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Research Article

The Influence of Modified Pluronic F127 Copolymers with Higher Phase Transition Temperature on Arsenic Trioxide-Releasing Properties and Toxicity in a Subcutaneous Model of Rats

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Abstract. Pluronic F127 (PF-127) shows thermoreversible property, which is of the utmost interest in optimizing drug formulation and delivery. However, its hitherto unresolved drawback of a low phase transition temperature (T_{tr}) has limited its application in injectable drug delivery systems. We have recently synthesized a new type of PF-127 copolymers with higher T_{tr} using a simple oxidative method. Here, we have investigated the drug-releasing feature of oxidized PF-127 and oxidized PF-127-containing silver nanoparticles (SNPs), carrying arsenic trioxide (ATO), in a subcutaneous model of rats. Injectable hydrogels prepared with oxidized PF-127s were less viscous and easier to inject, at the same concentration, than their precursor. Addition of SNPs further elevated T_{tr}, resulting in even lower viscosity of the injectable hydrogel prepared from SNP-containing oxidized PF-127. The oxidized PF-127 copolymers did not differ significantly in ATO-releasing ability, compared with parental PF-127, but the addition of SNPs altered the ATO-releasing feature of oxidized PF-127 to some extent. ATO-carrying oxidized PF-127s had similar toxicity, but the addition of SNPs enhanced the hepatotoxicity of ATO, as evidenced by elevated serum levels of alanine aminotransferase and aspartate aminotransferase and histological alterations, compared to parental PF-127. The results presented herein warrant further investigation of the modified PF-127 copolymers to deliver ATO or other drugs in the form of injectable hydrogels.

KEY WORDS: arsenic trioxide; drug delivery; hydrogel; Pluronic F127; toxicity.

INTRODUCTION

Pluronic F127 (PF-127), a nontoxic and nonionic polymer surfactant with excellent biocompatibility and thermosensitivity, has attracted tremendous attention as a promising carrier to deliver drugs for various diseases (1). The feature of reverse thermal gelation allows PF-127 to be used as a drug delivery vehicle for most administration routes including oral, topical, intramuscular, intranasal, vaginal, rectal, ocular, parental, transdermal, and subcutaneous administration (2). Its remarkable reverse gelation characteristic is based on its hydrophilic–hydrophobic–hydrophilic triblock molecular structure. This arrangement, where each of the hydrophilic blocks comprises 106 ethylene oxide units and the hydrophobic block has 70 propylene oxide units, provides a phase transition from sol to gel on heating (3,4). At low temperature, a hydration layer surrounds PF-127 molecules in aqueous solutions, while the hydrogen bonds between the solvent and polymer chains break when the temperature rises, resulting in desolvation of the hydrophilic blocks (5). This phenomenon favors hydrophobic interactions among the polyoxypropylene domains, leading to gel formation (6).

However, the low phase transition temperature (T_{tr}) of PF-127 limits its application in injectable drug delivery systems. PF-127 tends to gel before application or during injection into the body with a syringe below body temperature or even below ambient temperature, leading to needle blockage and failure of injection (7). To overcome this problem, we have recently used a simple oxidative method that significantly increased the T_{tr} of PF-127 (4). Partial oxidation, under mild conditions for 48 or 72 h, of the terminal hydroxy groups of PF-127 resulted in 50% or 80% increase of T_{tr}, respectively, for a 30 wt.% aqueous solution of the polymer, but did not affect the main chain structure (4). The resultant copolymers should have similar drug-releasing features and toxicity to their precursor since no other organics are introduced, but have a practical advantage when applied in injectable drug delivery systems due to their higher T_{tr} values. Consequently, we designed this study to test these possibilities by using the

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modified PF-127 as the carrier and arsenic trioxide (ATO) as the drug to prepare drug-injectable hydrogels, in a subcutaneous model of rats. ATO has been widely employed to treat acute promyelocytic leukemia (APL) since its original application at Harbin Medical University in China in the 1970s (8), and recently, to treat a variety of solid tumors as a promising anticancer agent (9). The hepatotoxicity of ATO has been extensively reported in APL patients (10), mice (11), and rats (12). Although liver appears to be the main target of arsenic, kidney and heart are also vulnerable to arsenic toxicity (11).

In addition, recent studies have shown that combination of chemotherapeutic agents and functional nanomaterials, including silver nanoparticles (SNPs), could achieve a more effective action of the agents (13). Therefore, we also examined another new type of partially oxidized PF-127 copolymer containing SNPs in this study.

MATERIALS AND METHODS

Preparation of Copolymers

Pluronic F-127 triblock copolymer (chemical formula PEO_{106}–PPO_{70}–PEO_{106}) was purchased from Sigma and is referred to hereinafter as S0. The oxidized PF-127 copolymers, prepared as described previously (4), are designated S1 and S2, corresponding to oxidation times of 48 and 72 h, respectively. The molecular structures of S0, S1, and S2 are shown in Fig. 1. A new type of PF-127 copolymer containing 0.1 wt.% of SNPs was prepared from S2 and is designated S3. The purpose of addition of SNPs was to investigate whether SNPs could alter the drug-releasing feature and toxicity of PF-127. We expected that the introduction of SNPs would provide antimicrobial ability to the oxidized PF-127 hydrogels, as silver has been widely used as an antimicrobial agent to reduce bioburden and prevent infection (14).

Observation of Chemistry Changes

The copolymers were characterized using attenuated total reflectance Fourier transform infrared spectroscopy and X-ray photoelectron spectroscopy on dried powders (4). Nuclear magnetic resonance spectra were recorded at ambient temperature in dimethylsulfoxide-d_6 with a DRX-400 spectrometer (Bruker Corporation). A transmission electron microscopy (TEM) image of SNPs in S3 copolymer was taken on a Cu grid with a layer of nitrile cellulose and carbon coating, using a FEI/Philips CM-12 instrument (Eindhoven, The Netherlands). Elemental analysis was performed at the Campbell Microanalytical Laboratory, Otago University, New Zealand.

Determination of Molecular Weight and Molecular Weight Distribution

Gel permeation chromatography (GPC) was used with tetrahydrofuran as the eluent, as described previously (4). Copolymer solutions of 3 mg/ml were filtered through 0.45-μm syringe filters. The columns and refractive index detector (Waters 2410 differential refractometer) were maintained at 35°C. The GPC system was connected to a DAWN-DSP MALLS light scattering detector (Wyatt Technologies Corporation, Santa Barbara, CA, USA) with laser wavelength of 632.8 nm. The detectors were calibrated using polystyrene standards (with mean number average molecular weight Mn=16,700 g/mol).

Rheology

Rheological measurements were performed using a rheometer (UDS 200, Paar Physica, Österreich, Germany) with a 25-mm diameter plate–plate measuring system in oscillation mode at 0.1% or 1% strain and angular frequency, 10/s. The strain and frequency used were determined in preliminary experiments to be within the linear viscoelastic region. The plate gap was kept at 0.5 mm and the temperature range was 0 to 40°C.

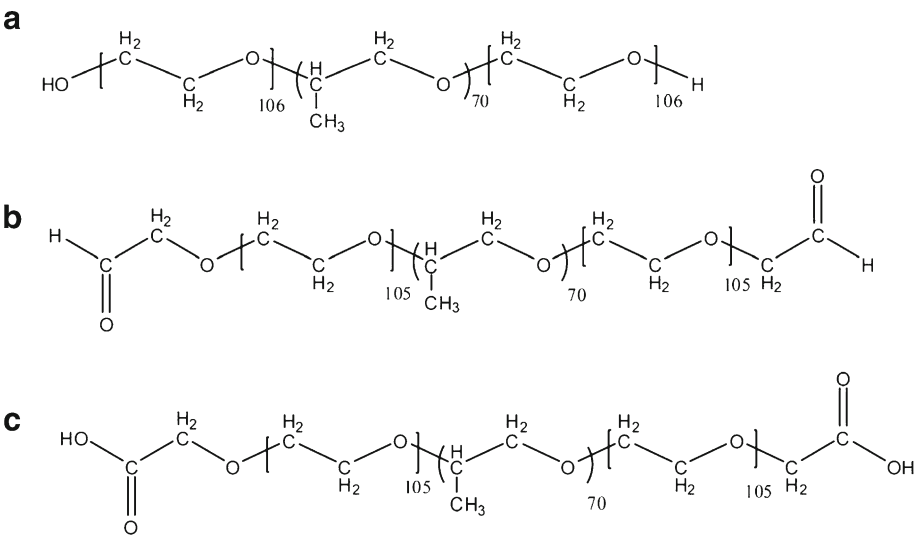

Fig. 1. Molecular structures of PF-127 polymers. The parental PF-127 polymer (S0) and oxidized PF-127 polymers (S1 and S2, prepared at 48 or 72 h of oxidation time, respectively) are shown in **a**, **b**, and **c**, respectively

Preparation of Injectable Hydrogels

The copolymers were mixed with ATO solution (Yida Pharmaceutical Co. Ltd, Harbin Medical University, China) at a ratio of 1:3 (*w*/*w*), stirred for 60 min, and incubated at 4°C overnight. The mixture was further stirred for 30 min and incubated at 4°C overnight to obtain injectable hydrogels containing 0.8 mg ml^{-1} of ATO and 25% *w*/*v* of PF-127 copolymers. All samples were stored at 4°C. The control solution was prepared by mixing physiological saline and ATO solution using the method described above.

Animal Experimental Design

Male Wistar rats (250–300 g) were supplied by the Animal Research Center at the First Affiliated Hospital of Harbin Medical University, Harbin, China. The animals were maintained under standard conditions and allowed free access to standard food and water. All the procedures and care administered to the animals had been approved by Animal Ethics Committee of the First Affiliated Hospital of Harbin Medical, and the animal experiments were conducted in full compliance with the Experimental Animal Regulations by the National Science and Technology Commission, China.

Thirty rats were randomly assigned to five groups (each group had six rats): control, S0, S1, S2, and S3, which underwent subcutaneous injection of ATO solution or injectable hydrogels containing mixtures of ATO with S0, S1, S2, or S3, respectively, on the left flank. Each rat received ATO at a dose of 6 mg/kg, as determined in previous reports and our preliminary experiments (15,16). An 18-gauge needle attached to a 3-ml syringe was used for the injection. The needle, syringe, ATO solution, and injectable hydrogels were kept on ice before injection. A sterile dry cotton pad was applied with pressure onto the injection site for 5 min to prevent leakage and bleeding. The rats were closely monitored. A 0.5-ml blood sample was collected from the tail of each rat at 4, 8, 12, 16, 20, 32, 48, 72, 96, and 120 h after injection and centrifuged at 2,000×*g* for 10 min to harvest the sera, which was stored at −80°C for measuring the concentration of ATO. After the last tail blood collection, the rats were euthanized, and 5 ml of blood was collected via cardiac puncture. The sera were harvested as above and stored at −80°C for measuring serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN), and creatinine (Cr) with an auto-biochemical analyzer (Toshiba, Japan). The liver, kidney, and heart were excised and fixed with 10% buffered formalin.

Measurement of ATO Concentration in Sera

The methodology of measuring ATO concentration has been described previously (16,17). Briefly, 3 ml of concentrated nitric acid and perchloric acid (4:1 *v*/*v*) were added to a covered beaker containing 0.1 ml of serum. After predigesting at room temperature overnight, the sample was heated at 80°C for 1 h. Distilled water (5 ml) was added to the cooled beaker, which was slowly heated to 280°C. When the solution changed from chartreuse to clear, the beaker was removed from the hotplate. A sample (1 ml) was transferred to a polypropylene centrifuge tube and mixed with 1 ml of 1% HCl and 2 ml of ascorbic acid–thiourea mixed solution (5% ascorbic acid and 5% thiourea). Digests were stored at room temperature for 20 min, and then, the concentration of ATO was measured with a hydride generation atomic fluorescence spectrometer (AFS-930, Beijing Titan Instruments Co. Ltd., China). A standard ATO solution (100 μg/ml, the National Institute of Metrology, Beijing, China) was diluted to 1, 2, 4, 6, 8, and 10.0 μg/ml and subjected to hydride generation atomic fluorescence spectrometry to plot a calibration curve.

Histological Analysis

The fixed specimens were embedded in paraffin. Sections of 4 μm were prepared, stained with hematoxylin–eosin, and then blindly examined under a light microscope. Histopathological scoring analysis was performed according to previously described methods (18–21).

Statistical Analysis

All the data are expressed as mean values ± standard deviation (SD). Analysis of variance was used to evaluate statistical significance. All statistical analyses were done with an SPSS 16.0 software. A value of less than 0.05 ($P<0.05$) was used for statistical significance.

RESULTS

Characteristics of Modified PF-127 Copolymers

The percentages of elements including carbon, hydrogen, oxygen, and silver in S0 and S3 are shown in Table I. S3 contained ~0.1% silver. The S0, S1, and S2 had similar molecular weights and molecular weight distributions, indicating that oxidation had not changed their main chains (Table II). The molecular weight and molecular weight distribution of S3 were not determined due to the interference of SNPs, but S3 was assumed to have similar molecular weight and molecular weight distribution to S2 as they had experienced similar reaction conditions. The addition of SNPs further increased T_{tr} of S3 to 32.0±2.0°C. The SNPs in S3 were examined with TEM, which showed that the diameter range was 10 to 25 nm. The characteristics of S1 and S2 have been reported previously (4). Here, we could further demonstrate that S2 had higher T_{tr} (13±0.2°C) than the parental PF-127 with T_{tr} 9±0.2°C.

Serum Levels of ATO

The serum level of ATO reached a peak of 1.82±0.09 mg/L 12 h after injection in the control group, while the serum levels

Table I. Elemental Analysis (Mean ± SD)

Sample	C (%)	H (%)	O (%)	Ag (%)
S0	56.58±0.03	9.60±0.02	33.82±0.00	0.00±0.00
S3	56.29±0.08	9.46±0.09	34.15±0.15	0.10±0.01

The values of percentage of elements were normalized based on carbon (C), hydrogen (H), oxygen (O), and silver (Ag). Five samples from each group were analyzed

Table II. Molecular Weight and Molecular Weight Distribution

Sample	Mn (g mol^{-1})	Mw (g mol^{-1})	Mw/Mn
S0	1.12×10^4	1.19×10^4	1.06
S1	1.08×10^4	1.19×10^4	1.10
S2	1.13×10^4	1.21×10^4	1.07

Five samples from each group were analyzed
Mn number average molecular weight, *Mw* weight average molecular weight, *Mw/Mn* molecular weight distribution index

of ATO reached peaked 16 h after injection at 1.41±0.03, 1.34±0.06, 1.50±0.10, and 1.57±0.20 mg/L, in the S0, S1, S2, and S3 groups, respectively, thus, the peak serum levels in four PF-127 gel groups were delayed for 4 h compared with the control (Fig. 2). Furthermore, the serum levels of ATO were only 0.61±0.07 and 0.25±0.06 mg/L in the control group, 20 and 32 h after injection, respectively, which were significantly lower than that in all the four PF-127 hydrogel groups at respective time points (Fig. 2), indicating high serum levels of ATO were maintained for a longer time, when an identical dose of ATO was formulated in PF-127 gels. In addition, the serum level of ATO in the S3 group was significantly ($P<0.05$) higher than that in the S0, S1, or S2 group 12 h after injection, and the serum level of ATO in the S3 group was significantly higher than in the S0 group, but not significantly different from that in the S1 or S2 group, 16 h after injection.

Toxicity

The function of liver and kidney was evaluated by measuring the serum levels of AST, ALT, BUN, and Cr. To investigate whether PF-127 hydrogels themselves have toxicity on rats, 15 rats received subcutaneous injections of PF-127 gels (S0, S1, S2, and S3) and phosphate-buffered saline (PBS) ($n=3$) and were killed 120 h after injection to harvest sera. The serum

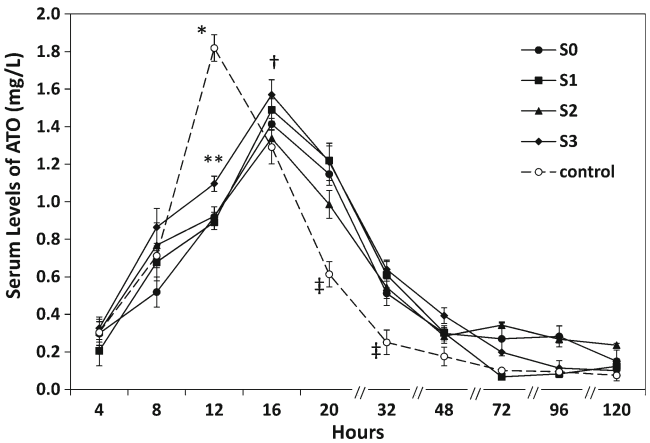

Fig. 2. Serum levels of ATO. Rats were subcutaneously injected with the control ATO solution or hydrogels containing ATO formulated in S0, S1, S2, or S3. The blood samples were collected at the indicated time points, and sera were harvested to measure the serum levels of ATO. *Single asterisk* indicates a significant higher level of ATO from the four hydrogel groups 12 h after injection. *Double asterisks*: A significant higher level from the other three hydrogel groups. *Dagger*: A significant higher level from the S0 group. *Double dagger*: A significant lower level from the four hydrogel groups, at the respective time points

levels of ALT, AST, BUN, and Cr in hydrogel-injected rats were all as normal as the PBS-injected controls (data not shown), indicating that the parental and modified PF-127 hydrogels are nontoxic, in accordance with previous published reports (1,2). However, the hydrogels carrying ATO impaired liver and renal functions and increased the toxicity of ATO. As shown in Fig. 3a, the serum levels of AST in the four hydrogels groups reached 228.5±57.5, 208.0±49.5, 208.5±13.4, and 273.0±56.4 U/L, respectively, which were significantly higher ($P<0.05$) than that in the control group (107.5±6.4 U/L). Similarly, the serum levels of ALT in the four hydrogels groups were 92.5±29.8, 107.0±34.9, 75.5±5.0, and 64.0±9.9 U/L, respectively, which were also significantly ($P<0.05$) higher than that in the control group (39.5±9.2 U/L). The impaired liver function could be caused by accumulated ATO in the body as the administration of PF-127 hydrogels led to prolonged and sustained serum levels of ATO, in accordance with a previous report (22), where ATO was found to accumulate continuously and gradually in hair and nails of APL patients. Although the serum levels of BUN and Cr were slightly higher in the four hydrogels groups than in the control groups, the difference did not reach significance (Fig. 3b). There was no significant difference in the serum levels of AST, ALT, BUN, and Cr among the S0, S1, and S2 groups, indicating that oxidative modification had no effect on the toxicity of the hydrogels. However, the serum levels of ALT and AST in the S3 group were significantly higher than in the other three hydrogels groups ($P<0.05$), possibly because of the higher serum levels of ATO in rats of the S3 group.

The serological changes were further confirmed by histological analysis. As shown in Fig. 4a, livers, kidneys, and hearts from all five groups had histological alterations including edema and vacuolization of hepatocytes (liver); loss of brush border, tubular epithelial cell fattening, interstitial edema, and cytoplasmic vacuolization (kidney); and degeneration of myocardiocytes and inflammation infiltration (heart). The histological alteration was semi-quantified to calculate histological scores. As shown in Fig. 4b, the histological scores of livers in the S0, S1, and S2 groups were slightly higher than that of the control group, but the difference did not reach significance. However, the histological score of livers in the S3 group was significantly ($P<0.05$) higher than in the control group, but was not significantly higher than in the other three PF-127 groups. There were no significant differences in histological scores of kidneys and hearts among the five groups.

DISCUSSION

The present study has for the first time investigated the drug-releasing property and toxicity of modified PF-127 copolymers, prepared using a simple oxidative method, in a subcutaneous model of rats. ATO had been initially regarded as a toxic drug until 1970s, when it was employed to treat APL at Harbin Medical University (8). ATO exerts antitumor effects as a result of its highly cytotoxic nature. Based on its cytotoxicity, we selected ATO as the target drug to evaluate the deliverability of the PF-127 delivery system. The area under the curve has been used to evaluate tolerance/toxicity in ATO-treated patients (23). The results herein have shown that subcutaneous injection of ATO formulated in PF-127 hydrogels delayed the peak serum levels of ATO up to 4 h, prolonged the sustained release, and maintained high serum

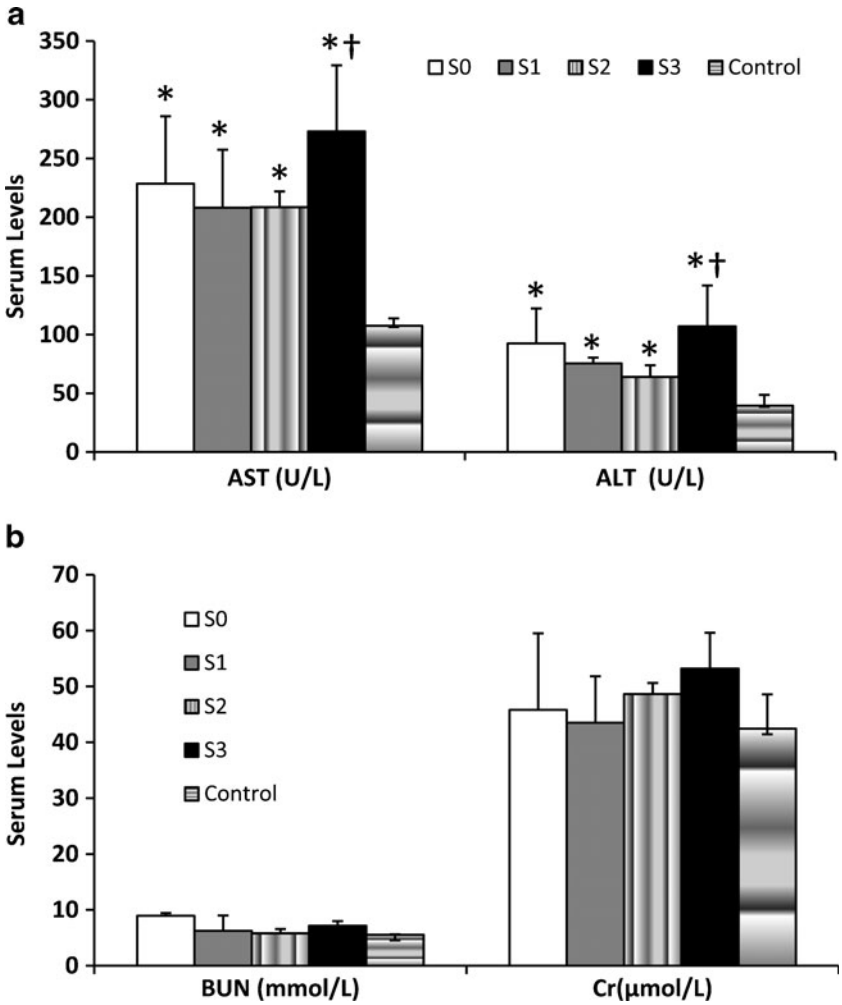

Fig. 3. Serum levels of AST, ALT, BUN, and Cr. The rats were killed 120 h after injection of the control ATO solution or hydrogels containing ATO formulated in S0, S1, S2, or S3, and blood samples were collected via cardiac puncture. The serum levels of AST and ALT (**a**) and BUN and Cr (**b**) were measured. Results are expressed as mean ± SD (n=6). A significant increase from the control group is denoted by an *asterisk* and a significant increase from the S1 or S2 groups by a *dagger*

levels of ATO in rats for a longer time compared with control ATO solution, in accordance with previous reports (24,25). The application of PF-127 delivery system resulted in significantly more hepatotoxicity than the control, evidenced by the elevated serum levels of AST and ALT. However, there was no significant difference in serum levels of ATO among the S0, S1, and S2 groups at all of the time points, indicating that oxidation had no significant impact on the ATO-releasing features of PF-127 copolymers. We prepared injectable hydrogels with 25% of PF-127 or its modified copolymers because the gelation temperature of 30% parental PF-127 (about 9°C) makes injection very difficult (26). With the lower concentration of 25%, the viscosity of parental PF-127 increased after being taken out of the ice box, but the solution had sufficient fluidity to allow injection into the rats at room temperature. In addition, in the preliminary study to assess the viscosity of the hydrogels, a 30% solution of parental PF-127 (S0) quickly gelled after being taken out of ice box, while 30% solutions of S1, S2, and S3 were sufficiently free flowing for injection, due to their higher values of T_{tr}. Furthermore, S3 hydrogel had even lower viscosity than S2, as addition of SNPs to S2 elevated the gelation temperature.

Further, the addition of SNPs to the copolymers influenced the ATO-releasing features moderately, which may be due to the higher gelation temperature and lower viscosity of SNP-containing oxidized PF-127. Although further investigation is required, caution may be taken in applications of SNPs with other cytotoxic drugs in relation to hepatotoxicity, as it has been reported that SNPs accumulated in organs, entered hepatocytes after administration (27), and induced oxidative stress and apoptosis of hepatocytes in adult zebrafish (28).

We have previously demonstrated that ATO has a narrow window of therapeutic opportunity in respect of dosage (15), as high doses lead to severe side effects while low doses stimulate tumor angiogenesis. Here, we have shown that delivery of ATO by PF-127 hydrogels reduced the peak but prolonged the duration of modest serum levels of ATO. The oxidized PF-127 copolymers prepared with our methods did not display significant differences in pharmacodynamics and toxicity of ATO in respect of liver and renal function and histological alteration of livers, kidneys, and hearts. The addition of SNPs to oxidized PF-127 slightly increased the serum levels of ATO at some time points, possibly due to the low viscosity. The aggravated liver dysfunctions and histological

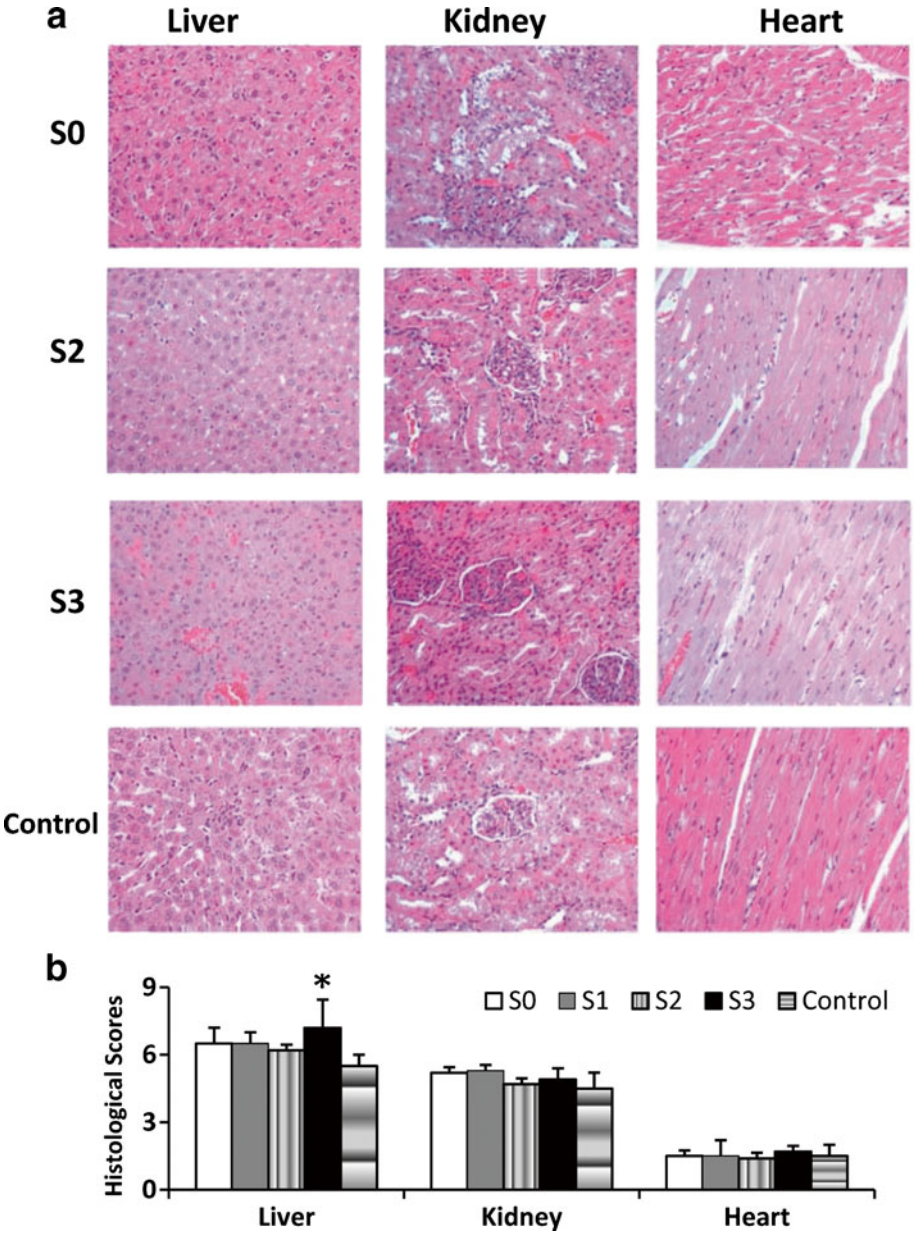

Fig. 4. Histological analysis of liver, kidney, and heart. **a** Representative photographs (×400 magnification) were taken from the sections of livers, kidneys, and hearts from the rats subcutaneously injected with the control ATO solution or hydrogels containing ATO formulated in S0, S1, S2, or S3, 120 h earlier. **b** Histological scoring was performed as described in "Materials and Methods" section. Data were represented by mean ± SD (n=6). A significant increase from the control group is denoted by an *asterisk* ($P<0.05$)

alterations in the hydrogel groups compared to the control group could be explained by the prolonged and sustained ATO release by the PF-127 hydrogels. Addition of SNPs to the oxidized PF-127 copolymers enhanced the toxicity of ATO especially in livers possibly because of higher serum levels of ATO released in rats. In addition to its efficient drug delivery feature, PF-127 has been shown to enhance the efficacy of doxorubicin to inhibit the growth of human ovarian carcinoma cells by targeting the glycoprotein P (29).

CONCLUSION

The modified Pluronic F127 copolymers with higher phase transition temperature have similar ATO-releasing feature and toxicity, compared to their precursor. The addition of SNPs influences the rheological properties of oxidized PF-127 and has stronger ATO-induced hepatotoxicity than the hydrogels without SNPs. The results presented herein warrant further investigation of these copolymers to deliver ATO or other

drugs in the form of injectable hydrogels, though more efforts should be put to investigate the toxicity of the modified PF-127 polymers themselves, how they are metabolized, and their influence on local tissues of injection sites, before they can be applied in clinic.

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Conflict of Interest The authors report no conflict of interest.

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