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pH- and temperature-sensitive, injectable, biodegradable block copolymer hydrogels as carriers for paclitaxel

Woo Sun Shim [a,1], Jong-Ho Kim [b,1], Kwangmeyung Kim [b], Yoo-Shin Kim [c], Rang-Woon Park [c], In-San Kim [c], Ick Chan Kwon [b], Doo Sung Lee [a,*]

[a] *Department of Polymer Science & Engineering, Sungkyunkwan University, Suwon 440-746, Republic of Korea*
[b] *Biomedical Research Center, Korea Institute of Science and Technology, Seoul 136-791, Republic of Korea*
[c] *Department of Biochemistry, School of Medicine, Kyungpook National University, Daegu 700-412, Republic of Korea*

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Abstract

Paclitaxel (PTX) was loaded into synthetic pH/*T*-sensitive block copolymer (OSM–PCLA–PEG–PCLA–OSM) solution with various concentrations. The phase diagram of PTX-loaded block copolymer solution shifted to lower temperature region compared to net block copolymer because of the salting-out effect of PTX. Release profiles of PTX showed sustained manner regardless of loading amount of PTX. To evaluate anti-tumor effect of PTX-loaded block copolymer, solutions were injected subcutaneously to tumor-bearing mice and TUNEL assay examined. PTX-loaded block copolymer hydrogel for *in vivo* use showed good anti-tumor effect for 2 weeks and induced strong apoptosis in tumor tissue. Therefore, we conclude OSM–PCLA–PEG–PCLA–OSM block copolymer as an effective injectable carrier of PTX.

Keywords: pH/*T*-sensitive block copolymer hydrogel; Injectable hydrogel; Paclitaxel; *In vivo* anti-tumor effect; TUNEL assay

1. Introduction

Paclitaxel (PTX) is one of the best anti-neoplastic drugs found in nature known for decades. It interacts with tubulin dimers in the G2 mitotic phase of cell division that promote microtubule polymerization to make highly stable microtubules, therefore, to prevent cell division (Horwitz, 1992). In addition to its anti-proliferative effects, PTX is a potent inhibitor of angiogenesis, cell migration, and collagenase production (Burt et al., 1995; Stearns and Wang, 1992). For another aspect, PTX is a hydrophobic molecule and therefore, a non-ionic polyethoxylated castor oil solubilizer, Cremophor® EL is used to enable its clinical administration. However, the large quantity administration of Cremophor® EL to deliver the required doses of PTX causes serious side effects, particularly hypersensitivity reactions, some of which are life-threatening (Onetto et al., 1993; Rowinsky et al., 1993; Dorr, 1994). In order to eliminate the toxicity of Cremophor® EL, to improve the drug's efficacy and to eliminate chances of premedication, recent research has been focused on developing new drug delivery systems. A variety of approaches have been investigated including emulsification (Kan et al., 1999; Constantinides et al., 2000; He et al., 2003), introducing microspheres (Harper et al., 1999; Mu and Feng, 2001), liposomes (Ceruti et al., 2000; Crosasso et al., 2000; Koshina et al., 2001), nanoparticles (Kim and Lee, 2001; Mu and Feng, 2003) and polymeric micelles (Miwa et al., 1998; Kim et al., 2001a). One of the novel alternate is the development of injectable formula that forms a semisolid implant.

There are countless reports on hydrogel-based injectable polymer delivery systems that have been developed for a variety of drugs, based on the polymeric materials possessing thermosensitivity and biodegradability (Jeong et al., 1997; Ha et al., 2006). The polymers exhibiting properties of reversible thermal gelation are triblock copolymers consisting of A-blocks and B-blocks, arranged as BAB or ABA, where A is poly(D,L-lactic-*co*-glycolic acid) (PLGA) and B is PEG. Aqueous solutions of these polymers undergo a reversible sol–gel transition and form free-flowing sol at room temperature, and become gel at body temperature (Jeong et al., 1997, 2000a; Kim et al., 2001b;

* Corresponding author. Tel.: +82 31 290 7282; fax: +82 31 292 8790.
E-mail address: dslee@skku.edu (D.S. Lee).
[1] These authors contributed equally to this paper.

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Zentner et al., 2001). PTX has been dissolved at high concentrations to aqueous solutions of ABA-type copolymers and injected into mice bearing human breast carcinoma xenografts resulting protracted clearance of PTX from the injection site (about 6 weeks) with minimal distribution to organs (Zentner et al., 2001). However, these thermo-sensitive hydrogels have several unresolved drawbacks, which make them difficult to be used for the injectable drug delivery systems. The temperature reversible hydrogels tend to transform to gel while they are moving in the warmed up needle, making it difficult to inject them into the body.

The novel pH- and temperature-sensitive hydrogel has been developed and that does not simply change forms from the free-flow sol to the gel only in response to increasing temperature (Shim et al., 2005). In the present study, we have characterized the novel hydrogel formulation loaded with PTX. The *in vitro* release profiles of PTX from within gel were first investigated, and the anti-tumoral activity of PTX released from the gel was then assessed *in vivo* using the B16F10 melanoma cell.

2. Materials and methods

2.1. Materials

Poly(ethylene glycol) (PEG) ($M_n = 1750$) was obtained from ID Biochem, Inc. (Seoul, Korea). D,L-Lactide, ε-caprolactone, stannous 2-ethyl-hexanoate, sulfamethazine, methacryloyl chloride, 3-mercaptopropionic acid, dicyclohexyl carboimide (DCC), 4-(dimethyl amino) pyridine (DMAP), *N*,*N*-dimethyl formamide, methylene chloride were used as received from Sigma–Aldrich (MO, USA). 2,2′-Azobisisobutyronitrile were supplied by Junsei Co., and was recrystallized from methanol twice prior to use. PTX (Genexol®) was obtained from Samyang Genex Co. (Seoul, Korea). All other chemicals were analytical grade and used without further purification.

2.2. Synthesis of pH- and temperature-sensitive block copolymer

The synthesis of pH/*T*-sensitive block copolymer, polymer characterization, and sol–gel transition behaviors were described in previous report (Shim et al., 2005). For pH/*T*-sensitive block copolymer synthesis, the poly(ε-caprolactone-*co*-lactide)–poly(ethylene glycol)–poly(ε-caprolactone-*co*-lactide) (PCLA–PEG–PCLA) block copolymer was first synthesized through a ring-opening copolymerization of D,L-lactide and ε-caprolactone with PEG as an initiator. The PCLA–PEG–PCLA block copolymer and sulfamethazine oligomer (OSM) were coupled using a coupling reagent, DCC and catalyst, DMAP.

2.3. Preparation of PTX-loaded pH- and temperature-sensitive block copolymer solution and sol–gel transition

The pH/*T*-sensitive block copolymer (OSM–PCLA–PEG–PCLA–OSM) was dissolved at 20 wt% concentration in a buffer solution for 1 day at 0 °C. The buffer solution was made of phosphate buffered saline (PBS) and NaOH (1.2 wt%). Then, the PTX was dissolved in an OSM–PCLA–PEG–PCLA–OSM block copolymer solution at a given concentration. The pH of the PTX-loaded block copolymer solution was adjusted to the specific pH required by adding small amounts of 5 M HCl solution at 2 °C. The sol (flow)–gel (no flow) was determined by a test tube inverting method (Shim et al., 2005; Jeong et al., 2000b; Wang et al., 2004). After equilibration at 4 °C for 30 min in a water bath, the vial was then slowly heated to temperature at intervals of 2 °C in water bath. The vial was held constant at each temperature for 10 min to equilibrate, and then laid down horizontally for 1 min. Inverting the vial determined a gel state when no fluidity in 1 min was visually observed. In addition, the viscosity changes of the block copolymer solutions were investigated by increasing the temperature with a reologica rheometer (REOLOGICA instruments AB®). It was operated at an oscillation frequency of 1 Hz and 1×10^{-3} strain with a 0.5 °C/min temperature ramp from 5 to 30 °C.

2.4. In vitro PTX release from pH- and temperature-sensitive block copolymer hydrogel

PTX was dissolved in a 20 wt% OSM–PCLA–PEG–PCLA–OSM block copolymer solution at 2.5, 5, and 10 mg/mL. The pH of the resulting solution was then adjusted to pH 7.4 at 2 °C, and the solution was allowed to form a gel in a shaking water bath (20 strokes/min) at 37 °C. After 10 min, release medium (3.5 mL, pH 7.4, 37 °C), comprised of PBS solution containing 2.4 wt% Tween 80 and 4 wt% Cremophor® EL, was added to each vial containing the PTX-loaded block copolymer hydrogel (0.5 mL). At predetermined time intervals, medium (1.5 mL) was withdrawn and replaced with an equal volume of fresh medium pre-warmed at 37 °C. The release study was performed in triplicate. The PTX concentration in each collected fraction was determined by HPLC (Shim et al., 2006).

2.5. In vivo anti-tumor activity of PTX-loaded pH- and temperature-sensitive block copolymer hydrogel

C57BL/6 male mice weighing 20 g were acclimatized for 7 days after arrival. Approximately, 1×10^6 B16F10 melanoma cells were injected subcutaneously into the hind right flank region of the C57BL/6 male mice and the tumors were allowed the growth. When the tumor reached 50 mm^3, the mice were randomly divided into five treatment groups of six each. The control group (group A) received 200 μL saline by subcutaneous injection. For the *in vivo* test, the pH/*T*-sensitive block copolymer solution was prepared to 20 wt% concentration and pH 8.0. The PTX-loaded and unloaded pH/*T*-sensitive block copolymer solution at pH 8.0 was subcutaneously injected to left flank region of tumor-bearing mouse, which had tumor on right flank region. The first treatment group (group B) received 200 μL of pH/*T*-sensitive of block copolymer solution subcutaneously into the hind left flank region (opposite site of tumor). The PTX treatment groups (groups C–E) received 200 μL of PTX-loaded (1.0, 2.5 and 5 mg/mL) pH/*T*-sensitive of block copolymer solution

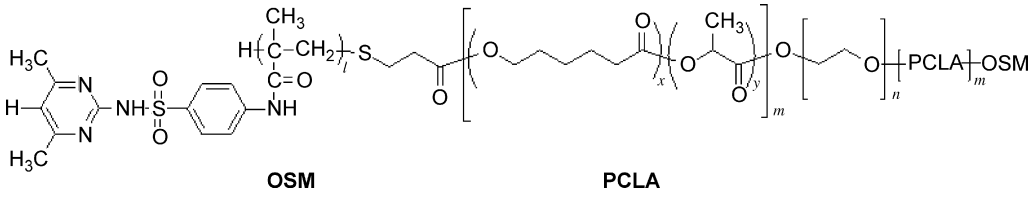

Fig. 1. Chemical structure of the OSM–PCLA–PEG–PCLA–OSM block copolymer.

(equivalent to 10, 25 and 50 mg/kg PTX dose) subcutaneously into the hind left flank region (opposite site of tumor), all on treatment day 0. The experimental groups for anti-tumor effect of PTX-loaded hydrogel were summarized in Table 1. At different time points, the tumor volume was determined by measuring the diameters of the tumor and calculated as follows:

$$\text{tumor volume (mm}^3) = \frac{ab^2}{2}$$

where a is the largest and b the smallest diameter (mm) of the tumor. The animals were sacrificed at predetermined interval of observation and the tumors collected for histological analysis.

2.6. Immunohistological examination

The histological examination was performed by terminal deoxynucleotidyl transferase-mediated nick and labeling (TUNEL) assay with a commercial apoptosis detection kit (Promega Corp., WI, USA) with the following modifications. Samples were fixed with 4% paraformaldehyde (methanol-free) for 10 min at room temperature. The samples were washed with PBS twice for 5 min each time and then incubated with 0.2% Triton X-100 for 15 min at room temperature. After the samples were washed twice more with PBS for 5 min each time, they were incubated with equilibration buffer from the Promega kit for 10 min at room temperature. The equilibration buffer was drained and a reaction buffer containing equilibration buffer, nucleotide mix, and TdT enzyme was added to the tissue sections, which then were incubated in a dark, humidified atmosphere at 37 °C for 1 h. The reaction was terminated by immersing the samples in 2× standard saline citrate for 15 min, and the samples then were washed three times for 5 min each to remove unincorporated fluorescein-TdT. TUNEL analyzed blindly at 600× magnification by use of a computer-aided light microscope. To confirm the findings of TUNEL assay, apoptotic and non-apoptotic cells also detected by 4,6-diamidino-2-phenylindole (DAPI) staining.

Table 1
Experimental groups for anti-tumor effect of PTX-loaded hydrogel

Group	PTX concentration (mg/mL)	Injection volume (μL)	PTX dose (mg/kg)
A	0	200	Saline
B	0	200	Hydrogel
C	1	200	10
D	2.5	200	25
E	5	200	50

3. Results and discussion

3.1. Characteristics of PTX-loaded pH/T-sensitive block copolymer solution

The PCLA–PEG–PCLA block copolymer had block lengths of 1642–1750–1642 (determined by ^{1}H NMR) and polydispersity = 1.39 (determined by gel permeation chromatography (GPC)). The molecular weights of PCLA–PEG–PCLA and OSM–PCLA–PEG–PCLA–OSM were 4844 and 5978 (determined by GPC with reference to poly(ethylene glycol), using tetrahydrofuran as an eluent), respectively. Fig. 1 shows the chemical structure of OSM–PCLA–PEG–PCLA–OSM block copolymer.

The sol–gel transition phase diagrams of the PTX-loaded and unloaded pH/*T*-sensitive block copolymer solutions were investigated under changing pH and temperature conditions. Fig. 2 shows the sol–gel transition phase diagrams of PTX-loaded and unloaded OSM–PCLA–PEG–PCLA–OSM block copolymer solutions. At high pH (pH 8.0), the sulfonamide group of the pH-sensitive moiety (OSM) is mainly present in its ionized state and the ionized OSM acts as a hydrophilic block in the OSM–PCLA–PEG–PCLA–OSM block copolymer regardless of temperature. Therefore, the block copolymer solution did not form a gel in high pH conditions as the temperature increased. However, at low pH (pH 7.4), the degree of ionization of the OSM decreases and the non-ionized OSM acts as a hydrophobic block in the copolymer. The block copolymer solution formed a gel in low pH conditions as the temperature increased to body temperature. As the portion of non-ionized OSM increases with decreasing pH of block copolymer solution, the hydrophobicity

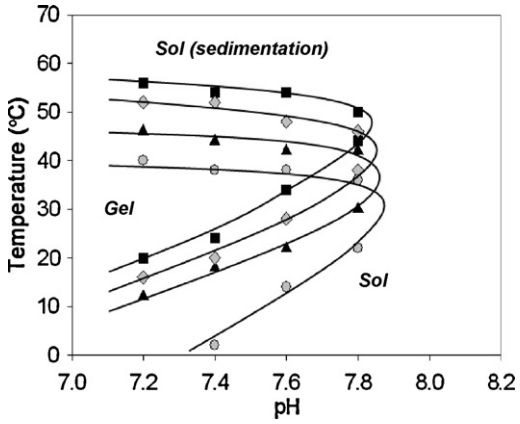

Fig. 2. Sol–gel phase diagrams of PTX-loaded block copolymer solutions at 20 wt% polymer concentration. Unloaded block copolymer solution (■); PTX-loading 2.5 mg/mL (◇); 5 mg/mL (▲); 10 mg/mL (◍).

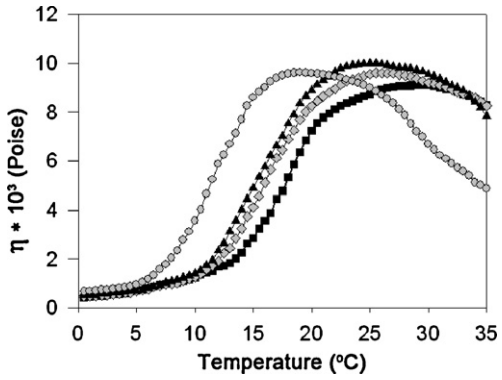

Fig. 3. Temperature dependence of the viscosity of PTX-loaded block copolymer solutions at 20 wt% polymer concentration. Unloaded block copolymer solution (■); PTX-loading 2.5 mg/mL (◈); 5 mg/mL (▲); 10 mg/mL (◌).

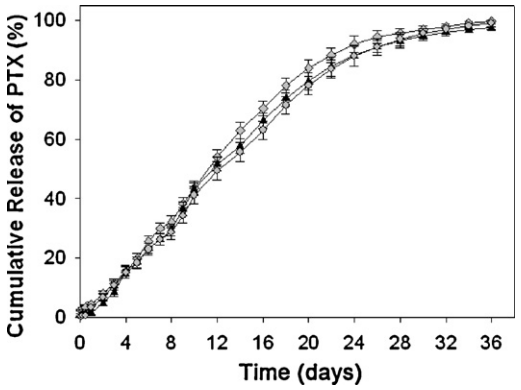

Fig. 4. *In vitro* release of PTX from the block copolymer hydrogel in PBS solution (pH 7.4) containing 2.4 wt% Tween 80 and 4 wt% Cremophor® EL at 37 °C. The results are represented as means ± S.D. ($n = 3$). The symbols are as follows: initial loading 2.5 mg/mL (◈); 5 mg/mL (▲); 10 mg/mL (◌).

of block copolymer increases, which induces gelling range of block copolymer is widen. The phase diagram of PTX-loaded block copolymer solution shifted to lower temperature region compare to unloaded block copolymer solution. It is ascribed to the salting-out effect of PTX in block copolymer solution (Pandit and Kisaka, 1996; Jorgensen et al., 1997). The sol–gel transition of PTX-loaded and unloaded OSM–PCLA–PEG–PCLA–OSM block copolymer solutions was also confirmed by viscosity change. Fig. 3 shows the temperature-dependent viscosity change of PTX-loaded and unloaded copolymers at pH 7.4. The viscosity increased rapidly in the sol–gel transition region. It is induced by the hydrophobicity of PCLA block, which generally increases as temperature arise. As the PTX concentration increased in the block copolymer solution, the viscosity is rapidly increased at lower temperature. According to the results on phase diagram and viscosity, the changes in properties of hydrogel were vivid, when PTX loading concentration was at 10 mg/mL. It suggests that the properties of hydrogel maintained until PTX loaded into hydrogel at 5 mg/mL concentration, meaning transition temperature at which viscosity rapidly increased was lower as PTX concentration was higher.

3.2. *In vitro* release of PTX from pH/*T*-sensitive hydrogel

In order to evaluate the ability of the pH/*T*-sensitive hydrogel to effectively deliver PTX, *in vitro* release studies were performed in PBS solution containing 2.4 wt% Tween 80 and 4 wt% Cremophor® EL at 37 °C, and concluded its sustained release. The PTX concentrations were calculated using a standard curve for its solution in acetonitrile and a linear correlation between peak area and PTX was observed (data not shown). Fig. 4 shows the release profile of PTX from the pH/*T*-sensitive hydrogel. PTX was continuously released from the pH/*T*-sensitive hydrogel, and the constant zero-order release rate was confirmed regardless of loading amount in initial state by 20 days. After zero-order release, the release of PTX from hydrogel was slowed down. In addition, the PTX-loaded hydrogel exhibited a low burst release with only 2–4% drug release within the first day. It should suggest that this hydrogel has a good potential for controlled drug delivery carriers. Based on the characteristics and release data, PTX-loaded hydrogel prepared with 5 mg/mL PTX concentration was chosen to evaluate anti-tumor effect *in vivo* since it showed reasonable sol–gel transition phase diagram suitable for using injectable system, which are maintenance of viscosity, and sustained manner of PTX release.

3.3. *In vivo* anti-tumor effect of PTX-loaded hydrogel

In vivo anti-tumor activity of PTX-loaded pH/*T*-sensitive hydrogel was done using C57BL/6 male mice bearing tumor on right flank region. PTX-loaded and unloaded solutions made the gel immediately (in 10 min) after subcutaneously injected

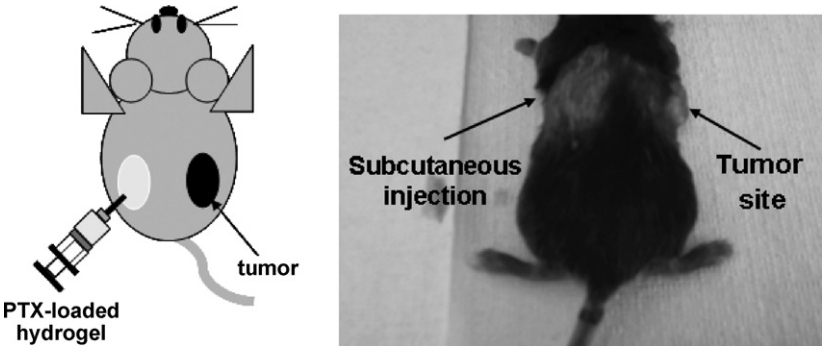

Fig. 5. Schematic diagram and photograph showing the location of subcutaneous injection of the PTX-loaded block copolymers.

into left flank. As shown in Fig. 5, injected site changed to be bulged since the block copolymer solution formed a gel into subcutis. The block copolymer solution transformed to gel-like plug, as the pH decrease and temperature increase by the tissue fluid surrounding injection site. It suggests that PTX will be released from hydrogel and then systemically circulated in body and eventually to act against tumor.

As Fig. 6 represents, the tumor volume changes in mice after injection of treatments, it was difficult to observe any significant differences between groups A and B. It could be inferred that hydrogel seldom affected the tumor. Among the other three groups that were PTX-treated mice, significant differences were observed compared to previous two groups. The rate of tumor volume increment was remarkably slower than that of non-treated mice. After 2 weeks, tumor volume of the saline-treated mice reached about 17 cm^3 while that of PTX-treated mice smaller than 7 cm^3. In addition, these anti-tumor effect of PTX-treated mice showed the dose dependence of PTX, to which tumor volume was smaller as dose of PTX increased. Fig. 7 shows the photograph of each group at 14 day after subcutaneous injection, and the mice are bearing tumor that are larger than their head as in groups A and B. However, in PTX-treated mice group, especially group E, shows the tumor size was similar to their ears, which could be concluded that PTX-loaded

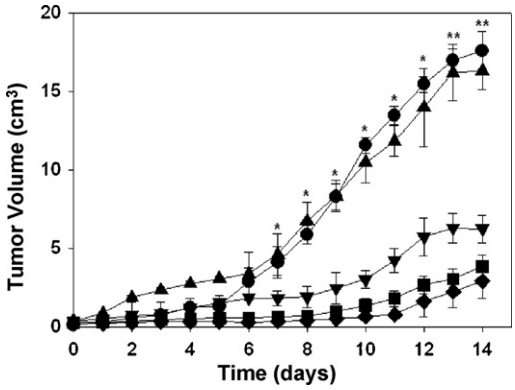

Fig. 6. *In vivo* anti-tumor activity of PTX-loaded block copolymers after subcutaneous administration. The results are represented as means ± S.D. ($n = 6$). Student's *t*-test ($^{*}p < 0.05$; $^{**}p < 0.01$). The symbols are as follows: group A (●); group B (▲); group C (▼); group D (■); group E (♦).

block copolymer hydrogel can effectively suppress the tumor development. It, in addition, suggests that PTX loaded in block copolymer hydrogel were released, dissolved and absorbed in body fluid, then systemically circulated to effect in tumor.

Fig. 8 shows the changes in body weight of tumor-bearing mice after injection. The body weight of saline and hydrogel

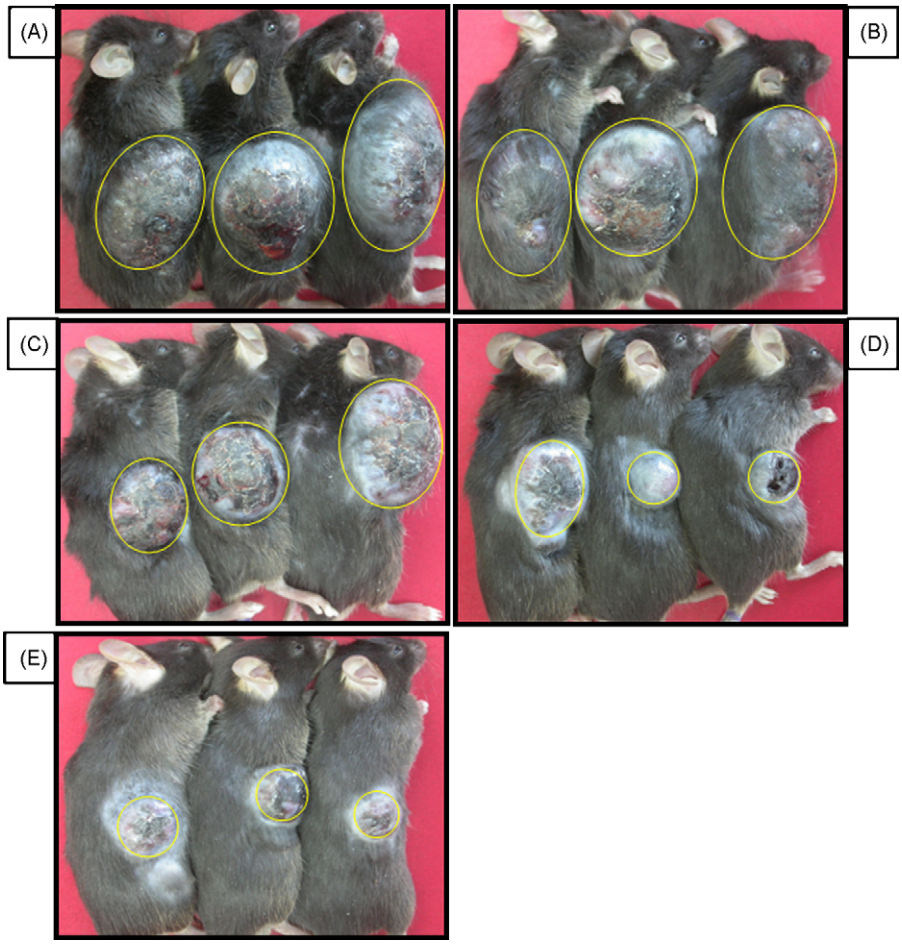

Fig. 7. Photographs of tumor-bearing mice at day 14 after PTX-loaded block copolymer hydrogel treatment.

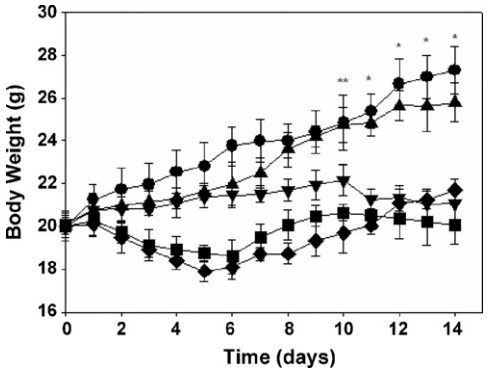

Fig. 8. Body weight changes of mice treated with PTX-loaded block copolymers after subcutaneous administration. The results are represented as means ± S.D. ($n = 6$). Student's t-test ($^{*}p < 0.05$; $^{**}p < 0.01$). The symbols are as follows: group A (●); group B (▲); group C (▼); group D (■); group E (♦).

injected groups (A and B) continuously increased due to the rapid growth of tumor volume as previously mentioned. In low dose (10 mg/kg) PTX-loaded hydrogel injected group (C), the body weight was maintained constantly for 2 weeks. However, the body weight decreased by 6 day and then slightly increased in groups D and E. An initial decreased period was caused by the therapy of tumor. As shown in Fig. 6, tumor volume hardly increased in same period. In other words, the toxicity of PTX, which suppressed the tumor, had an influence on body weight of mice.

3.4. Immunohistology by TUNEL assay

Fig. 9 shows the images of isolated tumor tissue in 14 day after subcutaneous injection that were subjected to DAPI staining and TUNEL assay following several treatment (groups A–E).

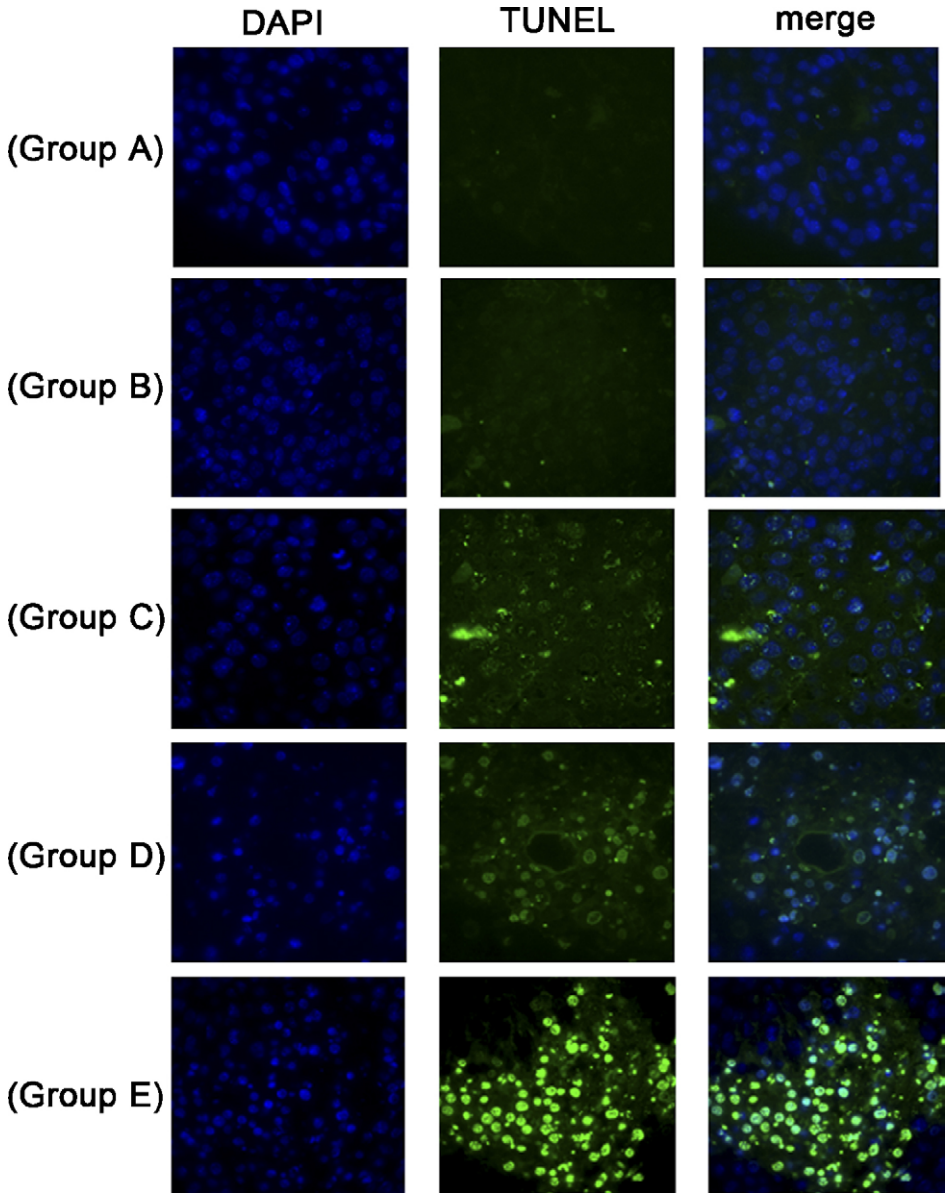

Fig. 9. Immunohistological detection of apoptotic cells in B16F10 melanoma tumors growing in the C57BL/6 mice treated with PTX-loaded block copolymers. Original magnification: 600×.

As shown in DAPI staining images, it is difficult to observe any significant difference among groups since the cell nuclei of live cells represented bright blue fluorescence attributed to DAPI staining. However, the TUNEL assay images showed the different results as it depends on treatment. The cell nuclei of non-apoptotic bodies did not exhibit the green fluorescence attributed to FITC-labeled TdT. In contrast, bright green fluorescence of the cell nuclei of apoptotic cells was observed. Therefore, the saline-only or hydrogel-only treated groups (A and B) hardly showed the TUNEL positivity while PTX-treated groups (C–E) represented the TUNEL positivity, bright green fluorescence, and it was brighter as PTX dose was higher. There are more effective merging of the two images, DAPI staining and TUNEL assay. In non-apoptotic groups, non-anti-tumor effective groups (A–B), DAPI staining and merging images were almost the same even though TUNEL assay was overlapped. However, the apoptotic cells which were blue in DAPI staining and green in TUNEL assay showed bright cyanine color in merging images. These TUNEL assay results could be supported the anti-tumor effect results of PTX-loaded block copolymer injectable hydrogel, which could suppress the tumor increasing, effectively.

4. Conclusion

PTX-loaded pH/*T*-sensitive block copolymer (OSM–PCLA–PEG–PCLA–OSM) solution showed the rapid sol–gel transition with pH and temperature changes. The phase diagram of PTX-loaded block copolymer solution shifted to lower temperature region by the salting-out effect of PTX. PTX-loaded block copolymer hydrogel showed the sustained release profile of PTX for 1 month without burst release, and the good anti-tumor effect by subcutaneous injection in tumor-bearing mice. Therefore, OSM–PCLA–PEG–PCLA–OSM block copolymer is considered to be an effective injectable carrier of PTX.

Acknowledgments

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