materials for a PEGylated drug. The PEGylated drug was found to alter the gelling points of such a polymeric biomaterial. Both in vitro and in vivo experiments were performed. A successful controlled release lasting for 1 month was achieved. The release mechanism was also discussed.

## 2. Materials and methods

### 2.1. Materials

Poly(ethylene glycol)s with two $M_W$ (PEG 1000 and PEG 1500; Sigma), monomethoxy-poly(ethylene glycol) (MPEG 5000; Sigma), DL-lactide (Purac), glycolide (Purac), stannous octoate (Aldrich), *N*,*N*′-dicyclohexyl carbodiimide (DCC; Aldrich), 4-dimethylamiopryidine (DMAP; Acros) were used as received. 20(S)-camptothecin (CPT, 95% purity) was purchased from Shanghai Junjie Biotechnology Co., Ltd. All other chemicals used were reagent grade and used as purchased without further purification.

### 2.2. Animals

S-180 sarcoma bearing Kunming mice (female, $19 \pm 2$ g) were supplied by the Shanghai Experimental Animal Center, Chinese Academy of Sciences (Shanghai, China). The animals were acclimatized at a temperature of $25 \pm 2$ °C and a relative humidity of $70 \pm 5$% under natural light/dark conditions for 1 week before dosing.

### 2.3. Synthesis of PLGA–PEG–PLGA triblock copolymers

PLGA–PEG–PLGA triblock copolymers were synthesized by a ring opening copolymerization as previously described (Zentner et al., 2001). Briefly, PEG was stirred and dried under vacuum at 150 °C in a three-necked flask for 4 h. Under argon atmosphere, DL-lactide (45 g) and glycolide (7.2 g) were added and stirred under vacuum at 120 °C for 30 min. After all the monomers were melted, the initiator, stannous octoate (0.2 wt%) was added. Then the reaction mixture was stirred, and the reaction proceeded for 8 h at 160 °C under an argon atmosphere. After 8 h, vacuum was used to remove any unreacted monomers in the reaction mixture for 30 min. Crude polymers were dissolved in ice cold water (5–8 °C). After completely dissolved, the polymer solution was heated to 80 °C to precipitate the polymer products and remove water-soluble impurities. The supernatant was decanted to separate the precipitated polymer. The above process was repeated once to obtain the purified copolymer. Finally, the residual water in the copolymer was removed by freeze-drying and stored for further use. Other triblock copolymers with different compositions were synthesized and purified in a similar way.

$^1$H NMR ($CDCl_3$): $\delta$ 1.55 (–OCH($CH_3$)CO–), $\delta$ 3.60 (–O$CH_2CH_2$–), $\delta$ 4.30 (–$OCH_2CH_2$OCO$CH_2$O–), $\delta$ 4.80 (–O$CH_2$CO–), and $\delta$ 5.20 (–O$CH$($CH_3$)CO–).

### 2.4. Synthesis of MPEG-CPT

First, MPEG-acid was, from the raw MPEG with one hydroxy end group, synthesized according to the previous method (Geckeler and Bayer, 1980). Then, the MPEG-acid (1 mmol) was dissolved in anhydrous methylene chloride (100 mL) at room temperature under argon atmosphere. The solution was cooled to 0 °C. DCC (2 mmol), DMAP (2 mmol) and CPT (2 mmol) were then added. The mixture was stirred in an ice bath for 2 h and then at room temperature overnight. The solution was concentrated to 50 mL and filtered. The filtrate was washed with 0.1 mol HCl, dried by using magnesium sulfate, and precipitated with excessive anhydrous ether. The product was further recrystallized from DMF/ether. The solid was filtered and washed with 2-propanol to obtain the final product. The percentage of CPT per MPEG molecule was determined by a UV assay similar to Greenwald et al. (1996).

$^1$H NMR ($CDCl_3$): $\delta$ 1.01 (t, H-18), $\delta$ 2.68 (t, –$OCH_2CH_2OCOCH_2CH_2COO$–), $\delta$ 2.85 (t, –$OCH_2CH_2OCOCH_2CH_2COO$–), $\delta$ 3.38 ($CH_3O$–), $\delta$ 3.60 (–$OCH_2CH_2$–), $\delta$ 4.24 (–$OCH_2CH_2OCOCH_2CH_2COO$–), $\delta$ 5.29 (S, H-5), 5.4–5.7 (ABq, H-17), $\delta$ 7.67 (t), $\delta$ 7.83 (t, H-10), $\delta$ 7.94 (d, H-9), $\delta$ 8.23 (d, H-12), and $\delta$ 8.40 (S, H-6).

### 2.5. $^1$H NMR analysis

A 500-MHz proton NMR spectrometer (Bruker, DMX500 spectrometer) was used for $^1$H NMR measurements in $CDCl_3$ to determine the chemical structure and composition of the copolymers and MPEG-CPT.

### 2.6. Gel permeation chromatography (GPC)

The molecular weights of copolymers and their molecular weight distributions were determined using an Agilent1100 GPC apparatus with a differential refractometer as detector. THF was used as eluent at a flow rate of 1.0 mL/min at 35 °C, and polystyrene standards were used as the calibration sample.

### 2.7. FT-IR study

The structure of MPEG-CPT was confirmed by Fourier-transform infrared spectroscopy (FT-IR spectra, Nicolet Magna-550). For FT-IR analysis, NaCl tablets were prepared by dissolving the sample in dichloromethane and evaporating the solvent under the IR light. CPT sample was measured in pellet form diluted with KBr powder.

### 2.8. MALDI-TOF mass spectrometry

The molecular weight of MPEG-CPT was determined by MALDI-TOF mass spectrometry. The sample was run on a Perceptive Biosystems model DERPMALDI/TOF mass spectrometer operated in the reflector mode, and positive ions were monitored. The matrix for the sample was α-cyano-4-hydroxycinnamic acid, and the beam line of a nitrogen laser at 337 nm was used. Hundred shots were averaged for every spectrum, and finally four spectra were overlap-add.

### 2.9. Transmission electronic microscopy (TEM)

The MPEG-CPT micelles were observed by transmission electron microscopy (TEM). In practice, the sample was prepared by placing 5 μL of micelle suspension of MPEG-CPT which had passed through a 450-nm filter on copper grids coated with a thin carbon film and observed at 80 kV in an electron microscope (Philips CM120).

### 2.10. Phase diagram of the block copolymers in water

Sol–gel or gel–sol transition behaviors of PLGA–PEG–PLGA block copolymers in water were investigated by the test tube inverting method (Tanodekaew et al., 1997). The temperature was increased by 1 °C per step. Each sample with a given concentration was prepared by dissolving the polymer in distilled water in a 4-mL vial. After equilibrating at 4 °C for 24 h, the vials containing samples were immersed in a water bath and held at each given temperature for 15 min to further equilibrate. The sample was regarded as a "gel" in the case of no flow within 30 s by inverting the vial.

The sol–gel transition of the copolymer aqueous solution was also investigated in a dynamic strain-controlled rheometer (ARES Rheometer Scientific) using a Couetter cell (Couette diameter, 34 mm; bob diameter, 32 mm; bob height, 33.3 mm; bob gap, 2 mm). Temperature was tuned with an accuracy of ±0.05 °C by an environment controller (Neslab). During temperature sweep experiments, strain amplitude was set at an appropriate value by preliminary tests to get both the linearity of viscoelasticity and large torque for detection. The heating and cooling rates were set as 0.5 °C/min while the angular frequency was set as 10 rad/s.

### 2.11. In vitro degradation of PLGA–PEG–PLGA

The copolymer solutions (25 wt%, 0.5 mL) were injected into a vial and incubated in a shaking bath (35 stroke/min) at 37 °C. After 10 min, 3.5 mL of phosphate buffer saline (PBS) solution (pH 7.4) was added to the formed gel. The PBS solution was replaced every 4 days to maintain medium pH. Some samples were taken out of vials every 4 days, and then freeze-dried. GPC measurements were used to determine the molecular weight of each taken sample as described in Section 2.6.

### 2.12. In vitro release of MPEG-CPT

MPEG-CPT (2.0%, w/w) was added to a 25 wt% copolymer aqueous solution and stirred at 4 °C till completely dissolved. Then, 0.5 g of the polymer solution containing drug was injected into a 10-mL test tube, and incubated in a shaking bath (35 stroke/min) at 37 °C for 10 min to form a gel. Six milliliters of PBS solution (pH 7.4) containing 0.02 wt% $NaN_3$ and 2 wt% Tween-80 was added. The system was then sealed to minimize evaporative loss. At designated time intervals, a 4-mL aliquot

was taken from the release media. The same amount of fresh buffer was then added in order to maintain the sink condition. Samples were collected at 1, 2, 4, 8, 16, 24, 48, and 72 h, and thereafter at a 48-h interval. The amount of MPEG-CPT in the released samples was determined by UV–vis assay at 368 nm associated with the biggest absorption peak of MPEG-CPT in PBS solution. The UV absorbance of the release media of drug-free hydrogels at each associated degradation time was taken as the control.

Samples of the release media were also subjected to high-performance liquid chromatography (HPLC) for analysis. HPLC (SPD-6AV) was equipped with a $C_{18}$ column (ODS $C_{18}$). The eluting solvent was acetonitrile–water with a ratio of 20:80. The flow rate was 1.0 mL/min.

The release data were analysed by the classic models. Diffusional drug release from a polymeric slab can be described by Higuchi equation (Higuchi, 1963) as written by

$$Q = k\sqrt{t} \quad (Q < 0.6) \tag{1}$$

Here, $Q$ is the fraction of drug released at time $t$, and $k$ is a constant related to diffusivity and also some structural and geometry parameters. A more complicated semi-empirical power relation was suggested by Peppas and Korsmeyer (Peppas, 1985; Peppas and Korsmeyer, 1986) as written by

$$Q = kt^n \quad \text{or} \quad \lg Q = k' + n \lg t \quad (Q < 0.6) \tag{2}$$

Obviously, Eq. (2) is reduced to Eq. (1) when the release exponent $n = 0.5$ for a slab geometry, which is called case I or Fickian diffusion transport, while $n = 1.0$ and $0.5 < n < 1.0$ refer to case II or swelling controlled transport and intermediate/anomalous transport, respectively.

### 2.13. In vivo release of MPEG-CPT from polymer hydrogels and anti-tumor tests in mice

Six groups of Kunming strain mice ($n = 8$) were examined, and sarcoma-180 (S-180) cells were implanted intradermally into the armpits of the mice. Twenty-four hours later, 0.6 mL of the polymer solution with a given drug-loading was subcutaneously injected into the backs of mice by a syringe with a 5.5-gauge needle. Groups 1 and 2 were treated with the physiological saline solution as the negative control; group 3, with 5-fluorouracil (5-Fu) as the positive control group (at the zeroth day and the third day, the drug was injected intravenously); group 4, with a polymer solution containing the virgin MPEG; groups 5–7, with the associated polymer solution mixed with different given drug-loadings. After 7 days, the mice were sacrificed. The anti-tumor effects against S-180 sarcoma were quantified according to the mean tumor weight defined as

$$\text{tumor inhibition ratio}\,(\%) = \frac{A_1 - A_2}{A_1} \times 100\%$$

Here, $A_1$ refers to the mean tumor weight of the negative control group without drug, and $A_2$, the mean tumor weight of the underlying drug-treated group. The standard of drug effect was set as follows: if the tumor inhibition ratio <40%, the result is of inefficacy; otherwise if the tumor inhibition ratio ≥40%, the result is of efficacy.

The anti-tumor test adheres to the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised 1985).

### 2.14. Data analysis

Statistical comparison was made using Student's $t$-test with $p < 0.01$ denoting a significant difference.

## 3. Results and discussion

### 3.1. Characterization of synthesized products

PLGA–PEG–PLGA triblock copolymers were obtained in an about 85% yield. The NMR peaks at 4.80, 3.60, 1.55 ppm were used to calculate the number average $M_W$ of the PLGA–PEG–PLGA triblock copolymer (Jeong et al., 1999b). The $M_W$ and their polydispersity indexes of the triblock copolymers and composition ratios of LA/GA or PEG/PLGA were determined via both GPC and $^1$H NMR. The results are shown in Table 1.

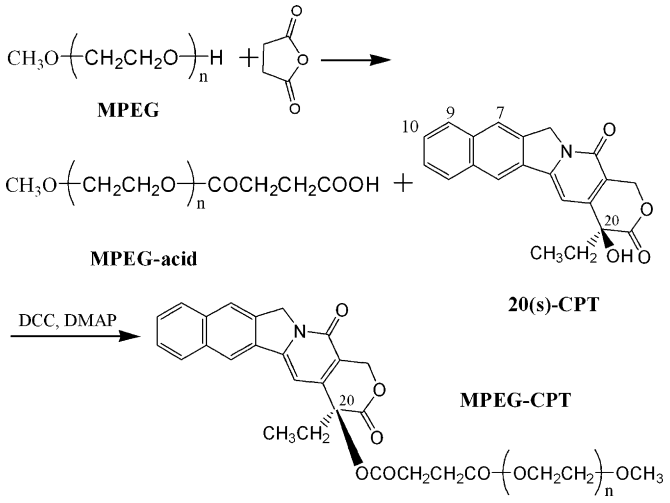
MPEG-CPT was synthesized by two steps as shown in Scheme 1. FT-IR spectra of MPEG, MPEG-acid, MPEG-CPT and CPT are shown in Fig. 1. Compared with MPEG, a new and strong carbonyl band was seen at 1735 cm$^{-1}$ in the FT-IR spectra of MPEG-acid, which confirms the formation of MPEG with carboxyl end group. On the spectra of MPEG-CPT, three characteristic peaks at 1617, 1666 and 1746 cm$^{-1}$ coming from CPT demonstrate the formation of MPEG-CPT.

The percentage of linked CPT per MPEG molecule is approximately 80% as determined by a UV assay similar to Greenwald et al. (1996). Fig. 2 shows the MALDI-TOF mass spectrum of the synthesized product, MPEG-CPT. The spectrum exhibits

Table 1
Samples of synthesized PLGA–PEG–PLGA triblock copolymers and MPEG-CPT

| Sample and $M_n$[a] | PEG or MPEG $M_n$[a] | LA/GA (mol/mol)[a] | $M_n$[b,c] | ($M_w/M_n$)[b,c] |
|---|---|---|---|---|
| Copolymer-1: 1730-1500-1730 | 1500 | 10 | 5310 | 1.19 |
| Copolymer-2: 1740-1500-1740 | 1500 | 5.0 | 5540 | 1.18 |
| Copolymer-3: 1400-1000-1400 | 1000 | 2.7 | 4460 | 1.21 |
| MPEG-CPT | 5000 | / | 4934[c] | 1.04[c] |

[a] The number-averaged molecular weight, $M_n$ of the central block PEG or MPEG was provided by Aldrich. The molar ratio of lactide/glycolide (LA/GA) and $M_n$ of PLGA blocks were calculated by $^1$H NMR.
[b] $M_n$ of triblock copolymers and their polydisperse indexes denoted by weight-averaged molecular weight $M_w$ over $M_n$ were measured via GPC.
[c] $M_n$ of MPEG-CPT and its polydisperse index were measured from MALDI-TOF mass spectrum.




Scheme 1. Synthesis scheme of MPEG-CPT.

two sets of peak: the strong one is associated with relatively high molecular weight, and a weak one, relatively low molecular weight. The strong peaks are ascribed to MPEG-CPT, while the weak peaks, to the virgin material MPEG. The population ratio of the two sets is consistent with the percentage of CPT per MPEG molecule measured by the UV assay.

CPT is a hydrophobic drug and insoluble in water with a solubility just about 1.3 μg/mL (Cortesi et al., 1997; Kang et al., 2002). The synthesized MPEG-CPT was, however, soluble in water over 150 mg/mL. So, the PEGylation enhanced the water solubility of CPT to a much large extent, which is nontrivial

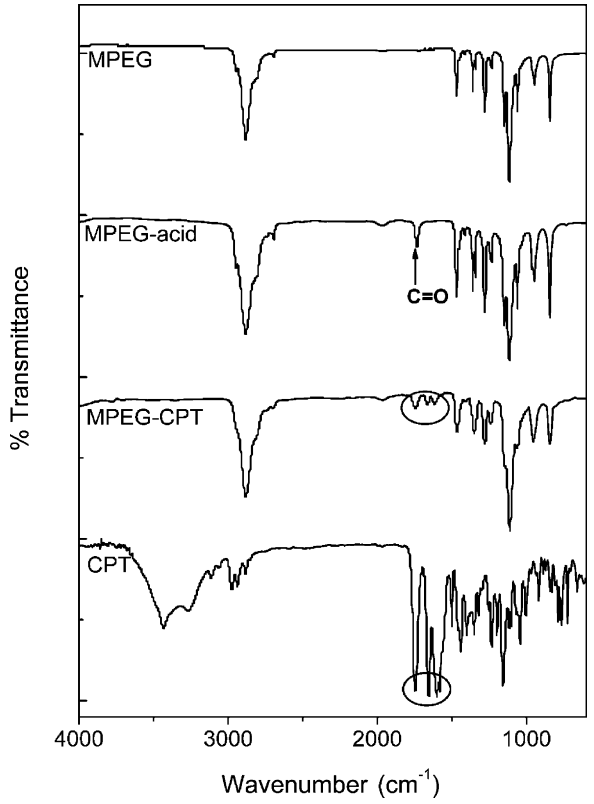



Fig. 1. FT-IR spectra of MPEG, MPEG-acid, MPEG-CPT and CPT.

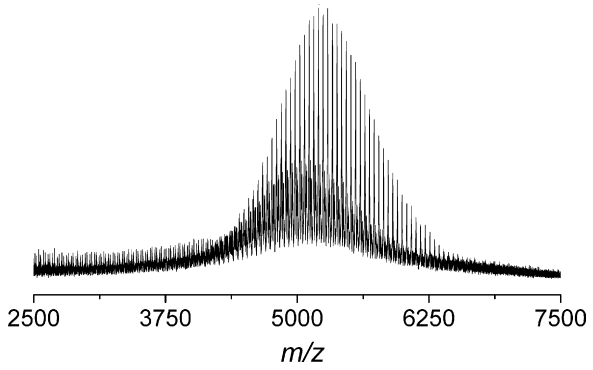



Fig. 2. The MALDI-TOF mass spectrum of MPEG-CPT.

for drug delivery. Since MPEG is hydrophilic, the MPEG-CPT is an amphiphile. The amphiphilic MPEG-CPTs tend to form micelles in water to reduce the free energy. The hydrophobic CPTs form cores, while the hydrophilic PEG blocks constitute coronas. The micelles of MPEG-CPTs were visualized by the TEM image as shown in Fig. 3. Hence, the apparent aqueous "solution" of MPEG-CPT is, actually, a nano-particle colloidal suspension.

### 3.2. Spontaneous gelling and biodegradation of PLGA–PEG–PLGA copolymers in water

The synthesized PLGA–PEG–PLGA triblock copolymers exhibited a temperature-dependent reversible sol–gel transition

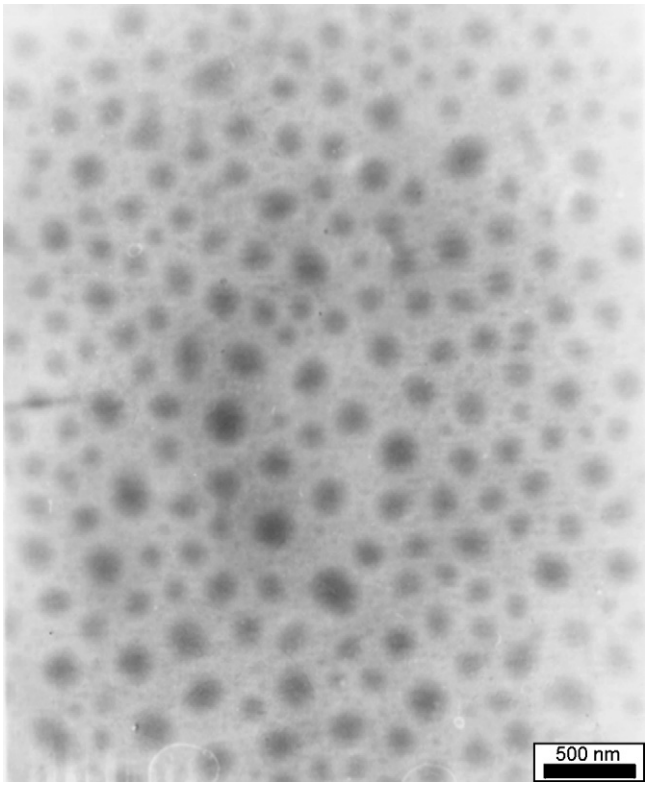



Fig. 3. A TEM image showing micelles of MPEG-CPT formed in an aqueous suspension at a concentration of 1 wt%.

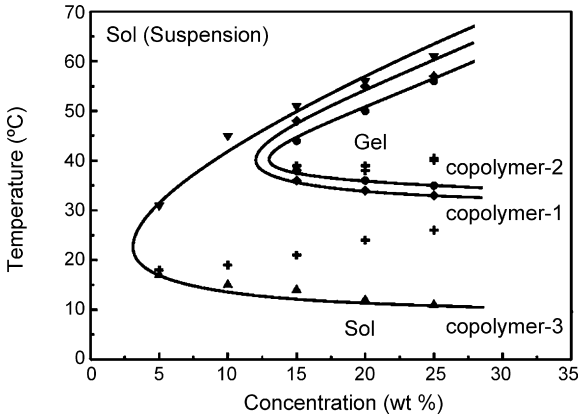



Fig. 4. Phase diagrams of PLGA–PEG–PLGA aqueous solutions. The legends represent copolymer samples listed in Table 1. Cross bars (+) indicates the temperatures above which the gel becomes opaque.

in water, if the polymer concentration was over a critical gellation concentration (CGC). The phase diagrams are shown in Fig. 4. In the examined temperature range (from 5 to 65 °C), the polymer/water mixtures took on three basic physical states: sol, gel and sol (suspension). As temperature increased, the solution (sol) became a hydrogel (gel), and finally entered into the state of "sol (suspension)" which looked like a sol at first but eventually precipitated after a sufficiently long time. The gel window was further divided into two regions associated with transparent gels and opaque gels.

The sol–gel transition exhibited LCST behaviors, which is meaningful for an injectable biomaterial. Fig. 4 indicates that the polymer composition influences CGC and LCST significantly. The sol–gel transition temperatures of copolymer-1 and copolymer-2 are much higher than that of copolymer-3. Copolymer-3 spontaneously gelled under the room temperature and thus led to difficulty in handling and injecting. On the other hand, an over high gelling temperature (higher than human body temperature) is also not desired—the polymer/drug/water mixture is, albeit injectable, unable to form gel and thus fail to encapsulate drugs. The critical gelling temperatures of copolymer-1 and copolymer-2 are between room temperature and body temperature, and thus suitable for drug delivery as an injectable material.

Besides capability of spontaneous gelling, another prerequisite of a synthesized polymer as an injectable and in situ-drug-encapsulating carrier is its biodegradability with an appropriate degradation rate. In vitro degradation of PLGA–PEG–PLGA triblock copolymers was thus examined. The results are shown in Fig. 5. The degradations of all of three samples lasted for more than 4 weeks. The difference of degradation rates among three samples might be accounted for mainly from the different LA/GA ratios in the PLGA blocks as given in Table 1. A higher LA/GA ratio leads to a stronger hydrophobility of PLGA and thus a lower degradation ratio which might lead to a slower sustained release.

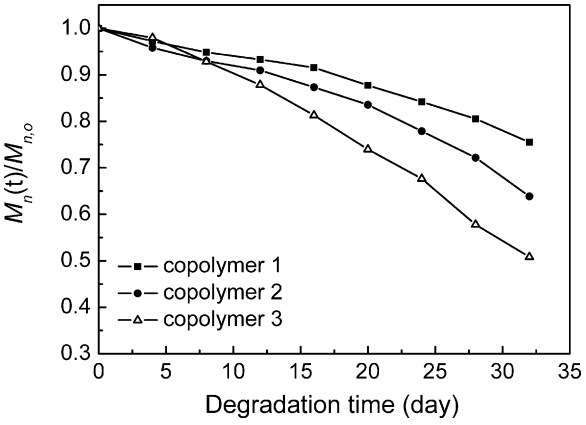



Fig. 5. Molecular weight of PLGA–PEG–PLGA as a function of in vitro degradation of the block copolymers (25 wt%) in PBS. The legends represent copolymer samples listed in Table 1.

### *3.3. The influence of the PEGylated drug to the gelling temperature and viscosity of PLGA–PEG–PLGA copolymers*

Our experiments so far indicate that the synthesized polymers seem suitable for a long-term drug delivery system, and drugs might be in situ entrapped by a simply syringe injection of their aqueous solutions at target sites. It is noteworthy to mention again that an appropriate sol–gel temperature of this kind of polymers is very important for its application as an injectable biomaterial and an in situ drug-loading carrier. Before we performed drug encapsulation and examined release kinetics, we addressed and tried to answer a question: whether or not a mixing with PEGylated drug might alter the gelling temperature of the biomaterial significantly.

Fig. 6 shows the effects of addition of MPEG-CPT on the sol–gel transition temperature. It is interesting that a PEGylated drug altered the gelling temperature indeed. The control experiment with addition of the same amount of PEG was also performed (another potential control experiment with just CPT added, but it is not soluble due to the poor solubility of the virgin CPT). This control experiment illustrates that PEG

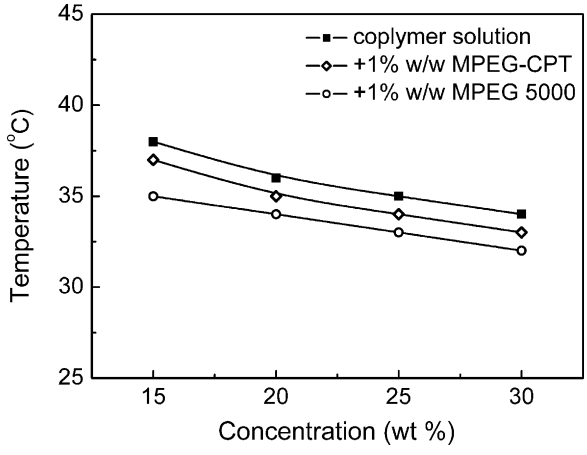



Fig. 6. Sol–gel transition temperatures of the PLGA–PEG–PLGA aqueous solutions with or without PEG additives. The solid squares refer to the copolymer 2 aqueous solution without external PEG additives; the black circles and diamond, with 1% (w/w) MPEG and 1% (w/w) MPEG-CPT added, respectively. The number 5000 denotes the molecular weight of the PEG used.

plays the important role in lowering sol–gel transition temperature of PLGA–PEG–PLGA aqueous solutions. Fig. 6 also indicates that the influence extent of MPEG-CPT is different from that of mere PEG. Such a difference might come from the self-assembly behaviors of the amphiphilic MPEG-CPT as demonstrated in Fig. 3. The detailed mechanism of the modification of gelling temperature of PLGA–PEG–PLGA aqueous solution by a PEGylated CPT is still open. Nevertheless, our experiments illustrates definitely that such an influence should be taken into consideration when we design a carrier system of PLGA–PEG–PLGA block copolymers for a PEGylated drug. Hence, although previous papers have reported the potential application of this kind of polymers as an injectable carrier of some small molecular drugs (Qiao et al., 2005; Zentner et al., 2001) and biomacromolecular drugs (Chen et al., 2005; Zentner et al., 2001), the present paper is, as the first report of encapsulation of a PEGylated drug into this material, has its own right to afford useful and important information to this kind of unique themosensitive intelligent hydrogels.

The rheological measurements also confirmed that the sol–gel transition temperature could be tuned via the addition of PEG and PEGylated chemicals (Fig. 7). Another important point is that the viscosity $\eta$ of the sol was increased with PEG or the PEGylated camptothecin added, which might lead to difficulty in injecting. Fortunately, Fig. 7 also indicates that the influence extent of MPEG-CPT is lower than that of mere PEG. Nevertheless, the unique properties of PEG additive effects on viscosity and LCST of PLGA–PEG–PLGA aqueous solutions must be taken into consideration in designing an appropriate formulation system and performing drug-loading/release experiments.

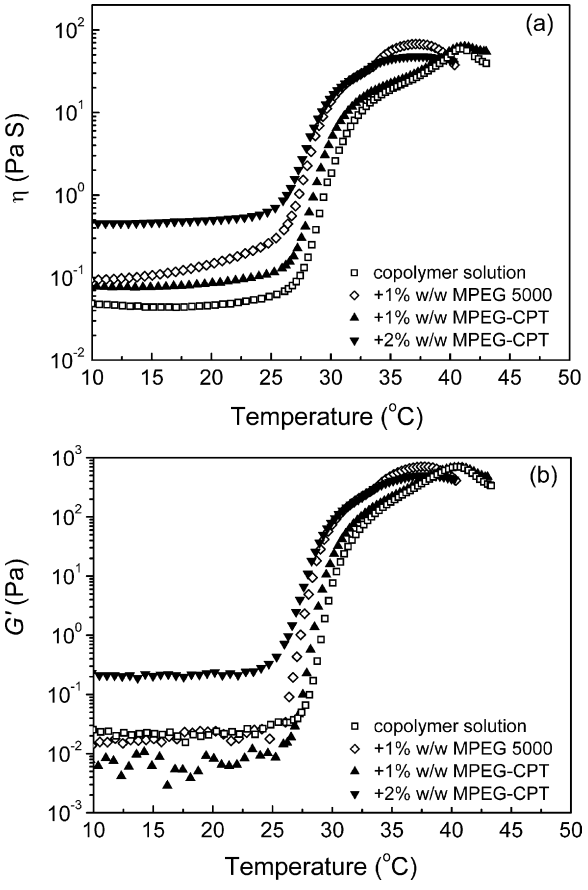



Fig. 7. (a) Viscosity $\eta$ and (b) storage modulus $G'$ as a function of temperature of the PLGA–PEG–PLGA aqueous solutions (25 wt%) with and without PEG additives. Heating rates: 0.5 °C/min; oscillatory frequency: 10 rad/s.

### 3.4. The influence of drug loading, composition and concentration of copolymers on in vitro drug release

Some typical in vitro release profiles of MPEG-CPT from the polymer hydrogels are shown in Fig. 8. The sustained release lasted for more than 1 month. In Fig. 8(a), the release rates of MPEG-CPT out of different polymeric hydrogels seem consistent with the degradation rates of the associated polymers with different compositions as shown in Fig. 5. So, the release of the drug is slowed down with the decrease of the degradation rate of the material.

We also examined in vitro release behaviors of MPEG-CPT from the hydrogels with different polymer concentrations and different drug loadings. Fig. 8(b) indicates that the release

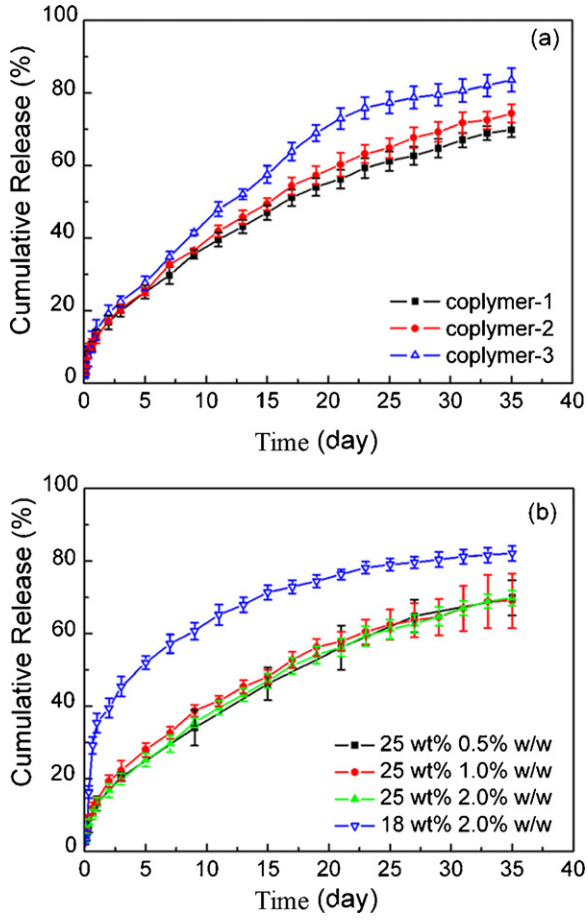



Fig. 8. In vitro release of MPEG-CPT release from PLGA–PEG–PLGA hydrogels in PBS at 37 °C with marked copolymer and drug loadings. (a) Effect of varying different compositions on the MPEG-CPT release. The legends represent copolymer samples listed in Table 1. The polymer concentration was 25 wt%, and the drug loading was 2.0% (w/w). (b) Effect of varying polymer concentrations and drug loadings on the MPEG-CPT release from copolymer-1 formulations. Each point represents the mean ± S.D.; $n = 3$.

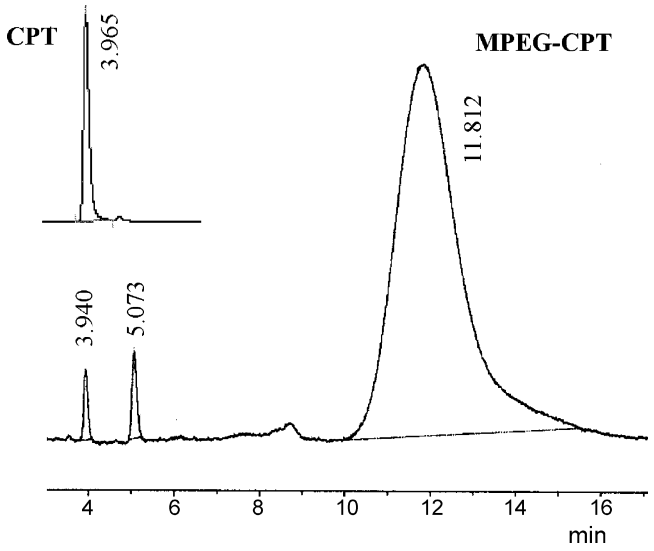



Fig. 9. The HPLC column elution profile of the released sample obtained at the fifth day from the release medium. The inlet shows the result from pure CPT and is taken as a control.

profile is less sensitive to drug loading than to polymer concentration. Therefore, it is quite flexible to select the drug amount to be encapsulated within a certain range as release rate is concerned. Nevertheless, the viscosity enhancement of a PLGA–PEG–PLGA aqueous solution after adding a PEGylated drug should be taken into consideration in order to keep injectability.

Here, we would like to mention again that the UV–vis method was employed to detect release of MPEG-CPT and the UV absorption comes from CPT instead of MPEG. So, it seems necessary to examine whether or not most of CPT are still linked to MPEG after released. The HPLC analysis of the medium after in vitro release of MPEG-CPT out of the block copolymer hydrogel (Fig. 9) indicates that the rate of hydrolysis of the PEGylation drug was low. Combination with more HPLC tests (data not shown) strengthens that the PEGylated CPT was basically stable in the gel and the release medium during the examination period of our in vitro release test. The UV absorbance of the release media of drug-free hydrogels has also, as the control, been taken into consideration to eliminate the interference of degradation products of copolymer, and Tween-80 etc. So, the UV–vis method is basically appropriate for determination of in vitro release profiles of this system.

### 3.5. Drug release mechanism

The release data of MPEG-CPT in the first release stage were assessed by Higuchi equation and Peppas-Korsmeyer equation. The results are shown in Table 2. Except a release system whose squared correlation coefficient value is 0.902, other release data of different release systems were well fitted to Higuchi equation or the Peppas-Korsmeyer equation with $n = 0.5$ with squared correlation coefficient values in the range 0.991–0.998. The exception cannot be fitted better by Peppas-Korsmeyer equation with an anomalous release exponent ($n = 0.629$) as indicated also in Table 2. Our analyses illustrates that the first-stage drug release from the hydrogels was basically diffusion-controlled, and this stage was quite long. On the other hand, comparison between the released profile of MPEG-CPT from polymeric hydrogels (Fig. 8) and the degradation kinetics of PLGA–PEG–PLGA polymers (Fig. 5) reveals that the mechanism of the sustained drug release in the late stage must be a combination of diffusion and degradation.

Table 2
Kinetic assessment of release data ($Q < 0.6$)

| Sample | Polymer concentration (wt%) | Drug loading (%, w/w) | $k$ | $R^{2a}$ |
|---|---|---|---|---|
| Copolymer-1[b] | 25 | 2.0 | 0.122 | 0.997 |
| Copolymer-2[b] | 25 | 2.0 | 0.128 | 0.995 |
| Copolymer-3[b] | 25 | 2.0 | 0.141 | 0.991 |
| Copolymer-1[b] | 25 | 0.5 | 0.122 | 0.997 |
| Copolymer-1[b] | 25 | 1.0 | 0.127 | 0.998 |
| Copolymer-1[b] | 18 | 2.0 | 0.232 | 0.902 |
| Copolymer-1[c] | 18 | 2.0 | 0.218 | 0.916 |

[a] Squared correlation coefficient.
[b] Fitted by Higuchi equation (Eq. (1)).
[c] Fitted by Peppas-Korsmeyer equation (Eq. (2)) with the best fitted release exponent $n = 0.629$.

Further, a direct visualization of the early stage release process of MPEG-CPT from the PLGA–PEG–PLGA hydrogel was also performed. Under the experimental conditions, the drug-loaded hydrogel was opaque while the associated drug-free hydrogel was transparent (Fig. 11). The opaque hydrogel became transparent with a clear "transparency frontier" during the presented time period. Now, we would like to interpret why the drug-loaded hydrogel could be opaque whereas the associated drug-free hydrogel was transparent. The phase diagram in Fig. 4 indicates that the PLGA–PEG–PLGA physical hydrogel was transparent immediately over the sol–gel transition boundary, but became opaque when the temperature is relatively far above the sol–gel transition temperature. The underlying mechanisms of the sol–gel transition and the transition form a transparent gel to an opaque gel are not fully understood so far, although some preliminary assumptions have been suggested by us (Yu et al., 2007). The present study demonstrated that the addition of a PEGylated drug decreased the sol–gel temperature significantly, as shown in Fig. 6. The result accordingly led to the decrease of the transparent–opaque transition temperature. So, the observation temperature (37 °C) was above the transparent–opaque transition temperature of the PEGylated-drug-loaded system while lower than that of the drug-free system. Such a PEG-induced transition temperature adjustment accounts for the opaque versus transparent behaviors in Fig. 10. The gradual change of drug concentration leads to the movement of the transparency frontier.

The quantitative measurements of height change of the transparent gel as a function of time was shown in Fig. 11. The underlying diffusion of our PEGylated drug in the block copolymer hydrogel is a typical case-I diffusion well described by Higuchi equation or the Peppas-Korsmeyer equation with the case-I release exponent ($n = 0.5$), as indicated in Fig. 12. A smooth line

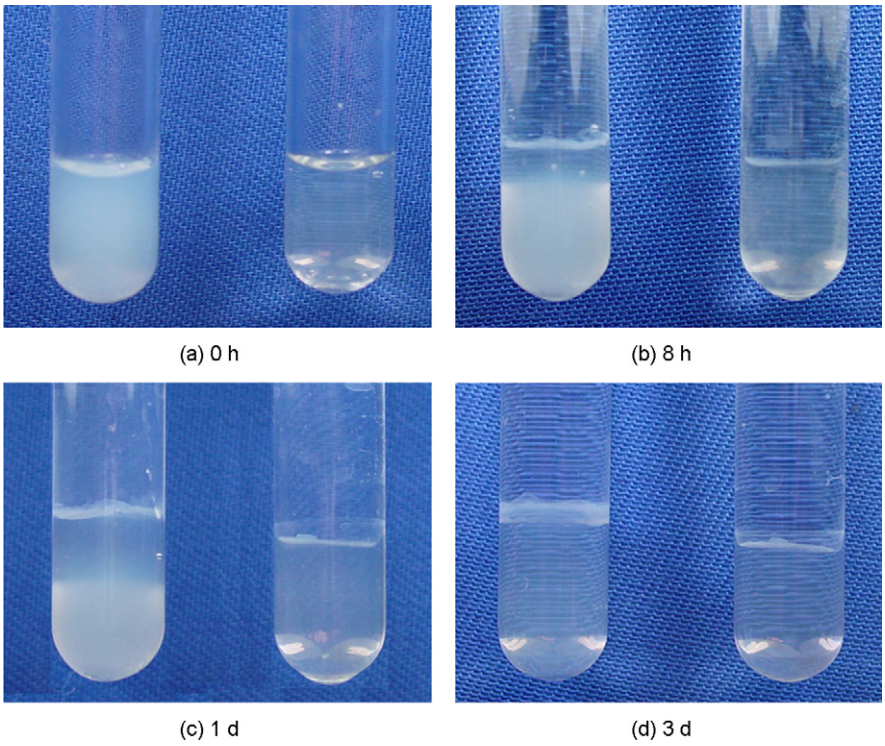



Fig. 10. Optical images of a drug loading hydrogel immersed in a large amount of PBS at 37 °C after the marked immersing time. The PBS level was highly above the upper edge of the hydrogel and thus beyond the display field in these images. The left vial is the release system (25 wt% copolymer-1, the MPEG-CPT-loading was 2%, w/w), while the right is the control with a drug-free hydrogel (25 wt% copolymer-1).

in the double-logarithm plot of $Q$ versus $t$ was observed in the whole time region of the first release stage, and no abrupt change was found after the whole hydrogel was transparent. Hence, both opaque and transparent hydrogels exhibit a similar drug release kinetics, and the transparency frontier has not led to significant complexity of the diffusion-controlled release. Our experiments as shown in Fig. 10 afford thus a good approach to visualize the release process of MPEG-CPT out of PLGA–PEG–PLGA hydrogels in the early stage, with employment of the effect of PEGylated drug on the transition temperature of the gellable system. The observation supports that the first stage of the drug release obeys a diffusion mechanism.

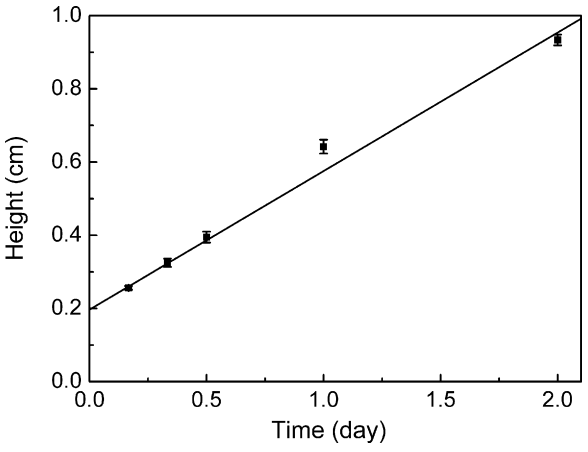



Fig. 11. Time evolution of height of transparent gel in the early stage of the in vitro drug release. The release system is shown in Fig. 10. $n = 3$ for statistics.

### 3.6. *In vivo anti-tumor effects of MPEG-CPT released from PLGA–PEG–PLGA hydrogels in mice*

The in vivo release of MPEG-CPT from the PLGA–PEG–PLGA hydrogel and associated anti-tumor effects against murine S-180 sarcoma was also observed. Fig. 13 shows the S-180 sarcomas extracted at the eighth day after a subcutaneous injection of PLGA–PEG–PLGA aqueous solution containing

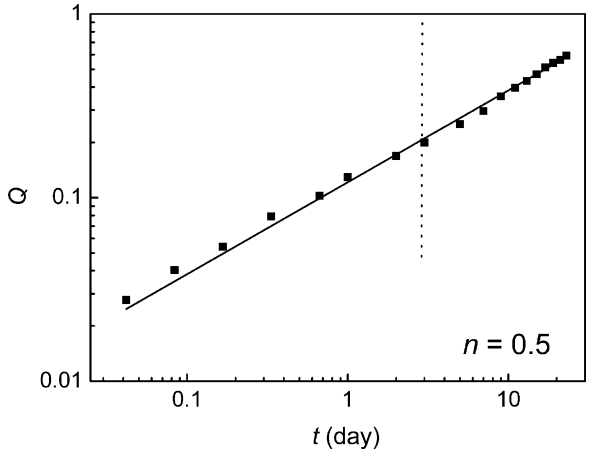



Fig. 12. The log–log plot of accumulated release of the PEGylated drug $Q$ vs. release time $t$ in PBS at 37 °C in the first stage with $Q < 0.6$. The release system is 25 wt% copolymer-1and 2% (w/w) MPEG-CPT-loading. The solid line denotes the theoretical result of the Peppas-Korsmeyer equation (Eq. (2)) with $n = 0.50$ or the Higuchi equation (Eq. (1)). The dashed line denotes the time after which the whole hydrogel as shown in Fig. 10 was transparent.

Table 3
In vivo anti-tumor effects of released MPEG-CPT against murine S-180 sarcoma

| Group[a] | Dose[b] | Route of medication[c] | Animals | | Mean weight of animal (g) | | Mean weight of tumor (g) | Tumor inhibition ratio (%) | $p^d$ |
|---|---|---|---|---|---|---|---|---|---|
| | | | Start | End | Start | End | $x \pm$S.D. | | |
| 1 + 2: NS | | i.v. | 16 | 16 | 19.5 | 34.3 | 2.08 ± 0.58 | | |
| 3: 5-FU | 50 mg/kg | i.v. | 8 | 8 | 19.3 | 28.9 | 0.81 ± 0.17 | 61.1 | <0.01 |
| 4: Copolymer-1 + MPEG | 3.0% (w/w) | s.c. | 8 | 8 | 19.4 | 29.5 | 1.43 ± 0.63 | 31.3 | |
| 5: Copolymer-1 + MPEG-CPT | 0.5% (w/w), ~9.31 mg/kg | s.c. | 8 | 8 | 19.3 | 29.0 | 1.61 ± 0.65 | 22.6 | |
| 6: Copolymer-1 + MPEG-CPT | 1.5% (w/w), ~28 mg/kg | s.c. | 8 | 8 | 19.4 | 28.1 | 0.73 ± 0.31 | 64.9 | <0.01 |
| 7: Copolymer-1 + MPEG-CPT | 3.0% (w/w), ~56 mg/kg | s.c. | 8 | 5 | 19.0 | 24.0 | 0.56 ± 0.33 | 73.1 | |

[a] NS: the negative control with just the physiological saline solution injected; 5-FU: the positive control with a sufficient amount of 5-FU injected twice.
[b] 3% w/w, 1.5% w/w, 0.5% w/w denote the MPEG-CPT drug-loading concentrations in 18 wt% of copolymer-1 aqueous solution; 56, 28, 9.31 mg/kg denote the whole drug dose (pure CPT content under consideration of CPT/MPEG-CPT = 1/16) relative to weight of mouse; 50 mg/kg denotes the 5-FU drug dose of every time, and at the zeroth and third days the drug was intravenously injected.
[c] i.v.: intravenous injection, s.c.: subcutaneous injection.
[d] The $p$ values of groups 4 and 5 were not calculated because the drug in the animal experiment was not of efficacy unless the tumor inhibition ratio ≥40%. The $p$ value of group 7 was neither calculated because nearly half mice died after 7 days due to toxicity of an over amount of CPT loaded.

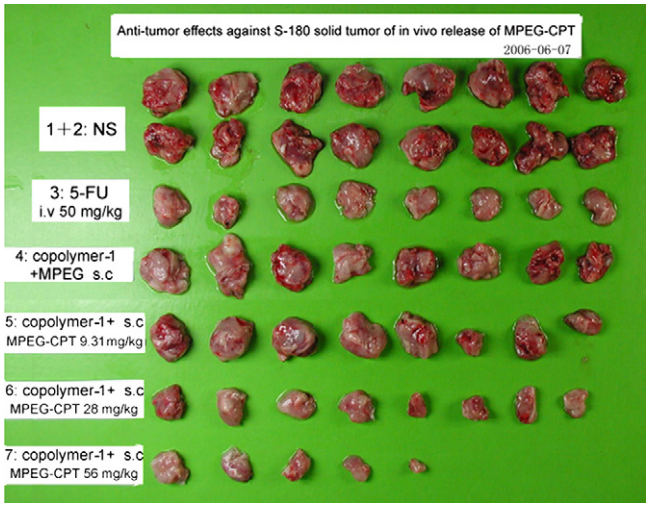



Fig. 13. S-180 sarcomas captured at the seventh day after a subcutaneous injection of PLGA–PEG–PLGA aqueous solution (18 wt% of copolymer-1) containing the marked amount of MPEG-CPT into mice. i.v.: intravenous injection; s.c.: subcutaneous injection; NS: the negative control with just the physiological saline solution injected; 5-FU: the positive control with a sufficient amount of 5-FU injected twice.

MPEG-CPT into mice. The quantitative measurements and associated statistics were also performed, as shown in Table 3. According to the mice experiments, the hydrogel matrix could control the in vivo release of MPEG-CPT. Significant anti-tumor effects were achieved under an appropriate drug loading (group 6 in Fig. 13 and Table 2).

The in vivo experiments also evidently demonstrate that the activity of CPT was retained well after chemically linked with MPEG, encapsulated into and released from the physical hydrogels. It is well-known that CPT is not very stable and the Δ-hydroxylactone ring of CPT could be opened to a carboxylate form in a pH-dependent equilibrium. At physiological pH, more than 80% of lactone ring of CPT are opened to form the carboxylate, whereas at pH below 5, all CPT is in the lactone form (Tong et al., 2003). Only the lactone form of CPT is of anti-tumor efficacy; but it is hard to keep CPT in an acid environment in mammals except in gastrointestinal tract. The degradation of PLGA leads to an acid local environment (Park, 1995; Wu and Ding, 2004; Zolnik et al., 2006). The acid degradation product is not good in most of cases as a biomaterial, but fortunately, it might be beneficial for keeping the activity of CPT encapsulated in the PLGA–PEG–PLGA hydrogel.

It seems worthy of noting that the subcutaneous injection site of the drug-loaded hydrogel in our mice experiments was different from the sarcoma location. So, after MPEG-CPT released from hydrogel, the MPEG-CPTs might enter into the circulation of body.

## 4. Conclusion

The biodegradable thermogelling PLGA–PEG–PLGA copolymers with the different compositions were synthesized as a sustained drug carrier, along with MPEG-CPT as a model of a PEGylated drug. By formation of a nano-particle, the

PEGylation of CPT enhanced the solubility of the hydrophobic CPT in water to several orders of magnitude. The sustained release of MPEG-CPT from PLGA–PEG–PLGA hydrogels was confirmed, which lasted for 1 month. The early release was sensitive to polymer concentration but less sensitive to drug loading. While the late release was a combination of diffusion and degradation in such a biodegradable hydrogel, the first-stage release was basically diffusion-controlled. In vivo experiments confirmed the anti-tumor efficacy of the PEGylated CPT released from the PLGA–PEG–PLGA hydrogels. Our in vitro observations also found that inclusion of the PEGylated drug modified the material gelling temperature. As a result, this paper has confirmed that the thermosensitive physical gelling of PLGA–PEG–PLGA aqueous solution could be extended as a good injectable biomaterial and a long-term sustained release carrier of a PEGylated drug. Meanwhile, our researches reveal that the unique properties of a PEGylated drug should be taken into consideration, especially the effects of the decrease of sol–gel transition temperature and the increase of viscosity on "injectability" of the drug-loaded polymer solution. Some other questions such as PEG length effects are still open and thus further studies are called for.

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