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# pH triggered injectable amphiphilic hydrogel containing doxorubicin and paclitaxel

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ABSTRACT

Injectable hydrogel with hydrophobic microdomains for incorporating both hydrophilic and hydrophobic drugs, herein doxorubicin hydrochloride (DOX) and paclitaxel (PTX), was synthesized through dynamic bonding of glycol chitosan and benzaldehyde capped poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol) via Schiff's reaction triggered by environmental pH. Rheology tests show that the inclusion of hydrophilic drug decreases the gelation time and gains more robust gel, while the addition of hydrophobic drug has opposite influences. Dual-drug release from the DOX + PTX loaded gels was observed and the release rate can be accelerated by decreasing the environmental pH from physiological (7.4) to weak acidic pH (6.8). In vivo investigation proved that the gels were able to diminish the amount of DOX in blood circulation and limit the DOX-induced cardiotoxicity. By intratumoral administration, the hydrogel-drug formulations resulted in efficient growth inhibition of subcutaneous tumor (B16F10) on C57LB/6 mouse model. The advantage of the current system for DOX + PTX combination therapy was demonstrated by a prolongation of survival time in comparison with the single drug therapy.
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## 1. Introduction

Doxorubicin (DOX) and paclitaxel (PTX) are common antitumor drugs used in clinical applications (Dombernowsky et al., 1995; Gianni et al., 1995). DOX acts as a DNA intercalating agent to inhibit further DNA and RNA biosynthesis (Goodman et al., 1977) and PTX stabilizes cellular microtubes polymerized in G2 mitotic phase thus prevents the cell division (Harper et al., 1999). Therefore the combination of DOX with PTX becomes a valid option in the chemotherapy of various kinds of solid tumors (Gehl et al., 1996). However, while the combination therapy indeed shows high activity, increasing toxic effects was found accompanied with the combination of the two drugs (Gianni et al., 1995; Harper et al., 1999), which could lead to the failure of the treatment (Gehl et al., 1996). It becomes a key issue to establish new advanced drug delivery systems (DDS) (Ahmed et al., 2006). Up to now the challenge still remains on efficiently hampering the non-specific uptake of antitumor drugs to target healthy organs when dosed by intravenous injection (Al-Abd et al., 2010). Therefore localized delivery was considered as an alternative way to diminish the drug level in the non-targeting sites (Al-Abd et al., 2010; Ta et al., 2008). In recent years, a number of intratumoral delivery systems have been developed based on polymers, including hydrogels (Al-Abd et al., 2010; Cho et al., 2009; Chun et al., 2009a,b; Lee et al., 2010; Yu et al., 2008), microparticles (Lin et al., 2000; Xie et al., 2007), nanoparticles (Chang et al., 2010) and nanofibers (Xu et al., 2009) etc.

Injectable hydrogels have demonstrated numerous advantages compared to conventional dose forms (Hoffman, 2002; Jeong et al., 1997; Qiu and Park, 2001; Yu and Ding, 2008) and been extensively studied for localized antitumor drug delivery (Hoffman, 2002; Qiu and Park, 2001). Such in situ forming gel systems can provide drug localization within the tumor site, perform sustained or stimuli triggered drug release, and at the same time reduce the surgical invasiveness and the frequency of administration. To date, different kinds of injectable gels have been synthesized using polymers which can crosslink through either physical interaction in response to external stimuli such as pH and temperature (Chang et al., 2009; Chiu et al., 2009; Khutoryanskaya et al., 2008; Kissel et al., 2002; Ruel-Gariepy and Leroux, 2004) or the in situ formation of chemical bonds under physiological conditions (Ding et al., 2010; Nishi and Jayakrishnan, 2007; Tan et al., 2009; Weng et al., 2008). And the co-delivery of DOX using the injectable gels with other drugs like mitoxantrone (Bouhadir et al., 2001), cisplatin (Konishi et al., 2005), aspirin (Wei et al., 2009), 5-fluorouracil (5FU) and

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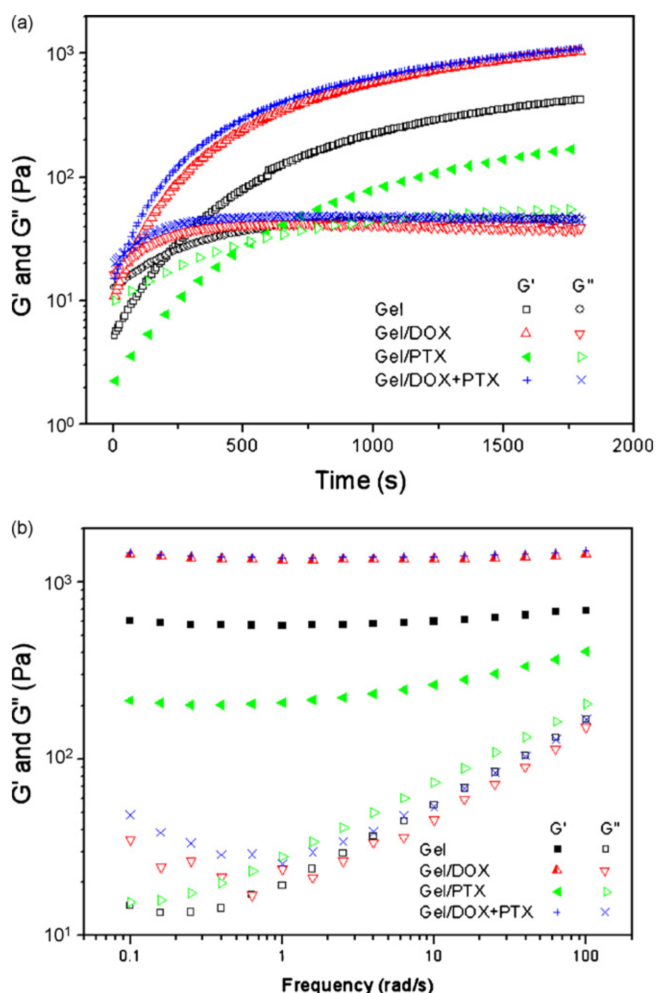



**Fig. 1.** Time (a) and frequency (b) sweeping of neat GC/OHC-PEO-PPO-PEO-CHO gel, DOX loaded gel, PTX loaded gel and DOX + PTX loaded gel at pH 7.4. Drug loading: 7.5 mg/mL for each drug.

leucovorin calcium (Zhang et al., 2010) has also been addressed. However the use of injectable gel for the delivery of DOX and PTX was rarely reported probably due to the distinct solubility characteristics of the two drugs. While DOX is a water soluble drug, PTX is water insoluble and requires to formulate with solubilizer and solvents, or to conjugate on water soluble polymers in order to improve the solubility (Lee et al., 2010). For topical delivery of PTX, in situ gelling amphiphilic block copolymers were frequently used (Chun et al., 2009b; Shim et al., 2007; Zentner et al., 2001). Otherwise, Obara et al. has used Cremophor® EL to enhance the loading of PTX in their photocrosslinked chitosan hydrogels (Obara et al., 2005). Nevertheless, with the aim of combination chemotherapy, it is desirable for the two drugs being carried simultaneously in a single formulation.

In previous study, we developed chemically crosslinked injectable gels based on glycol chitosan (GC) and benzaldehyde terminated poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol) (OHC-PEO-PPO-PEO-CHO) (Ding et al., 2010). The two polymers crosslink through an imination reaction triggered by the increase of environmental pH from acidic to neutral. In vitro characterization proved that the formed hydrogels were able to encapsulate and release either hydrophilic or hydrophobic drugs and in vivo tests revealed that the gel was degradable in approximately a month in rats. In the present work, we attempt to further investigate the GC/OHC-PEO-PPO-PEO-CHO injectable gel on the combination delivery of anti-tumor drugs, i.e. DOX and PTX. The gelation and drug release behavior of the GC/OHC-PEO-PPO-PEO-CHO formulations were studied in vitro and the antitumor activity was evaluated on mouse model.

**Table 1**
Influence of DOX and PTX content on the gelation time and elastic modulus of GC/OHC-PEO-PPO-PEO-CHO formulations at 37 °C and pH 7.4.*

| DOX conc. (mg/mL) | PTX conc. (mg/mL) | Gelation time (s) | $G'$ (kPa)** |
|---|---|---|---|
| 0 | 0 | 217 | 0.6 |
| 4.5 | 0 | 250 | 0.9 |
| 7.5 | 0 | 58 | 1.3 |
| 0 | 4.5 | 378 | 0.5 |
| 0 | 7.5 | 524 | 0.2 |
| 7.5 | 7.5 | 45 | 1.4 |

* Polymer concentration was kept at 5 wt% for GC and 3.35 wt% for OHC-PEO-PPO-PEO-CHO.
** Value at a frequency of 1 rad/s in the frequency sweeping test.

## 2. Experimental

### 2.1. Materials and animals

Glycol chitosan (GC, Mw ~ 125 kDa), doxorubicin hydrochloride (DOX), Dulbecco's modified Eagle's medium (DMEM) and phosphate buffered saline (PBS) were purchased from Sigma–Aldrich (St. Louis, USA). Paclitaxel (PTX) was purchased from Beijing Norzer Pharmaceutical Co. Ltd. Fetal bovine serum (FBS) was purchased from Hangzhou Sijiqing biological engineering material Co. Ltd., China, and was inactivated under 50 °C for half an hour before use. Benzaldehyde capped poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol) (OHC-PEO-PPO-PEO-CHO, 40 wt% EO, Mn ~ 3000 Da) was synthesized according to our former work (Ding et al., 2010). Solvents and other compounds were all obtained form Beijing Chemical Reagents Company, China.

Female C57LB/6 mice (19 ± 2 g) were obtained from the Beijing Xing-Long Laboratory Animal Co. Ltd., China, and raised under normal conditions with free access to food and water. All care and handling of animals were performed according to the Guiding Principles for the Care and Use of Experiment Animals in Beijing Obstetrics and Gynecology Hospital.

### 2.2. Methods

#### 2.2.1. Preparation of polymer-drug formulation

GC and OHC-PEO-PPO-PEO-CHO was dissolved in phosphate buffer (pH 7.4) at a desired concentration. PTX/OHC-PEO-PPO-PEO-CHO solution was prepared by further dispersed the drug powder in the OHC-PEO-PPO-PEO-CHO solution by probe sonication for 5 min using a JY-96 probe sonicator (Zhejiang Xinzhi, China, output 150 W). Meanwhile, DOX/GC mixture was prepared by stirring known amount of DOX in the GC solution. For investigating the gelation behavior and drug release, formulations were made by mixing equal value of GC and OHC-PEO-PPO-PEO-CHO solutions or their drug dispersions (0.5 mL each) by a vigorous stirring for 10 s. For in vivo tests, the GC/OHC-PEO-PPO-PEO-CHO formulations were prepared under the same procedure as described above, except the pH of the polymer solutions were adjusted to be 5.0 by adding small amount of 2 M HCl in order to keep the mixture in sol state before injection. The final concentration of GC and OHC-PEO-PPO-PEO-CHO for the in vivo experiments was kept at 5 and 3.35 wt% respectively.

#### 2.2.2. Characterization

Rheological behavior of the GC/OHC-PEO-PPO-PEO-CHO formulations was characterized with a TA AR2000ex stress-controlled

rheometer (TA Instruments, USA), equipped with a parallel plate of 20 mm in diameter. For each measurement, the mixture of GC and OHC-PEO-PPO-PEO-CHO solutions or the polymer-drug dispersions prepared in the former step were immediately poured onto the plate preheated at 37 °C. The measurement was then started with a gap of 0.7 mm. Both elastic (G′) and loss (G″) moduli were monitored as a function of time to characterize the gelation property of the formulation. The time at the crossover of G′ and G″ was defined as the gelation time. The frequency sweeping tests were performed from 0.01 to 100 rad/s at a given strain amplitude of 2%, ensuring a linear viscoelastic region, after stable gel was formed. Microstructure of the formed hydrogels was observed using a Hitachi S-4300 scanning electron microscope (SEM) on freeze-dried samples.

### 2.2.3. *In vitro drug release*

DOX/GC (0.5 mL) and PTX/OHC-PEO-PPO-PEO-CHO (0.5 mL) dispersions were mixed in a test vial and stood for 30 min to ensure the formation of hydrogel. The formed drug incorporated hydrogels were immersed into 10 mL of 0.01 M PBS (pH 7.4 or 6.8) containing 0.1% (v/v) tween-80 at 37 °C (Lee et al., 2002). 2 mL of the release medium was collected periodically and replenished with 2 mL of fresh PBS. PTX was extracted three times from the release media with dichloromethane and the organic phase was dried under nitrogen flux. The residue was reconstituted in 0.5 mL of mobile phase and quantitated using reversed-phase high performance liquid chromatography (RP-HPLC, Agilent 1200, Agilent Technologies Inc., USA) with a UV detector set at 227 nm. 20 μL of sample was injected into a ZORBAX Eclipse Plus C18 column (150 mm × 4.6 mm, 5.0 μm, Agilent Corp., USA) and eluted with the mobile phase consisted of acetonitrile/water/methol (48/41/11, v/v/v) at a flow rate of 1 mL/min. The amount of DOX in the release medium was determined spectrophotometrically at a wavelength of 485 nm. The release results were plotted as mean value of three repeated tests.

### 2.2.4. *Tumor implantation*

B16F10 cells were cultured using DMEM supplemented with 10% FBS under 37 °C in 5% $CO_2$, 95% humidified atmosphere in an incubator and subcultured every 48 h. The cells were harvested by trypsinization using a 0.05% (w/v) trypsin/0.03% (w/v) EDTA solution and were subsequently centrifugal washed by sterile PBS three times to remove protein and then re-dispersed in PBS. The suspension of B16F10 cells ($1.2 \times 10^6$ cells in 0.2 mL of sterile PBS) were injected subcutaneously in the right flank of C57LB/6 mice using 26G needles. After 7 days the tumor was palpable and reached a size of ca. 300 $mm^3$ and the mice were randomly divided into groups ($n$ = 5–6) for in vivo tests described below.

### 2.2.5. *Plasma pharmacokinetics and cardiotoxicity*

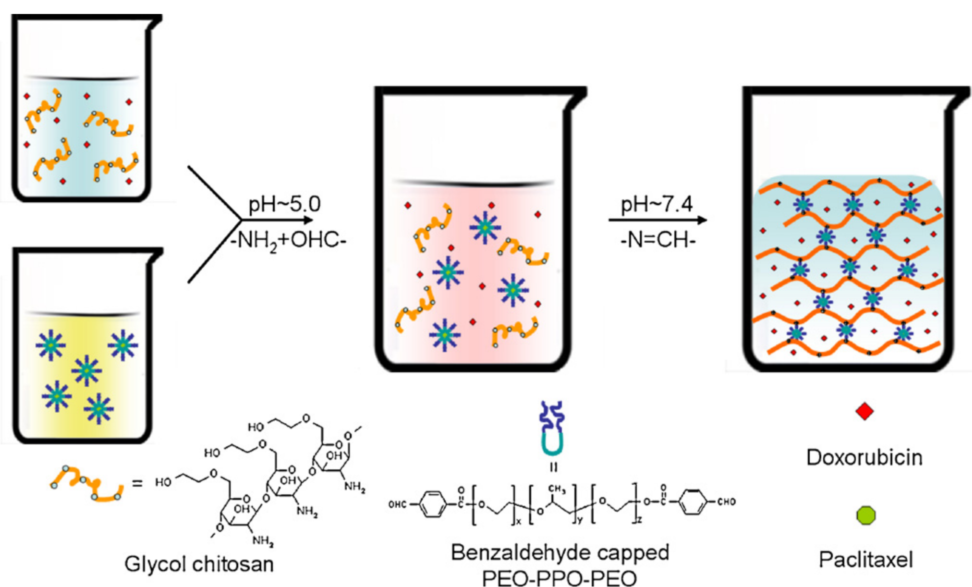
Fifteen healthy C57LB/6 mice were randomly divided in 3 groups ($n$ = 5), and were administrated by a single dose of DOX formulations at 20 mg/kg through (1) intravenous injection (i.v.) of DOX solution (0.1 mL in saline) via tail vein (Group A), (2) subcutaneous injection (s.c.) of the drug solution (0.1 mL in saline) in the right flank (Group B) and (3) subcutaneous injection of 0.1 mL of the mixture of DOX loaded GC/OHC-PEO-PPO-PEO-CHO solution (pH = 5.0) in the right flank (Group C). Besides, five tumor bearing mice were also dosed intratumorally by 0.1 mL of the DOX incorporated GC/OHC-PEO-PPO-PEO-CHO solution (pH = 5.0) (Group D).

After the injection, 100 μL blood was collected using heparinized tubes at predicted times from the tail vein of the mice (Etrych et al., 2008). The blood samples were immediately centrifuged at 3000 rpm for 10 min and the separated plasma were stored at −20 °C until the analysis of DOX content using HPLC (Wang et al., 2006). For the analysis, 150 μL of acetonitrile was added to 50 μL of plasma. The precipitates in the mixture were removed by centrifugation and the concentration of DOX was measured by injecting 20 μL of the supernatant into the HPLC. A ZORBAX Eclipse Plus C18 column was used for the measurements and the UV detector set at 485 nm. The mobile phase was consisted of acetonitrile and water (32:68, v/v) with pH adjusted to 3.0 using phosphoric acid. The flow rate was of 1 mL/min and the limit of quantitation (LOQ) was determined to be 1 ng/mL.

The animals were sacrificed at day 12 post administration. Heart and tumor was isolated and fixed in 10% neutral-buffered formalin, then embedded in paraffin, sectioned at 4–6 μm and stained with hematoxylin and eosin (H&E) using standard techniques for histopathological examination.

### 2.2.6. *Antitumor activity*

The B16F10 tumor bearing mice were randomly divided into five groups ($n$ = 6) and received following treatments. Group 1, mice were untreated as control group; Group 2, mice received an intra-




**Scheme 1.** Preparation of injectable GC/OHC-PEO-PPO-PEO-CHO gel containing DOX and PTX.

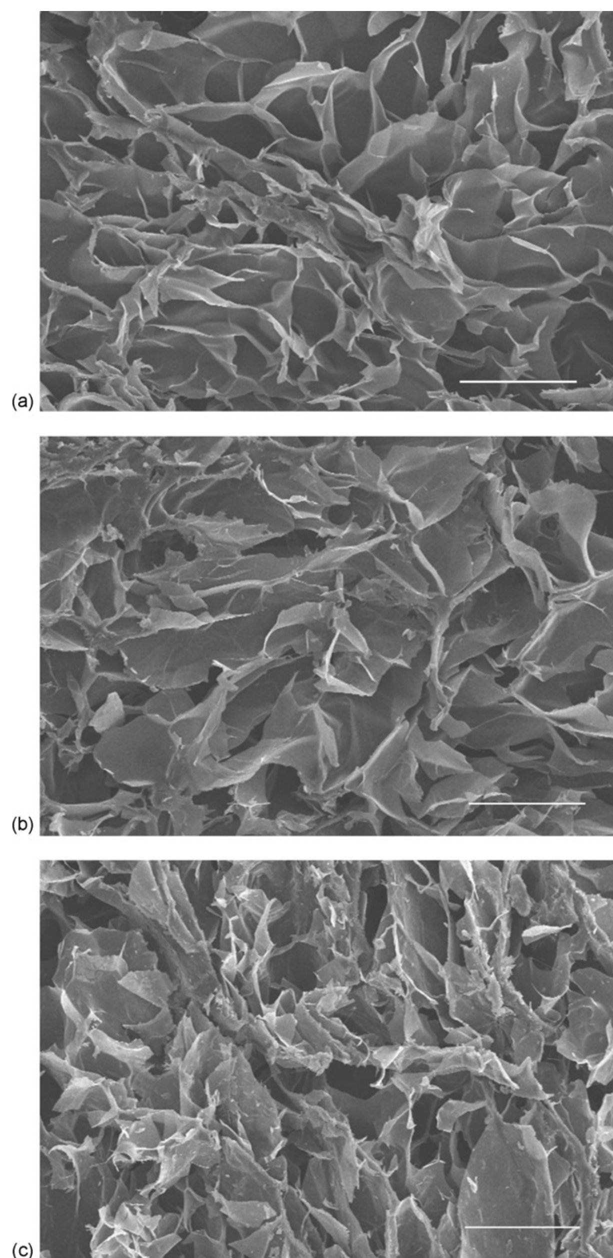



**Fig. 2.** SEM images of freeze-dried GC/OHC-PEO-PPO-PEO-CHO gel (a), DOX loaded gel (b) and PTX loaded gel (c). Scale bar = 100 μm.

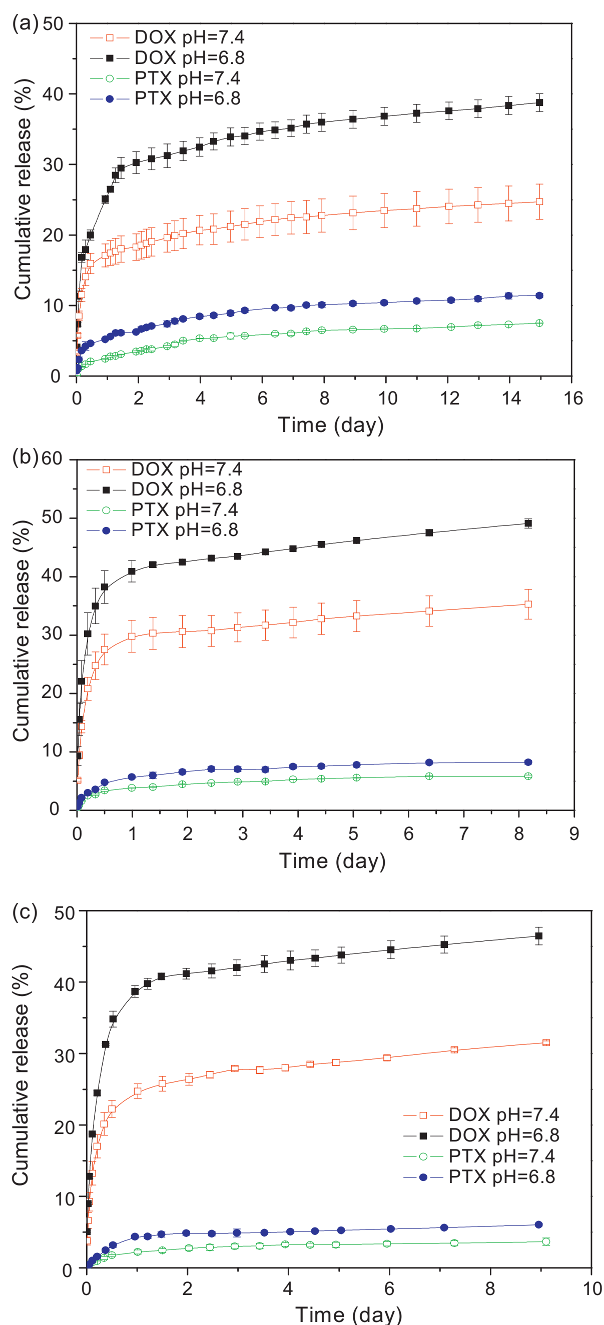



**Fig. 3.** Cumulative release of DOX and PTX from the dual-drug loaded hydrogels immersed in 10 mL of saline at different pHs. During gel preparation, the eventual GC concentration was 5 wt% with an OHC-PEO-PPO-PEO-CHO content of 3.35 wt% (a and b) and 2.35 wt% (c). The DOX and PTX loadings in each formulation were equal which were 7.5 mg/mL (a and c) and 4.5 mg/mL (b).

tumoral injection of 0.1 mL of GC/OHC-PEO-PPO-PEO-CHO solution at pH 5.0; Group 3, mice received an intratumoral injection of DOX incorporated GC/OHC-PEO-PPO-PEO-CHO solution (40 mg/kg in 0.1 mL); Group 4, mice received an intratumoral injection of DOX and PTX incorporated GC/OHC-PEO-PPO-PEO-CHO solution (40 mg/kg for each drug in 0.1 mL); Group 5, mice received an intratumoral injection of PTX incorporated GC/OHC-PEO-PPO-PEO-CHO solution (40 mg/kg in 0.1 mL). The mice were weighted and tumors were measured using a caliper for 20 days post administration. The tumor volume was calculated using the formula $a^2 \times b/2$, where a is the short diameter and b is the long diameter.

### 2.2.7. Statistics analysis

Statistical significance was assessed using Student's *t*-test and Kaplan–Meier analysis. The difference was considered to be statistically significant if the probability value was less than 0.05 ($p < 0.05$).

## 3. Results and discussion

### 3.1. Gel formation

Injectable gels have been developed as a promising system for the topical dose of antitumor drugs (Chun et al., 2009b; Yu and Ding, 2008). However, while the in situ gelling systems for carrying either DOX or PTX have been reported respectively and evaluated both in vitro and in vivo, few works have addressed the development of injectable gel with flexible ability to deliver DOX and PTX. In the present work, the GC/OHC-PEO-PPO-PEO-CHO injectable gel is able to in situ incorporate both the drugs due to the inclusion

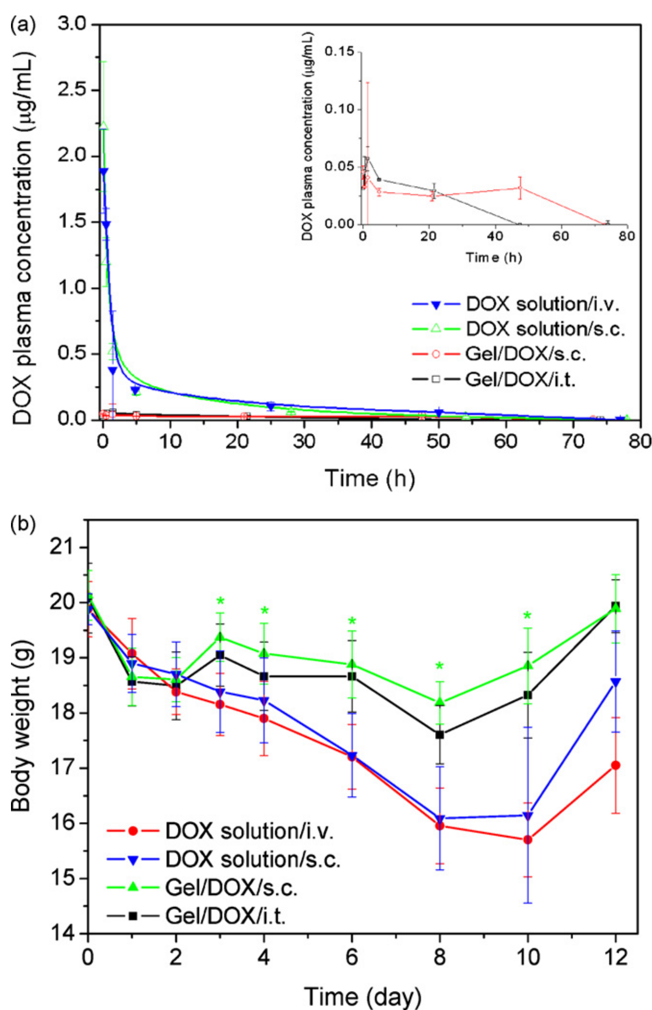



**Fig. 4.** Plasma DOX concentration–time profiles (a) and body weight (b) of mice received intravenous injection, subcutaneous injection of free DOX solution, and subcutaneous injection, intratumoral injection of DOX loaded GC/OHC-PEO-PPO-PEO-CHO formulations. The drug dose was 20 mg/kg. Statistics analysis: $^{*}p < 0.05$, Gel/DOX/s.c. vs. DOX solution/s.c.

of amphiphilic macromolecular crosslinker (Ding et al., 2010). The incorporation procedure is illustrated in Scheme 1. PTX was first solubilized by the OHC-PEO-PPO-PEO-CHO micelles, forming stable dispersion up to a drug concentration of 15 mg/mL in the polymer solution (3–7 wt%) with no PTX precipitates observed up to several hours. DLS measurements confirmed that the size of OHC-PEO-PPO-PEO-CHO aggregation swelled from $403 \pm 4$ nm to $527 \pm 57$ nm (15 mg/mL PTX in 6.7 wt% polymer solution) after the addition of PTX, indicating the drug solubilization (Huh et al., 2005). Thus the PTX was eventually incorporated in the formed hydrogels. Meanwhile, DOX could be well dissolved in 10 wt% GC solution and then was encapsulated in the aqueous phase of the gels.

The gelation behavior of the GC/OHC-PEO-PPO-PEO-CHO formations was investigated by rheology (Fig. 1). By mixing the GC and OHC-PEO-PPO-PEO-CHO solutions, hydrogel formed at ca. 200 s at 37 °C and pH 7.4 with an eventual concentration of 5 wt% and 3.35 wt% respectively, while it kept in sol state if the solution pH was adjusted to 5.0. The addition of drugs in the polymer solutions influenced the gelation time as well as the moduli of the formed hydrogels (Fig. 1 and Table 1). As listed in Table 1, it is revealed that the galtion was faster in case DOX was mixed in the solution at 7.5 mg/mL, whereas it became slower with the addition of PTX at 4.5 and 7.5 mg/mL. Meanwhile, the elastic modulus (G′) of the hydrogels increases with the DOX content, but decreases with PTX incorporation. This is because the solubilization of PTX increases of the aggregation number of the OHC-PEO-PPO-PEO-CHO micelle, hence decreases the crosslink points during gel formation, and therefore affects the microstructure of the hydrogel. SEM observation shows that the pore size is less uniform in the PTX loaded hydrogel, when compared to the neat and DOX loaded gels (Fig. 2). However, due to the chemical crosslinkage, i.e. the benzoic-imine bond, the G′ of the PTX loaded gels is independent with shear frequency from 0.1 to 100 rad/s indicating that the gel is robust (Fig. 1b). The loading of DOX resulted in more robust gels, probably due to the electrostatic repulsion and the formation of hydrogen bond between DOX and the GC backbone. Comparably, the gelation behavior of the DOX + PTX loaded gel is close to that of the DOX loaded gel, implying that the DOX–GC interaction is dominating in the drug loaded gels.

### 3.2. In vitro drug release

Drug release from the DOX + PTX GC/OHC-PEO-PPO-PEO-CHO hydrogels is plotted in Fig. 2. Sustained release of the two drugs is observed for up to two weeks despite the change of polymer composition and initial drug loading in the gels. The release rate of DOX is much faster than that of PTX, and was more obviously influenced by the change of GC/OHC-PEO-PPO-PEO-CHO ratio and drug loading (Fig. 3). A 30% decrease of crosslinker content (Fig. 3b) or a 40% decrease of drug loading (Fig. 3c) led to the increase of DOX cumulative release from 22% (Fig. 3a) to 31 and 34% respectively at day 7. Comparably, the change of PTX release rate is relatively minor. The results confirm that PTX was well embedded in the matrix (Xu et al., 2009). On the contrary, most of DOX molecules were dispersed in the aqueous phase and easily diffused out. The release of the two drugs could be simultaneously accelerated by decreasing the media pH from 7.4 to 6.8, due to the characteristics of the benzoic-imine linkage in the hydrogel, i.e. stable at physiological pH and labile at acidic pH (Ding et al., 2010), while the solubility of the drugs has limit difference in this pH interval. As shown in Fig. 3a, the DOX release increases from 22% to 35% and the PTX release increases from 6% to 10% in the first week upon the slight drop of pH. The pH sensitivity of the release should be positive for the system applied in tumor treatment because the extracellular pH of solid tumors is lower than normal tissues (Wike-Hooley et al., 1984). Calculation based on Fig. 3 showed that the PTX release followed non-Fickian mechanism whereas DOX was released by Fickian diffusion (Lynch and Dawson, 2004). The release profile of single drug loaded gels was found similar to that of the dual drug loaded gels, besides the initial burst release of DOX was less serious in comparison with the DOX + PTX loaded gel. For example, 17% of DOX releases at 24 h and pH 7.4 as shown in Fig. 3a whereas it was 11% from the DOX loaded gel with same polymer composition (Ding et al., 2010).

### 3.3. Cardiotoxicity

The toxicity of antitumor drugs in the chemotherapy was commonly related to the drug exposure in plasma (Shikanov et al., 2008). Therefore even by localized administration it may also face a maximum tolerated dose (MTD) due to the possible diffusion of drug from the implantation site to the blood circulation. This might be more serious for the water soluble drugs like DOX. Thus the toxicity of the injectable gel regimen was examined using DOX formulations. PTX is poorly water-soluble and releases in slow release rate from the gels therefore would unlikely cause serious systematic toxicity if under a moderate dose as in this work (Zentner et al., 2001). The DOX loaded GC/OHC-PEO-PPO-PEO-CHO gel was implanted subcutaneously to the flank of healthy mice and intratumorally to tumor bearing mice at a single dose of 20 mg/kg. As controls, DOX solution was also injected to healthy mice both intra-

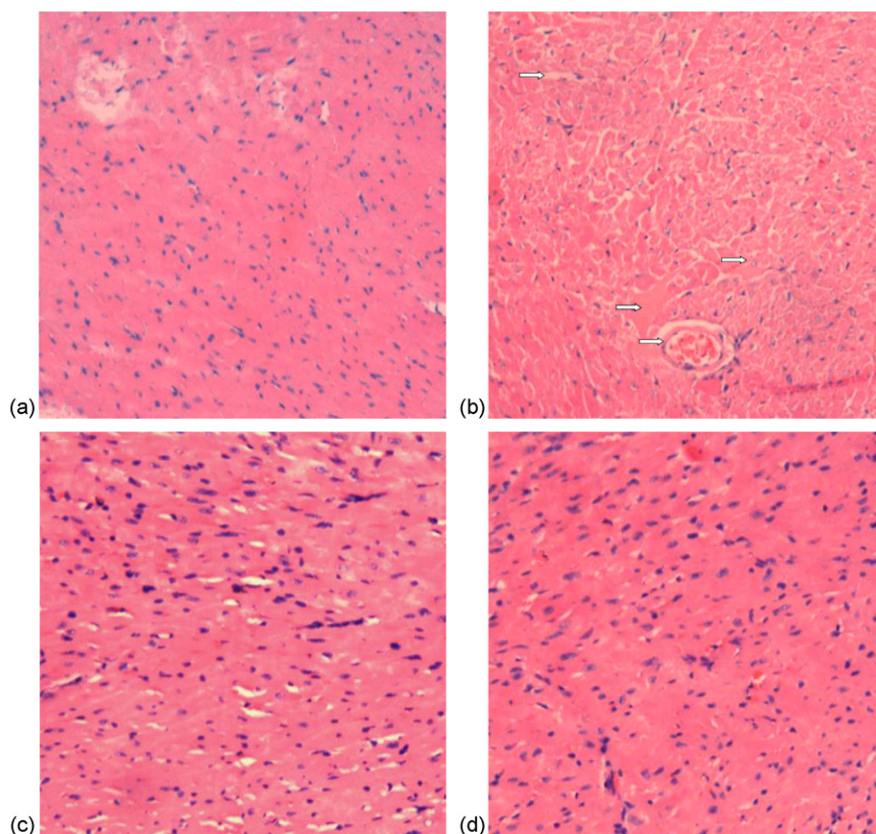



**Fig. 5.** Representative H&E stain of heart tissues isolated from untreated healthy mouse (a), and the mice 12 days after a single dose of DOX at 20 mg/kg via subcutaneous injection of drug solution (b), subcutaneous injection of DOX loaded GC/OHC-PEO-PPO-PEO-CHO formulation (c), and intratumoral injection of DOX loaded GC/OHC-PEO-PPO-PEO-CHO formulation (d). Magnification: 100×.

venously and subcutaneously at the same level. All animal received the gel treatment survived during the 12 days' observation whereas two mice died in the group received the s.c. injection of free drug. The pharmacokinetics in plasma is shown in Fig. 4a. High blood DOX concentration, i.e. $C_{max}$ = 2.2 μg/mL, was detected in the mice right after the s.c. injection of free drug (Group B) and it quickly reduced within 80 h period. This concentration–time profile is similar to that of the mice received the i.v. administration (Group A). In contrast, the mice treated by the gel formulations, no matter via s.c. or i.t. pathway, had much lower DOX concentration in plasma (Fig. 4a). The $C_{max}$ is around 0.05 μg/mL after the s.c. administration with the injectable gel (Group C), 40 times lower than that of free drug (Group B), which leads to a 4-fold decrease of the area under curve ($C_{0-\infty}$).

Cardiotoxicity was normally used for evaluating the toxicity of DOX formulations (Klein et al., 2000). As expected, the histology image shows marked myofibrillar loss, cytoplasmic vacuole and mild perivascular fibrosis in the heart of mice treated by s.c. injection of free drug (Fig. 5b). The cardiac damage is typical as that induced by systematic administration of free DOX which is understandable because their pharmacokinetics was similar. In contrast, no obvious damage in the cardiac tissue is observed from mice injected by the DOX-loaded injectable gels (Fig. 5c and d), attributed to less drug released to the circulation system. Correspondingly, the animals dosed by free DOX had serious weight loss, whereas limited changes in body weight was observed in the gel injected groups (Fig. 4b). In addition to the drug holding capacity, the injectable gels could release the drug at an efficient level to gain therapeutic effect because serious necrosis of tumor tissue was observed from the tumor bearing mice that received an i.t. injection of the DOX loaded gel (Group D) (Fig. 6). Thus, the GC/OHC-PEO-PPO-PEO-CHO gel shows promising potentials for tumor treatment by controlled delivery of toxic therapeutic agents.

### 3.4. Antitumor activity

The antitumor activity of drug loaded GC/OHC-PEO-PPO-PEO-CHO injectable gels was accessed on C57BL/6 mouse model bearing B16F10 murine melanoma cancer. B16F10 is known as an aggressive tumor which leads to high death rate of animals if not being treated (Veronese et al., 2005). In this work, a high dose of DOX, i.e. 40 mg/kg (Al-Abd et al., 2010), together with a moderate dose of PTX, i.e. 40 mg/kg (Shim et al., 2007), was applied for investigating the anticancer activity. As shown in Fig. 7, the tumor in the untreated mice (Group 1) grew very fast (Fig. 7a) and all animals died within four weeks after the subcutaneous tumor-cell implantation, with a median survival time (mST) of 12 days from starting the test (Fig. 7b). It is noted that the administration of neat gel could inhibit the tumor growth in the first week (Fig. 6a, Group 2 vs. Group 1, $p < 0.05$ on days 6–8), possibly due to the antibacterial activity of chitosan (Chun et al., 2009b), but subsequently the tumor grew again. The tumor growth rate was significantly inhibited for at least 20 days after the administration of various drug loaded gels (Fig. 7a), demonstrating the flexibility of the injectable gel system in making formulations. Despite this, the survival rate was not improved by the gels formulated with individual DOX or PTX when compared to the untreated control group (Fig. 7b). The median survival time of the mice dosed by the PTX (Group 5) and DOX (Group 3) loaded gels was 10 and 14 days respectively. PTX formulation obtained moderate antitumor efficiency due to the dose level and slower release rate (Zentner et al., 2001). The death of mice could be majorly attributed to the growth of tumor although

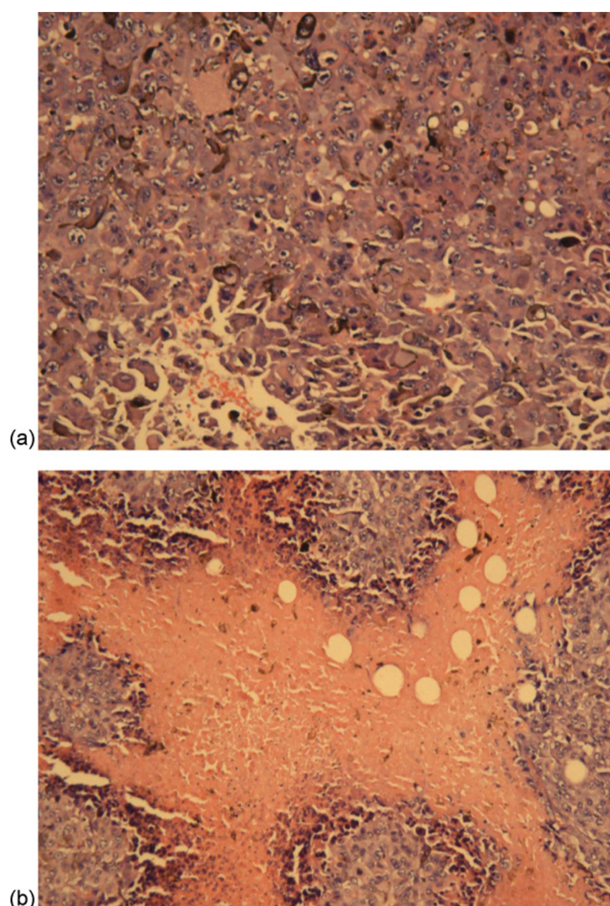



**Fig. 6.** Histological images of H&E stained tumor tissue isolated from untreated mouse (a), and mouse after an intratumoral injection of DOX loaded GC/OHC-PEO-PPO-PEO-CHO formulation at a drug dose level of 20 mg/kg for 12 days (b) where serious necrosis of tumor tissue can be seen in the center region. Magnification: 100×.

the detailed reasons are to be clarified. On the other hand, with high level of drug dose, the DOX formulations achieved more efficient tumor inhibition according to the tumor size measurements. However, the higher dose of DOX should also contribute to more drug release to the blood circulation with no therapeutic gain to the tumor, which caused toxic effect hence the death of the mice in Group 3.

It is interesting that when DOX and PTX were dosed simultaneously using the GC/OHC-PEO-PPO-PEO-CHO gel, no increasing toxic effect was observed. The animals in this group were relatively well tolerated under the extremely high drug dose, evidenced by no significant weight loss compared to the mice treated by single drug (Fig. 7c). Moreover, the median survival time of Group 4 is 23 days, meaning that the combination of the two drugs significantly improved the survival (Fig. 7b, $p < 0.01$, Group 4 vs. Group 1), besides the treatment gained similar tumor inhibition as the DOX loaded gel. As mentioned previously, former researches have proven that by systematic administration, the combination of DOX with PTX could achieve better response rate and increased tumor regression (Gehl et al., 1996; Klein et al., 2000; Sledge et al., 2003), however together with enhanced toxic effect (Gehl et al., 1996; Gianni et al., 1995), which resulted in no survival benefit (Sledge et al., 2003). Therefore our results might be important. While the therapeutic mechanism needs to be further understood, as a presumption, the gel system may lead to an enhancement on the incorporation effect of the two drugs. Compared to the systematic dose, even though the gel could not prevent the drug molecules from transferring to the blood circulation, it would significantly reduce the extent of drug spreading (Fig. 4). At the same time, the bioavailability of the drugs was preserved due to the sustained release manner (Fig. 3). Furthermore, by the localized delivery the drug molecules faced fewer barriers for penetrating into the tumor cells (Jain, 1996). Therefore more tumor cells rather than healthy cells were targeted simultaneously by the two drugs therefore the combination therapy was favored. Besides, the difference in the release kinetics of DOX and PTX should be also taken in account. In

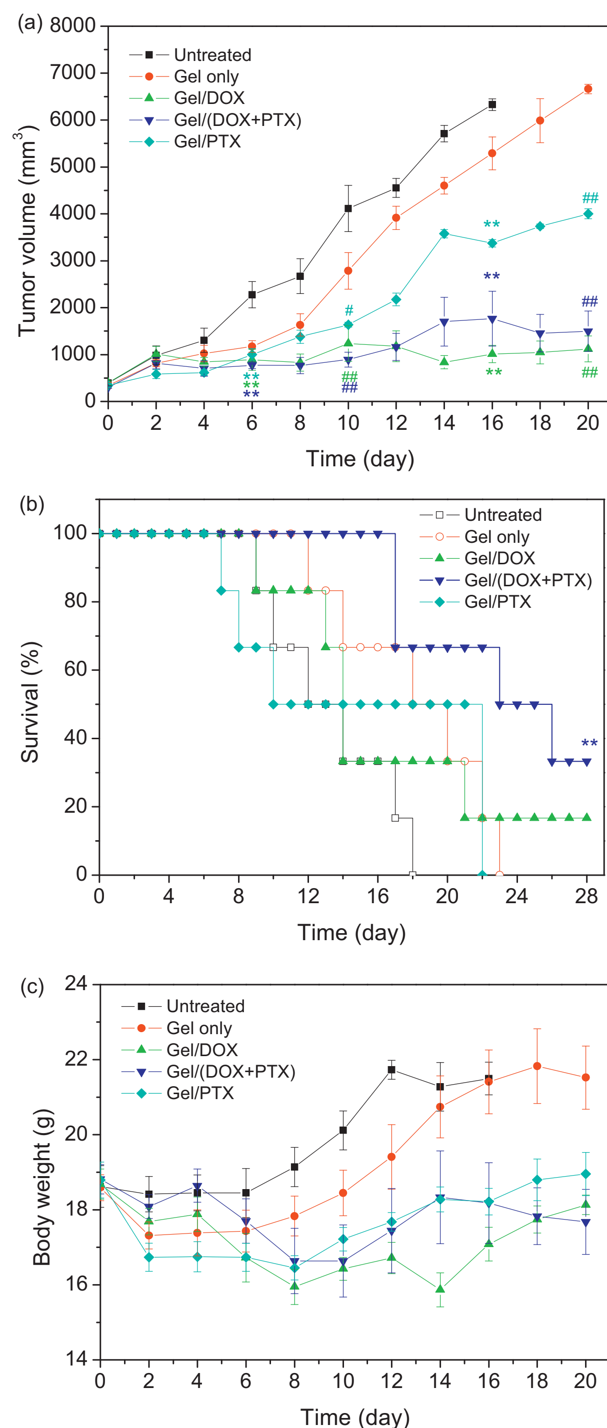



**Fig. 7.** Tumor size (a), survival rates (b) and body weight (c) of B16F10 tumor bearing mice after treated with different GC/OHC-PEO-PPO-PEO-CHO formulations. The dose level was 40 mg/kg for each drug. Statistics analysis: $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. gel only group.

the initial stage, more DOX released from the dual drug loaded gels to cause the inhibition in tumor size, whereas the PTX release was kept in longer period to improve treatment in the later stage.

## 4. Conclusions

GC/OHC-PEO-PPO-PEO-CHO injectable gel was prepared as a flexible vehicle for intratumor delivery of anticancer drugs with distinct solubility characteristics, e.g. DOX and PTX. And the drug release rate was faster at tumor pH than physiological pH. In vivo tests proved the ability of the gel as a depot to diminish the amount of drug transferring to blood circulation, hence to reduce the drug-induced toxic effect. The gel formulations exhibited tumor inhibition efficiency, no matter loaded by single or dual drugs. The activity of the current system for combined DOX and PTX therapy was demonstrated by a significant prolongation of the survival rate of the tumor bearing mice.

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## References


Ahmed, F., Pakunlu, R.I., Brannan, A., Bates, F., Minko, T., Discher, D.E., 2006. Biodegradable polymersomes loaded with both paclitaxel and doxorubicin permeate and shrink tumors, inducing apoptosis in proportion to accumulated drug. J. Control. Release 116, 150–158.

Al-Abd, A.M., Hong, K.Y., Song, S.C., Kuh, H.J., 2010. Pharmacokinetics of doxorubicin after intratumoral injection using a thermosensitive hydrogel in tumor-bearing mice. J. Control. Release 142, 101–107.

Bouhadir, K.H., Alsberg, E., Mooney, D.J., 2001. Hydrogels for combination delivery of antineoplastic agents. Biomaterials 22, 2625–2633.

Chang, G., Li, C., Lu, W., Ding, J., 2010. N-boc-histidine-capped plga-peg-plga as a smart polymer for drug delivery sensitive to tumor extracellular pH. Macromol. Biosci. 10, 1248–1256.

Chang, G., Yu, L., Yang, Z., Ding, J., 2009. A delicate ionizable-group effect on self-assembly and thermogelling of amphiphilic block copolymers in water. Polymer 50, 6111–6120.

Chiu, Y.L., Chen, S.C., Su, C.J., Hsiao, C.W., Chen, Y.M., Chen, H.L., Sung, H.W., 2009. pH-triggered injectable hydrogels prepared from aqueous N-palmitoyl chitosan: in vitro characteristics and in vivo biocompatibility. Biomaterials 30, 4877–4888.

Cho, Y.I., Park, S., Jeong, S.Y., Yoo, H.S., 2009. In vivo and in vitro anti-cancer activity of thermo-sensitive and photo-crosslinkable doxorubicin hydrogels composed of chitosan–doxorubicin conjugates. Eur. J. Pharm. Biopharm. 73, 59–65.

Chun, C., Lee, S.M., Kim, C.W., Hong, K.Y., Kim, S.Y., Yang, H.K., Song, S.C., 2009a. Doxorubicin-polyphosphazene conjugate hydrogels for locally controlled delivery of cancer therapeutics. Biomaterials 30, 4752–4762.

Chun, C., Lee, S.M., Kim, S.Y., Yang, H.K., Song, S.C., 2009b. Thermosensitive poly(organophosphazene)-paclitaxel conjugate gels for antitumor applications. Biomaterials 30, 2349–2360.

Ding, C., Zhao, L., Liu, F., Cheng, J., Gu, J., Shan-Dan, Liu, C., Qu, X., Yang, Z., 2010. Dually responsive injectable hydrogel prepared by in situ cross-linking of glycol chitosan and benzaldehyde-capped PEO-PPO-PEO. Biomacromolecules 11, 1043–1051.

Dombernowsky, P., Gehl, J., Boesgaard, M., Jensen, T.P., Jensen, B.W., Ejlertsen, B., 1995. Treatment of metastatic breast cancer with paclitaxel and doxorubicin. Semin. Oncol. 23, 13–17.

Etrych, T., Chytil, P., Mrkvan, T., Sírová, M., Ríhová, B., Ulbrich, K., 2008. Conjugates of doxorubicin with graft HPMA copolymers for passive tumor targeting. J. Control. Release 132, 184–192.

Gehl, J., Boesgaard, M., Paaske, T., Jensen, B., Dombernowsky, P., 1996. Combined doxorubicin and paclitaxel in advanced breast cancer: effective and cardiotoxic. Ann. Oncol. 7, 687–693.

Gianni, L., Munzone, E., Capri, G., Fulfaro, F., Tarenzi, E., Villani, F., Spreafico, C., Laffranchi, A., Caraceni, A., Martini, C., Stefanelli, M., Valagussa, P., Bonadonna, G., 1995. Paclitaxel by 3-hour infusion in combination with bolus doxorubicin in women with untreated metastatic breast cancer: high antitumor efficacy and cardiac effects in a dose-finding and sequencefinding study. J. Clin. Oncol. 13, 2688–2699.

Goodman, M.F., Lee, G.M., Bachur, N.R., 1977. Adriamycin interactions with T4 DNA polymerase: two modes of template-mediated inhibition. J. Biol. Chem. 252, 2670–2674.

Harper, E., Dang, W., Lapidus, R.G., Garver, R.I., 1999. Enhanced efficacy of a novel controlled release paclitaxel formulation for local–regional therapy of lung cancer tumor nodules in mice. Clin. Cancer Res. 5, 4242–4248.

Hoffman, A.S., 2002. Hydrogels for biomedical applications. Adv. Drug Deliv. Rev. 54, 3–12.

Huh, K.M., Lee, S.C., Cho, Y.W., Lee, J., Jeong, J.H., Park, K., 2005. Hydrotropic polymer micelle system for delivery of paclitaxel. J. Control. Release 101, 59–68.

Jain, R.K., 1996. Delivery of molecular medicine to solid tumors. Science 271, 1079–1080.

Jeong, B., Bae, Y.H., Lee, D.S., Kim, S.W., 1997. Biodegradable block copolymers as injectable drug-delivery systems. Nature 388, 860–862.

Khutoryanskaya, O.V., Mayeva, Z.A., Mun, G.A., Khutoryanskiy, V.V., 2008. Designing temperature-responsive biocompatible copolymers and hydrogels based on 2-hydroxyethyl(meth)acrylates. Biomacromolecules 9, 3353–3361.

Kissel, T., Li, Y.X., Unger, F., 2002. ABA-triblock copolymers from biodegradable polyester A-blocks and hydrophilic poly(ethylene oxide) B-blocks as a candidate for in situ forming hydrogel delivery systems for proteins. Adv. Drug Deliv. Rev. 54, 99–134.

Klein, J.L., Maroto Rey, P., Dansey, R.D., Karanes, C., Du, W., Abella, E., Cassells, L., Hamm, C., Peters, W.P., Baynes, R.D., 2000. Cardiac sequelae of doxorubicin and paclitaxel as induction chemotherapy prior to high-dose chemotherapy and peripheral blood progenitor cell transplantation in women with high-risk primary or metastatic breast cancer. Bone Marrow Transplant. 25, 1047–1052.

Konishi, M., Tabata, Y., Kariya, M., Hosseinkhani, H., Suzuki, A., Fukuhara, K., Mandai, M., Takakura, K., Fujii, S., 2005. In vivo anti-tumor effect of dual release of cisplatin and adriamycin from biodegradable gelatin hydrogel. J. Control. Release 103, 7–19.

Lee, J.Y., Kim, K.S., Kang, Y.M., Kim, E.S., Hwang, S.J., Lee, H.B., Min, B.H., Kim, J.H., Kim, M.S., 2010. In vivo efficacy of paclitaxel-loaded injectable in situ-forming gel against subcutaneous tumor growth. Int. J. Pharm. 392, 51–56.

Lee, K.E., Kim, B.K., Yuk, S.H., 2002. Biodegradable polymeric nanospheres formed by temperature-induced phase transition in a mixture of poly(lactide-co-glycolide) and poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) triblock copolymer. Biomacromolecules 3, 1115–1119.

Lin, W., Tsai, S.C., Hsieh, J.F., Wang, S.J., 2000. The effects of $^{90}$Y-microspheres on liver tumors: comparison of intratumoral injection method and intra-arterial injection method. J. Nucl. Med. 41, 1892–1897.

Lynch, I., Dawson, K.A., 2004. Release of model compounds from “plum-pudding”-type gels composed of microgel particles randomly dispersed in a gel matrix. J. Phys. Chem. B 108, 10893–10898.

Nishi, K.K., Jayakrishnan, A., 2007. Self-gelling primaquine-gum arabic conjugate: an injectable controlled delivery system for primaquine. Biomacromolecules 8, 84–90.

Obara, K., Ishihara, M., Ozeki, Y., Ishizuka, T., Hayashi, T., Nakamura, S., Saito, Y., Yura, H., Matsui, T., Hattori, H., Takase, B., Ishihara, M., Kikuchi, M., Maehara, T., 2005. Controlled release of paclitaxel from photocrosslinked chitosan hydrogels and its subsequent effect on subcutaneous tumor growth in mice. J. Control. Release 110, 79–89.

Qiu, Y., Park, K., 2001. Environment-sensitive hydrogels for drug delivery. Adv. Drug Deliv. Rev. 53, 321–339.

Ruel-Gariepy, E., Leroux, J.C., 2004. In situ-forming hydrogels-review of temperature-sensitive systems. Eur. J. Pharm. Biopharm. 58, 409–426.

Shikanov, A., Shikanov, S., Vaisman, B., Golenser, J., Domb, A.J., 2008. Paclitaxel tumor biodistribution and efficacy after intratumoral injection of a biodegradable extended release implant. Int. J. Pharm. 358, 114–120.

Shim, W.S., Kim, J.H., Kim, K., Kim, Y.S., Park, R.W., Kim, I.S., Kwon, I.C., Lee, D.S., 2007. pH- and temperature-sensitive, injectable, biodegradable block copolymer hydrogels as carriers for paclitaxel. Int. J. Pharm. 331, 11–18.

Sledge, G.W., Neuberg, D., Bernardo, P., Ingle, J.N., Martino, S., Rowinsky, E.K., Wood, W.C., 2003. Phase III trial of doxorubicin, paclitaxel, and the combination of doxorubicin and Paclitaxel as front-line chemotherapy for metastatic breast cancer: an intergroup trial (E1193). J. Clin. Oncol. 21, 588–592.

Ta, H.T., Dass, C.R., Dunstan, D.E., 2008. Injectable chitosan hydrogels for localised cancer therapy. J. Control. Release 126, 205–216.

Tan, H., Chu, C.R., Payne, K.A., Marra, K.G., 2009. Injectable in situ forming biodegradable chitosan–hyaluronic acid based hydrogels for cartilage tissue engineering. Biomaterials 30, 2499–2506.

Veronese, F.M., Schiavon, O., Pasut, G., Mendichi, R., Andersson, L., Tsirk, A., Ford, J., Wu, G., Kneller, S., Davies, J., Duncan, R., 2005. PEG-doxorubicin conjugates: influence of polymer structure on drug release, in vitro cytotoxicity, biodistribution, and antitumor activity. Bioconjug. Chem. 16, 775–784.

Wang, J.C., Liu, X.Y., Lu, W.L., Chang, A., Zhang, Q., Goh, B.C., Lee, H.S., 2006. Pharmacokinetics of intravenously administered stealth liposomal doxorubicin modulated with verapamil in rats. Eur. J. Pharm. Biopharm. 62, 44–51.

Wei, L., Cai, C., Lin, J., Chen, T., 2009. Dual-drug delivery system based on hydrogel/micelle composites. Biomaterials 30, 2606–2613.

Weng, L., Romanov, A., Rooney, J., Chen, W., 2008. Non-cytotoxic, in situ gelable hydrogels composed of N-carboxyethyl chitosan and oxidized dextran. Biomaterials 29, 3905–3913.

Wike-Hooley, J.L., Haveman, J., Reinhold, H.S., 1984. The relevance of tumour pH to the treatment of malignant disease. Radiother. Oncol. 2, 343–366.

Xie, M., Zhou, L., Hu, T., Yao, M., 2007. Intratumoral delivery of paclitaxel-loaded poly(lactic-co-glycolic acid) microspheres for Hep-2 laryngeal squamous cell carcinoma xenografts. Anticancer Drugs 18, 459–466.

Xu, X., Chen, X., Wang, Z., Jing, X., 2009. Ultrafine PEG-PLA fibers loaded with both paclitaxel and doxorubicin hydrochloride and their in vitro cytotoxicity. Eur. J. Pharm. Biopharm. 72, 18–25.
Yu, L., Chang, G., Zhang, H., Ding, J., 2008. Injectable block copolymer hydrogels for sustained release of a PEGylated drug. Int. J. Pharm. 348, 95–106.
Yu, L., Ding, J., 2008. Injectable hydrogels as unique biomedical materials. Chem. Soc. Rev. 37, 1473–1481.
Zhang, H., Zhao, C., Cao, H., Wang, G., Song, L., Niu, G., Yang, H., Ma, J., Zhu, S., 2010. Hyperbranched poly(amine-ester) based hydrogels for controlled multi-drug release in combination chemotherapy. Biomaterials 31, 5445–5454.
Zentner, G.M., Rathi, R., Shih, C., McRea, J.C., Seo, M.H., Oh, H., Rhee, B.G., Mestecky, J., Moldoveanu, Z., Morgan, M., Weitman, S., 2001. Biodegradable block copolymers for delivery of proteins and water-insoluble drugs. J. Control. Release 72, 203–215.