Injectable Hydrogel–Microsphere Construct with Sequential Degradation for Locally Synergistic Chemotherapy

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Supporting Information

ABSTRACT: In recent years, in situ chemotherapy mediated by biodegradable polymer platforms has attracted increased attention. Herein, an advanced drug delivery system, combretastatin A-4 (CA4) and docetaxel (DTX)-loaded microsphere embedded in injectable thermosensitive polypeptide hydrogel (i.e., hydrogel–microsphere (Gel–MP) construct), was reported for sequential release of drugs with different mechanisms to treat osteosarcoma synergistically. The Gel–MP construct showed sequential biodegradability and excellent biocompatibility. CA4 was preferentially released from hydrogel with faster degradation to disturb the vascular structure of the tumor and reduce the exchange of nutrients between the tumor and surrounding tissues, which created interstitial space in the tissue for DTX penetration to inhibit tumor cell proliferation. The in vivo treatment with Gel/CA4–MP/DTX efficiently suppressed the growth of mouse K7 osteosarcoma compared to other formulations. More importantly, by systematical study of histopathology and immunohistochemistry, the Gel–MP construct can significantly upregulate antiproliferation effect and reduce toxicity of drugs. Therefore, this injectable and locally sequential delivery system has a bright prospect in clinical application of in situ synergistic chemotherapy.

KEYWORDS: thermosensitive hydrogel, microsphere, sequential drug release, osteosarcoma, synergistic chemotherapy

1. INTRODUCTION

Osteosarcoma is a primary malignant bone tumor in childhood and adolescence,1,2 with the incidence rate of ∼4.0 or ∼5.0 for the ranges of 0–14 or 0–19 years per year per million persons, respectively, which ranks eighth in the incidence of childhood malignancies.3 Currently, the combination of preoperative, operative, and post-operative neoadjuvant chemotherapy constitutes the standard treatment program of osteosarcoma.4 After standard treatment, the 5-year survival rate for osteosarcoma without metastasis is ∼60%–70%, which is limited to only 30% for those with metastasis.5 Although chemotherapy is still considered as the first choice to treat osteosarcoma, the disappointing pharmacokinetics and systemic toxicity of small molecular drugs (SMDs) are the major concerns for patient compliance, which encourage researchers to exploit efficacious system to address these issues.6–9

Recently, many studies have focused on the in situ drug delivery systems, such as polymer thermosensitive hydrogels (Gels).10,11 Chemotherapy drugs can be facilely dispersed into the thermoresponsive polymer solution at low temperature (e.g., 4 °C), without the usage of organic solvent, and the drug release depot is automatically formed in situ under the stimulus of body temperature. The local injection is easily manipulated using a syringe and could minimize the surgical trauma. Compared with the systemic administration of chemotherapy drugs, the local and continuous drug release from the Gels in the tumor site can effectively improve the efficacies and reduce the systemic toxicity of drugs.12–14

Microspheres (MPS) are widely used in controlled drug delivery to treat various diseases.15 Currently, several polymers have been used to prepare MPS.16,17 Among them, the biodegradable synthetic polymer, poly(lactic-co-glycolic acid) (PLGA), has received widespread attention.18 Previous studies have shown that the PLGA MPs can entrap a variety of SMDs, such as 5-fluorouracil (5-FU), cisplatin (CDDP), dexametha-
sone (DMS), docetaxel (DTX), doxorubicin (DOX), and paclitaxel (PTX). The SMDs-loaded MPs can steadily release the encapsulated drugs with the gradual degradation of PLGA backbone to achieve a long-term sustained administration.

Nowadays, the combination of vascular disrupting agents and cytotoxic drugs has been reported to have improved antitumor efficacy, which can induce the wholly regional apoptosis of tumors. Combretastatin A-4 (CA4) can destroy the structure of blood vessel by binding the tubulin of endothelial cells and finally induce tumor necrosis by blocking the transmission of oxygen and other necessary nutrients. CA4 played a significant role on the proliferation stage of the blood vessel endothelial cells in vitro, but had little effect in the quiescent stage. Since the antitumor effect of CA4 can only act on the internal tumor tissues with rich peripheral vascular proliferation and has no proliferation inhibition effect on tumor cells, it is often the cause of tumor treatment failure and recurrence. Fortunately, the cells at the edge of the tumor, with rich blood vessels, are sensitive to traditional chemotherapy drugs, and it enables us to explore the alternative strategy to sequentially deliver CA4 and cytotoxic drugs. However, the synergistic therapeutic effects are often unsatisfied by intravenous injection, which mainly result from short circulating half-life in vivo and unselective biodistribution. In addition, the sequence of administration is also important in the combination of CA4 and cytotoxic drugs. If the peripheral tumor cells were killed by cytotoxic drugs first, the tumor cells within the solid tumors will be survived, which will cause the tumor recurrence and metastasis.

For this issue, an injectable construct of the MP-loaded thermosensitive Gel with stepwise degradation was prepared to sequentially deliver CA4, a vascular disrupting drug, and docetaxel (DTX), a cytotoxic agent, for the treatment of osteosarcoma in this study. As shown in Scheme 1, CA4 is released preferentially, which destructs neovascularization and inhibits the formation of new blood vessels, followed by reducing exchange of nutrients between tumor and surrounding tissues to kill the tumor cells inside. The sustained release of DTX then leads to the destruction of surface cells of the tumor and causes the wholly regional apoptosis of the tumor. Our work demonstrates that the construct of hydrogel–microsphere (Gel–MP) has a bright application prospect in the in situ chemotherapy of malignancies.

2. MATERIALS AND METHODS

2.1. Materials. All the materials used in this work have been described in the Supporting Information.

2.2. Fabrication and Characterizations of Gel–MP Construct. The copolymer of poly(β-alanine-co-β-phenylalanine)-block-poly(ethylene glycol)-co-poly(β-alanine-co-β-phenylalanine) (PLAF-b-PEG-b-PLAF) was synthesized through the ring-opening polymerization (ROP) of β-alanine N-carboxyanhydride (β-Ala NCA) and β-phenylalanine N-
carboxanhydrase (L-Phe NCA) using amino-terminated poly- (ethylene glycol) (NH2−PEG-NH2) as a macrominitiator, as depicted in Scheme S1 in the Supporting Information. The blank and DTX-loaded MPs were prepared through a water-in-oil-in-water (W1/O/W2) double emulsion technique. The detailed procedures for preparations and characterizations of Gel and MP were described in the Supporting Information.

2.2.2. Preparation of Drug-Loaded Gel−MP. The concentration of DTX-entrapped PLGA MP (MP/DTX) was decided based on its drug loading content (DLC) (9.88 wt %) and the required dose of DTX at 15.0 mg per kg body weight (mg (kg BW)^−1) for tumor inhibition in vivo. The concentration of CA4 was determined based on the used dose of CA4 at 25.0 mg (kg BW)^−1. For the preparation of Gel/CA4−MP/DTX, 8.0 wt % PLAF-b-PEG-b-PLAF with 3.9 wt % MP/DTX and 0.5 wt % CA4 was dissolved together in phosphate-buffered saline (PBS). The mixed system was stirred at 4 °C for 3 days to obtain a homogeneous solution. Gel/CA4−MP and Gel−MP were prepared by dispersing different components with the same proportion.

2.2.3. In Vitro Degradation and Release Profiles of Gel/CA4−MP/DTX. 0.5 mL of Gel/CA4−MP/DTX solution was added to the vial with the inner diameter of 11 mm and placed at 37 °C for 30 min. After gelation, Gel/CA4−MP/DTX was weighed, and then 2.0 mL of PBS without or with 2.0 mg mL^−1 of elastase, mimicking the degradation microenvironment in vivo, was slowly injected into the upper layer of the Gel. Subsequently, the vial was placed into the oscillation at 37 °C with continuous shaking at 70 rpm. The upper buffers were collected and replaced by fresh buffer, and the remaining Gel was weighed every other day. The weight change of Gel/CA4−MP/DTX in the entire process was collected to show the degradation profile. After the completion of Gel/CA4−MP/DTX degradation, the concentrations of two drugs in the reserved degradation solution were measured by high-performance liquid chromatography (HPLC) (Waters, Model 1525 system with a Waters, Model C18 column and a Waters, Model 2489 ultraviolet/visible (UV/vis) detector; Waters, Milford, MA, USA). Acetonitrile−water (60:40, V/V) was used for elution with a flow rate of 1.0 mL min^−1. The absorption wavelength (λmax) was set at 230 nm, and Breeze software was used for datum analyses. The peak of DTX appeared at 6 min and the peak of CA4 appeared at 6.5 min.

2.2.4. In Vivo Degradation and Biocompatibility of Gel−MP Construct. Sprague–Dawley rats were used for the evaluation of in vivo degradation and biocompatibility of Gel−MP construct. The polypeptide with 5.0 wt % MP was dissolved in 8 wt % PBS. The solution was stirred at 4 °C for 3 days. Then, 500.0 μL of the Gel−MP solution was subcutaneously injected into the backs of rats. The rats were sacrificed at the predetermined time points (30 min, and 7, 14, 21, 28, 35, and 42 days). The Gel-located skin was collected and fixed in a 4.0% (W/V) paraformaldehyde solution. The tissues were embedded into paraffin and cut into sections of 5 μm. Tissue sections were stained with hematoxylin and eosin (H&E) for pathology and organ damage analyses.

2.3. Tumor-Bearing Mouse Model Assessments. 2.3.1. Animal Procedures. Eighty-eight-week-old BALB/c male mice were purchased from the Experimental Animal Center of Jilin University and divided into eight groups. The animal experiments were performed according to the protocol approved by the Institutional Animal Care and Use Committee of Jilin University. The tumor-bearing mice were prepared through subcutaneous injection in the armpits of right anterior limbs with 0.1 mL of cell suspension containing 2.0 × 10^6 mouse K7 osteosarcoma cells in PBS.

2.3.2. In Vivo Antitumor Efficacy. The tumor-bearing mice weighing ~20 g were raised under the same condition. When the tumors grew to ~150 mm³, 100.0 μL of different formulations were peritumorally injected. The formulations were abbreviated as follows:

- Group 1: PBS as control group (control);
- Group 2: blank MP-loaded Gel group (Gel−MP);
- Group 3: group of 5.0 mg mL^−1 free CA4 in PBS (CA4);
- Group 4: group of 3.0 mg mL^−1 free DTX in PBS (DTX);
- Group 5: group of free CA4 and free DTX at a concentration of 5.0 and 3.0 mg mL^−1 in PBS, respectively (CA4+DTX);
- Group 6: CA4 and blank MP-loaded Gel group (Gel/CA4−MP);
- Group 7: group of Gel with DTX-loaded MP (Gel−MP/DTX);
- Group 8: CA4 and MP/DTX coloaded Gel (Gel/CA4−MP/DTX).

The treatment doses of CA4 and DTX were 25.0 and 15.0 mg (kg BW)^−1, respectively. The tumor sizes were measured every 2 days by vernier calipers. The tumor volume (V, in mm³) was calculated using eq 1.

Figure 1. Morphologies and scales of MP, Gel, and Gel−MP: (A) SEM microimage of MP, (B) average size of MP, (C) SEM microimages of Gel, and (D) Gel−MP.
The histological and immunohistochemical alterations carried out following the previously reported immunocytochemistry (NMR) spectrum (Figure S1 in the Supporting Information). The degrees of polymerization (DPs) of the L-Ala and L-Phe were determined to be 6720 g mol$^{-1}$ at 4.67 and 4.41 ppm, relative to that at 3.67 ppm, respectively. The paraformaldehyde fixation was carried out overnight and then embedded in paraffin. The paraffin-embedded tumors were cut into sections with dimensions of $\sim$5 $\mu$m for H&E staining and sections with dimensions of $\sim$3 $\mu$m for immunohistochemical analyses. Immunohistochemical staining was carried out following the previously reported immunocytochemistry protocol. The histological and immunohistochemical alterations were detected by a microscope (Nikon, Model Eclipse Ti, Optical Apparatus Co., Ardmore, PA, USA) and subsequently analyzed with ImageJ software (National Institutes of Health, Bethesda, MD, USA).

3. RESULTS AND DISCUSSION

3.1. Preparation and Characterization of Injectable Gel–MP Construct. As shown in Scheme S1 in the Supporting Information, PLAF-b-PEG-b-PLAF was synthesized through the ROP of L-Ala NCA and L-Phe NCA in the presence of NH$_2$-PEG-NH$_2$ and the chemical structure was characterized by proton nuclear magnetic resonance ($^1$H NMR) spectrum (Figure S1 in the Supporting Information). The degrees of polymerization (DPs) of the L-Ala and L-Phe units were analyzed to be 30 and 4, via integration of the peaks at 4.67 and 4.41 ppm, relative to that at 3.67 ppm, respectively. The number-average molecular weight ($M_n$) and molecular weight distribution (polydispersity index, PDI) were determined to be 6720 g mol$^{-1}$ and 1.47 by $^1$H NMR and gel permeation chromatography (GPC), respectively. The PLGA MP was prepared through the W$_1$/O/W$_2$ double emulsion technique. The scanning electron microscopy (SEM) microimage showed that MP exhibited an excellent balling effect and a smooth surface (Figure 1A), and the average size of MP was $\sim$2.94 $\mu$m (Figure 1B). DTX can be efficiently loaded into MP with 9.88 wt % of DLC and 98.8 wt % of drug loading efficiency (DLE). Therefore, the PLGA MP is considered as a promising platform to encapsulate and deliver DTX.

The PLAF-b-PEG-b-PLAF solution underwent sol–gel precipitation transition with the increase of temperature, and the transition temperature was dependent on the concentration of polypeptide solution. As shown in Figure 2A, the sol–gel transition temperature decreased as the polypeptide concentration increased, and the gel–precipitation temperature showed an opposite trend. The polypeptide solution with a concentration of 8 wt % existed as fluid with low viscosity, and underwent a fast sol–gel transition at $\sim$29 °C, both of which revealed the great potential as an injectable material. The phase diagram of Gel–MP construct is similar to that of the blank gel, and the addition of MP into polypeptide solution decreased the gelation temperature, which was attributed to the enhanced interaction between Gel and MP (Figure 2B). The rheology changes of polypeptide solutions without/with MP were also evaluated (see Figures 2C and 2D), and the storage moduli ($G'$) increased as the temperature increased. Moreover, the gelation temperatures obtained from the cross-point of $G'$ and loss moduli ($G''$) were consistent with those obtained using a vial inversion method. In addition, the addition of drugs also suppressed the gelation temperature and upregulated $G'$ and $G''$, as shown in Figure S2 in the Supporting Information. The results should be ascribed to the increased hydrophobic interactions among various components.

SEM was further used for the observation of microstructure of the resulting Gel and Gel–MP construct. As shown in Figure 1C, there was an interconnected pore structure in the polypeptide Gel, which allowed the infiltration of interstitial fluid to accelerate the degradation of polypeptide backbone and also was beneficial to the adhesion of MP. As shown in Figure 1D, MP was observed to be uniformly dispersed on the surface and inside of the Gel after incorporation with Gel (Figure 1D). The size and shape of the MP, as well as the Gel, did not change after the mixing. Stirring for 3 days at 4 °C did not

\[ V (\text{mm}^3) = \frac{L \times S^2}{2} \]  

Here, $L$ is the largest tumor diameter (mm) and $S$ is the smallest tumor diameter (mm).

In addition, the body weight and survival rate were monitored in 26 days after all treatments, and all the rest of the mice were sacrificed. The entire tumor and major organs (i.e., the heart, liver, spleen, lung, and kidney) were isolated.

2.4. Histological and Immunohistochemical Analyses. The collected tumors and major organs were fixed in 4% (W/V) PBS-buffered paraformaldehyde overnight and then embedded in paraffin. The paraffin-embedded tumors were cut into sections with dimensions of $\sim$5 $\mu$m for H&E staining and sections with dimensions of $\sim$3 $\mu$m for immunohistochemical analyses. Immunohistochemical staining was carried out following the previously reported immunocytochemistry protocol. The histological and immunohistochemical alterations were detected by a microscope (Nikon, Model Eclipse Ti, Optical Apparatus Co., Ardmore, PA, USA) and subsequently analyzed with ImageJ software (National Institutes of Health, Bethesda, MD, USA).
destroy the three-dimensional (3D) structure of MP, which was beneficial to the hydrophobicity and stability of the PLGA matrix. All the above results indicated that the Gel–MP construct is suitable as an injectable platform for in vivo application.

3.2. Degradation of Gel–MP Construct and Sequential Drug Delivery. In vitro degradation of Gel–MP construct was performed in different media, that is, PBS and PBS with 2.0 mg mL⁻¹ of elastase (Figure 3A). The degradation rate of Gel–MP construct in the presence of elastase was accelerated and resisted for 50 days, compared to 60 days for the PBS group. The in vivo degradation testing was also performed in the rat model. As shown in Figure 3B, the MP-mixed Gel was observed after 30 min through subcutaneous injection, and gradually degraded for 42 days. The degradation rate in vivo was faster than that in vitro, which may be caused by the diverse enzymes in subcutaneous layer that accelerated the degradation of Gel–MP construct. Moreover, the slightly local acidic microenvironment caused by the degradation of PLGA was also due to the accelerated degradation.27–29 At the same time, the biocompatibility of Gel–MP construct was studied by H&E staining. As shown in Figure 3B, after 30 min of the injection, negligible inflammation was detected, although redness of the skin was visible in the image. The inflammation turned to be serious after 7 days, based on the increase in inflammatory cells, and the phenomenon was gradually alleviated through degradation of the implantation. After 42 days, inflammatory cells were no longer found in the section. The results indicated that there is no safety concern for the use of Gel–MP construct as subcutaneous implantation.

In vitro release of CA4 and DTX was conducted in PBS without and with elastase (Figure 3C). Gel/CA4–MP/DTX in PBS with elastase exhibited an accelerated drug release rate, because of fast degradation of the Gel–MP construct. Approximately 54% of the loaded DTX was released from DTX Gel/CA4–MP/DTX within 48 days, and no burst release was observed compared to the usage of PLGA MP only as a drug carrier (Figure S3 in the Supporting Information).30 The constant release of DTX ensured the effective drug concentration in situ and avoided the serious side effects. On the other hand, 90% of CA4 was released from Gel/CA4–MP/DTX in the same period (i.e., 48 days), and a slightly initial burst release was observed in the first 8 days resulted from its fast diffusion from the Gel surface. CA4 was preferentially released from the Gel to disturb the vascular structure of the tumor and reduce the exchange of nutrients between the tumor and the surrounding tissues, which also created tissue interstitial space for the penetration of subsequent released DTX to inhibit tumor cell proliferation.

3.3. Gel/CA4–MP/DTX for Locally Synergistic Chemotherapy of Osteosarcoma. The synergistic effect of the combination of CA4 and DTX in the Gel–MP system was evaluated in the osteosarcoma-xenograft mice model. As shown in Figure 4, the same trend of tumor growth was observed in the control and Gel–MP groups with tumors steadily deteriorating, indicating that the Gel–MP system had no therapeutic effect on osteosarcoma. Compared to the single injection of CA4 or DTX, although the coadministration of CA4 and DTX suppressed tumor growth efficiently, the safety concern was also detected regarding to the obvious loss in body weight. One thing is worth noting: the therapeutic effect of free CA4 was superior to that of free DTX, probably because of the high CA4 concentration in the initial blood of CA4 group, which resulted in the shutdown of subcutaneous tumor blood vessels with tumor necrosis. In addition, the embedding of MP/DTX or CA4 (Gel/CA4–MP or Gel–MP/DTX) in the Gel showed enhanced tumor-growth inhibition, because of the sustained release of the loading drug in a long period. The best

Figure 3. Degradation, biocompatibility, and drug release. (A) In vitro weight remaining profiles of Gel/CA4–MP/DTX incubated in PBS at pH 7.4 without or with elastase. (B) In vivo biodegradation and biocompatibility of Gel–MP construct; the Gel–MP solution was subcutaneously injected into mice. (Photographs around the implants were taken at 0 (30 min), 14, 28, and 42 days post-injection. H&E staining images around the implanted Gel after 0, 14, 28, and 42 days of subcutaneous injection of the Gel.) (C) In vitro CA4 and DTX release from Gel/CA4–MP/DTX incubated in PBS without or with elastase. (Statistical data were represented as mean ± standard deviation (SD) (n = 3).)
tumor-growth suppression was achieved by the treatment of Gel/CA4−MP/DTX, which was attributed to the locally sequential and sustained release of two drugs. The collected tumor was fixed and sectioned for analyzing the antitumor effect of different formulations via the morphology of histopathology by H&E staining. The results were supportive of the highest tumor-suppression efficiency of Gel/CA4−MP/DTX. As shown in Figure 5A, large cell nuclei, and deeply colored and an increased number of split-phase cells were observed in the control and Gel−MP groups, suggesting the rapid proliferation of tumor cells. However, for other groups, nuclear condensation and nuclei dissolution were observed, indicating the progression of tumor cell necrosis. Especially, the fewest tumor cells were found in the group treated with Gel/CA4−MP/DTX, and the necrosis area of tumor tissue was much broader than other groups, both of which supported the best tumor suppression efficiency. Moreover, NIS-Elements imaging software (Nikon Instruments SpA, Florence, Italy) was used to quantify the necrosis area of tumor tissue. Consisting with H&E section results, the largest necrotic area of ~90.5% was achieved in the Gel/CA4−MP/DTX group, which was two times greater than that of the CA4+DTX group (45.5%; see Figure 5B). Some other observations are also worth noting. First, the areas of necrosis in the control and Gel−MP groups were 2.8% and 3.6%, respectively, which might belong to the spontaneous necrosis changes of tumor cells occurring during the excessive development of the tumor. Second, in the CA4 and DTX groups, the necrotic areas were only 6.1% and 14.7%, respectively, probably due to the fast diffusion and low concentration of drugs locally. Third, the Gel−MP/DTX and Gel/CA4−MP groups also showed promising tumor-growth suppression with the necrotic area of 47.9% and 54.1%, respectively. The utilization of injectable Gels as in situ drug carrier could reduce the burst release of drugs and maintain a high drug concentration at the tumor site, to inhibit tumor cell proliferation.

To further confirm the superior antitumor effect of Gel/CA4−MP/DTX, ex vivo immunohistochemical detections were carried out. Poly(ADP-ribose) polymerase (PARP) and proliferating cell nuclear antigen (PCNA) were applied to analyze the effect of tumor inhibition. Platelet endothelial cell adhesion molecule-1 (CD31) was stained for the vascular

Figure 4. Tumor volume change. Variation of tumor volume in K7 osteosarcoma-grafted mice after peritumoral injection of PBS as control, Gel−MP, CA4, DTX, CA4+DTX, Gel/CA4−MP, Gel−MP/DTX, or Gel/CA4−MP/DTX. (Data were represented as mean ± SD (n=10; *** P < 0.001).)

Figure 5. Histopathological analyses and necrotic area statistics. (A) Ex vivo apoptosis analyses of tumor sections in H&E staining and (B) percentage of necrotic area after treatment with PBS as control, Gel−MP, CA4, DTX, CA4+DTX, Gel/CA4−MP, Gel−MP/DTX, or Gel/CA4−MP/DTX. (Statistical data were represented as mean ± SD (n = 3; (*** P < 0.001).)
disrupting efficacy. The vascular structures of the tumor can be clearly observed through marking the blood vessel epithelial cells by CD31 immunohistochemical staining. As shown in Figure 6A, the groups without CA4 treatment had intensive tumor vascularity, whereas the groups with CA4 treatment showed decreased and irregular blood vessels. The numbers and areas of blood vessels in the control and Gel−MP groups were reduced from 16.45 and 15.88 per mm^2, to 3.66 per mm^2 of the Gel/CA4−MP/DTX group (Figure 6B). Similarly, the vessel area decreased from $8.97 \times 10^3 \mu m^2$ and $8.31 \times 10^3 \mu m^2$ in the control and Gel−MP groups to $2.03 \times 10^3 \mu m^2$ in the Gel/CA4−MP/DTX group (Figure 6C), which might be
caused by the decrease of capillary blood flow and the blood vessel squeeze by the surrounding tumor cells. Anti-PARP antibody was often used to evaluate the chemotherapeutic drug-induced cell apoptosis, as shown in

Figure 8. Variations of body weight and survival rate. (A) Change of body weight and (B) survival rate of K7 osteosarcoma-grafted mice after treatment of PBS as control, Gel–MP, CA4, DTX, CA4+DTX, Gel/CA4–MP, Gel–MP/DTX, or Gel/CA4–MP/DTX. (Data were represented as mean ± SD (n = 10; (*) P < 0.05).)

Figure 9. Security in vivo. Ex vivo histological analyses of main organ sections after treatment with PBS as a control, Gel–MP, CA4, DTX, CA4+DTX, Gel/CA4–MP, Gel–MP/DTX, or Gel/CA4–MP/DTX.
Figures 7A and 7B. The fluorescence intensity of control and Gel–MP groups was higher than those of all drug-loaded formulations. For example, the fluorescence intensity of the DTX and CA4 groups was 39.7% and 53.6%, respectively, relative to the control group. The intensifying PARP inhibition signal was observed in the Gel/CA4–MP and Gel–MP/DTX groups, which was consistent with the changes of their tumor volumes. The PARP expression of Gel/CA4–MP/DTX group was reduced to 7.3%, compared to that of the control group. Similarly, the PCNA intensity in the groups treated with Gel/CA4–MP/DTX was higher than that of the other group, indicating the effective inhibition of tumor cell proliferation (see Figures 7A and 7C).

3.4. Security of Gel/CA4–MP/DTX In Vivo. Safety is one of the most important factors in the potential clinical applications of any advanced chemotherapy drug formulations. In this study, the security of all formulations was established by monitoring the changes of body weight, survival rate, and histopathological morphology of visceral organ section.

Throughout the experiment, the body weights in all groups showed a slight downward trend (see Figure 8A). Especially, the mice in the CA4+DTX group had the fastest and most obvious decline. The fastest decline occurred in the first 8 days and then gradually slowed down, reflecting the toxicity of the large injection amounts of the two drugs. Furthermore, the mice of the three Gel–MP groups had no significant decline, indicating that Gel–MP had no obvious toxicity to the mice and could decrease the side effects of antitumor drugs.

At the same time, the survival rates were monitored during the experiment, as shown in Figure 8B; the results indicated that the survival rates of control and Gel–MP groups were lower than that of all the groups with the application of antitumor drugs. Most fascinatingly, the treatment with Gel/CA4–MP/DTX exhibited the complete survival attributed to the excellent antitumor efficacy and high safety in vivo.

To further evaluate the security of all tested formulations, the histopathological analyses of main internal organs (e.g., the heart, liver, spleen, lung, and kidney) were performed through H&E staining after all treatments (Figure 9). The histological morphologies of each organ were observed. The organs of control and Gel–MP groups showed the normal histological morphologies. The results suggested that the Gel–MP construct had no toxicity to body organs. However, all the drug formulation groups exhibited pathological changes in the H&E-stained sections (more or less). The organ pathological changes were obviously more serious in the three free drug groups than the three drug-loaded formulation groups. More specifically, direct injection of free CA4 had the most serious damage to liver cells. The hepatocyte turned edema and vacuolation. The CA4+DTX group had the most severe pulmonary edema and renal capsule cavity shrank, and the most serious inflammatory cell infiltration in myocardium, hepatocyte, and alveolar walls, because of the combination toxic effects of the two drug-free groups. This experiment showed that the Gel–MP construct reduced the damage to vital organs, indicating the good prospects for clinical application.

4. CONCLUSIONS

An injectable Gel–MP construct with PLAF-b-PEG-b-PLAF and PLGA as the matrices of Gel and MP, respectively, was fabricated for the sequential delivery of CA4 and DTX. After subcutaneous injection, the Gel–MP construct degraded completely in 42 days, and it had good biocompatibility. For in vivo application, after peritumoral injection, CA4 was first released from Gel/CA4–MP/DTX to destroy the blood vessels in the tumor, and then DTX was sustainably diffused to inhibit cell proliferation, inducing the internal and external apoptosis of osteosarcoma, respectively. The results demonstrated that the construct of Gel–MP with sequential degradation exhibited a great potential for stepwise release of double antitumor drugs with different mechanisms for locally synergistic chemotherapy.

ASSOCIATED CONTENT

# Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.6b15245.

Detailed experimental section; synthetic route and 1H NMR spectrum of PLAF-b-PEG-b-PLAF; rheological analysis of Gel/CA4–MP/DTX; DTX release from MP (PDF)

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