

Implantable Polyurethane Scaffolds Loading with PEG-Paclitaxel Conjugates for the Treatment of Glioblastoma Multiforme

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Electronic Supplementary Information

Abstract Improvement of the treatment for Glioblastoma multiforme (GBM) especially the development of *in situ* controllable drug release is still a major concern. In this study, we developed waterborne biodegradable polyurethane (WBPU) scaffolds incorporated with redox-sensitive and RGD-decorated paclitaxel (PTX) polymer-drug conjugates (PDCs) for targeted GBM therapy *in situ*. The drug scaffolds could be implanted at residual GBM site post-operation. Dual-targeting PTX-PDCs were obtained through step-by-step conjugation of disulfide linked PTX, poly(ethylene glycol) (PEG), and arginine-glycine-aspartic acid (RGD). The RGD-modified PTX-PDCs were spherical nanoparticles (NPs) that would be released from scaffolds and identified GBM cells actively. Internalized redox-sensitive PTX-PDCs would be decomposed and release PTX inside GBM cells under the circumstances of glutathione (GSH). The release profiles of PTX from the scaffolds with/without GSH were investigated. *In vitro* cytotoxicity assay revealed that the dual-targeting PTX-PDCs from scaffolds could specifically kill GBM cells and protect normal cells, suggesting that dual-targeting PTX-PDC-loaded scaffolds may have the potential to repair tumor-induced brain injury. *In vivo* anti-recurrence assay indicated that the PTX-PDC-scaffolds could deliver PTX-PDCs to the GBM cells followed by inhibiting tumor growth and inducing apoptosis. In general, the PTX prodrug-loaded devices exhibited significant anti-GBM effects and normal tissue protection simultaneously, indicating that the WBPU scaffolds incorporated with dual-targeting PTX-PDCs may be a promising strategy for local therapy of GBM.

Keywords GBM; PDCs; WBPU; Scaffolds; Controlled release

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INTRODUCTION

Glioblastoma multiforme (GBM) is the most common malignant primary brain tumor in adults with a relatively poor prognosis.^[1] Surgical resection is a preferred treatment option to relieve symptoms and obtain pathological diagnosis of GBM.^[2] However, the aggressive and highly angiogenic GBM often leads to local recurrence even after maximizing extent of resection.^[3] What is worse, because blood brain barrier (BBB) and blood brain tumor barrier (BBTB) prevent most drugs from entering the central nervous system, the effect of post-operative chemotherapy is often poor.^[4] How to improve the therapeutic efficacy of GBM is still a major concern. Over the past few decades, a variety of approaches have been investigated to improve drug delivery into the GBM, including drug modification and drug carriers.^[5] Specifically, nanomaterial-

based delivery carriers are able to increase drug solubility, bioavailability, and BBB penetrability and to some extent overcome drug resistance of GBM.^[6,7] Still, these nano-vehicles also face a drawback of rapid blood clearance by the reticuloendothelial system (RES), leading to a failure in the clinical treatment of GBM.

In view of this, it is currently believed that local delivery of therapeutic agents to fight GBM may be safer and more effective than systemic administration.^[8] Specifically, the design of a suitable device to carry chemotherapy drugs, cytokines, and cells for local implantation that bypass BBB has been investigated for adjuvant therapy of GBM after surgical resection. It has been well known that the polymeric Gliadel[®] is the most famous implantable biomaterial studied for the treatment of GBM.^[2] Gliadel[®] is a commercial polyanhydride copolymer (p(CPP:SA) wafer loading with bis-chloroethylnitrosourea (BCNU)), and has been demonstrated to release drug over a period of 3 weeks after implanted into resection cavity of GBM.^[9] Although a promising device, Gliadel[®] failed to prevent the recurrence of GBM clinically due to the resistance of BCNU.^[10]

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Recent studies have shown that the introduction of microspheres or nanoparticles (NPs) into implantable devices to form a composite drug delivery system may control the release of different drugs and reduce drug resistance.^[11–14] Fourniols and colleagues reported a photocross-linkable hydrogel that was made from polyethylene glycol dimethacrylate (PEG-DMA).^[15] The hydrogel was used for delivering temozolomide-loaded polymeric micelles (M-TMZ) to GBM in mice. It was shown that the drug-loaded hydrogel was intracranially injected into resected GBM to realize controllable release of TMZ and growth inhibition of tumor cells. However, in most cases, the drug-encapsulated delivery platform may face less controlled formulation, low drug loading, and less controlled drug release, making it difficult to achieve therapeutically effective concentration after released from implantable devices at tumor site.^[16]

Polymer-drug conjugate (PDC) was introduced as a unique type of polymeric drug delivery system in the 1950s and have aroused the interest of scientists ever since.^[17,18] In contrast to the encapsulate nanomaterial carriers, PDCs therapeutics refer to macromolecular constructs with therapeutic agents covalently bonded to polymeric carriers.^[17] The versatility of stimuli-responsive bonds between drugs and polymers confers spatiotemporal control over the release of drugs under specific circumstances.^[19] As a consequence, PDCs have several advantages compared to nanoparticulate carriers, including improved drug loading, prolonged half-life, enhanced cell uptake, controlled drug release, and reduced side effects.^[17]

In this study, the concept of site-specific drug release for Paclitaxel (PTX) was designed and confirmed. $\alpha v \beta 3$ integrin-targeted and redox-sensitive PTX-PDCs were explored and loaded on polyurethane scaffold for the treatment of GBM. PTX, a previously demonstrated anti-GBM chemo drug,^[20] was conjugated to poly(ethylene glycol) (PEG) *via* disulfide linkage to achieve the redox-responsive release of PTX. Arginine-glycine-aspartic acid (RGD), a well-studied $\alpha v \beta 3$ integrin targeted peptide,^[21] was introduced in the PEG-ss-PTX conjugates to form GBM stroma-targeting PTX-PDCs. Waterborne biodegradable polyurethane (WBPU), a widely applied biomedical material, has been demonstrated in our previous work with good properties in drug delivery and tissue engineering.^[7,22,23] Here, WBPU was prepared and fabricated into 3D porous scaffold to carry and release dual-targeting PTX-PDCs at residual GBM site. The structure and morphology of the PTX-PDC-loaded scaffolds were characterized. The drug release profile, *in vitro* and *in vivo* anti-GBM effects were studied.

MATERIALS AND METHODS

Materials

PTX ($\geq 99.5\%$) was purchased from Shanghai Jinhe Biotechnology Company. Taxol was purchased from Yangzijiang Pharmaceutical. 3,3'-Dithiodipropionic acid, *N*-hydroxysuccinimide (NHS), dicyclohexylcarbodiimide (DCC), 1-hydroxybenzotriazole (HOBT) and 4-dimethylaminopyridine (DMAP) were purchased from Shanghai Aladdin Reagent Company. NH_2 -PEG₂₀₀₀-Mal and cyclic peptide RGD were purchased from Beijing Huawei Ruike Chemical Corporation. Tetrahydrofuran (THF) was purchased from Chengdu Kelong Chemical Co., Ltd., dried with sodium

metal (Na) under reflux, and stored with 4 Å molecular sieve. Acetyl chloride, toluene, ether, chloroform and other common reagents were all purchased from Chengdu Kelong Chemical Co., Ltd. Lysine diisocyanate (LDI, analytical grade) was purchased from Nantong Dahong Chemical Co., Ltd., and re-evacuated under reduced pressure before use. 1,3-Propanediol (PDO, analytical grade) was purchased from Chengdu Kelong Chemical Co., and re-evacuated under reduced pressure before use. Poly(ethylene glycol) (PEG1450, analytical grade) was purchased from Dow Chemical, and dehydrated at 90 °C for 1.5 h before use. Polycaprolactone (PCL2000, analytical grade) was purchased from Japan Daicel, and dehydrated at 90 °C for 1.5 h before use. L-lysine (Lys, analytical grade) was purchased from Ronggao (Sichuan). 3-(4,5-Dimethylthiazole-2)-2,5-diphenyltetrazolium bromide (MTT, Sigma) was used in the cytotoxicity test. Phosphate buffered saline (PBS), DMEM high-glucose medium, MEM medium, fetal bovine serum, and trypsin were purchased from Thermo Fisher Scientific (China). Penicillin, streptomycin, paraformaldehyde, Triton X-100, 4',6-diamidino-2-phenylindole (DAPI), and Rhodamine-labeled Phalloidin were purchased from Solarbio Life Science Co., Ltd. Mouse glioma cells GL261, mouse fibroblasts L929, mouse microglia BV2, and human umbilical vein endothelial cells (HUVEC) were provided by West China Hospital. SPF female C57BL/6 mice (18–20 g, 6–8-week-old) were purchased from Laboratory Animal Center of Sichuan University.

Synthesis of PTX-ss-PEG-RGD

To synthesize PTX-ss, dithiodipropionic anhydride (DTDPA), an anhydride form of 3,3'-dithiodipropionic acid (DTPA), was synthesized at first through dehydration according to previous reports.^[24] PTX (0.1 g, 0.12 mmol), DTDPA (0.02 g, 0.12 mmol), HOBT (0.05 g, 0.36 mmol) and DMAP (0.04 g, 0.36 mmol) were dissolved in THF (20 mL). The mixture was stirred at room temperature (RT) for 24 h to get PTX-ss (80%). Then, PTX-ss (0.039 g, 0.04 mmol) was activated by DCC (0.024 g, 0.12 mmol), HOBT (0.016 g, 0.12 mmol) and NHS (0.014 g, 0.12 mmol) under ice-salt bath conditions for 6 h. After that, NH_2 -PEG-Mal (0.06 g, 0.03 mmol) was added, and the mixture was stirred at room temperature (RT) for 24 h. The products were purified by chromatography column (trichloromethane:methanol=30:1, yield 70%). Finally, c(RGDfc) (0.002 g, 0.004 mmol) and PTX-DTDPA-PEG (0.015 g, 0.005 mmol) were dissolved in a PBS solution (pH=7.3) and stirred at RT for 24 h to obtain PTX-ss-PEG-RGD with a yield of 65%. The products were dissolved in DMSO- d_6 and the chemical structure was determined using ^1H -NMR analysis. As a control, redox non-sensitive PTX-cc was synthesized through a similar method, using adipic anhydride instead of DTDPA.

Preparation of PTX-PDCs NPs and Scaffolds

To evaluate the dual-targeting capability (extracellular integrin and intracellular redox) towards tumor cells, four PTX-PDCs NPs were prepared: both integrin-targeted and redox-sensitive PTX-ss-PEG-RGD NPs (named PDPR), only redox-sensitive PTX-ss-PEG NPs (named PDP), only integrin-targeted PTX-cc-PEG-RGD NPs (named PAPR), and both integrin non-targeted and redox non-sensitive PTX-cc-PEG NPs (named PAP). The solvent evaporation method was used to prepare the PTX-PDCs NPs. Typically, 10 mg of PTX-PDCs was dissolved in 1 mL of THF. Then, the mixture was added dropwise into 10 mL of distilled deionized water with vigorous stirring for 2 h. Finally, the micellar solution was

evaporated under reduced pressure at 30 °C to remove THF and obtain PTX-PDCs NPs. Before cell and animal experiment, the micellar solution was passed through 0.22 µm bacteria filter.

WBPU emulsions were prepared according to our previous report, and a series of drug-loaded scaffolds with porous structures were formed by a freeze-drying method. Firstly, L-lysine diisocyanate (LDI) and 1‰ of organic bismuth was added into the stirred PEG ($M_w=1450$) and poly-3-caprolactone (PCL, $M_n=2000$) at 80 °C under a dry nitrogen atmosphere. Then, 1,3-propanediol (PDO), as a chain extender, was added to melting reaction mixture for 2 h at 65 °C. Secondly, the prepolymer was emulsified by mixing with L-lysine solution for 3 h. Meanwhile, the pH of the system was adjusted to 8.0 using dilute sodium hydroxide. After that, 12 wt% of emulsion was blended with various formulations of PTX (Taxol, PAP, PAPR, PDP, and PDPR) and sent to freeze-drying. The blended emulsion and blank emulsion were dropped into a 96-well plate and precooled in the refrigerator at 4 °C for 4 h, then the plate was transferred to −24 °C for 24 h before freeze-drying. The pre-frozen emulsion in 96-well plate was lyophilized in a freeze-dryer (Boyikang, Beijing) for 48 h to dry completely. The scaffolds were sterilized by gamma radiation with a dose of 20 kGy and immersed in sterile PBS overnight before *in vitro* and *in vivo* experiments.

Determination of Particle Size and Zeta Potential

The particle sizes, polydispersity indexes (PDI) and zeta potential of PTX-PDCs NPs and WBPU emulsion were determined using a laser light scattering technique (Malvern, UK). Size measurements were taken at an angle of 90° at 25 °C.

Transmission Electron Microscopy (TEM)

Transmission electron microscopy (TEM) of PTX-PDCs NPs, WBPU emulsion, and blended emulsion were performed on a Hitachi model H-600-4 transmission electron microscope (Japan) with an accelerating voltage of 75 kV. A drop of each solution stained by 0.01 g/mL of phosphotungstic acid was placed on a copper grid with Formvar film, and then the liquid was blotted off and air-dried before measurement.

Scanning Electron Microscopy (SEM)

The microstructure of the PTX-PDC-scaffolds was observed by a scanning electron microscope (SEM) (Inspect F50, FEI company, USA) with a 20-kV accelerating voltage. Before morphological observation, the specimens were fractured in liquid nitrogen and coated with gold. The pore size and porosity of the scaffolds were determined by analyzing the SEM images using Image J.

Water Absorption

The PTX-PDC-scaffolds with various concentrations of PTX prodrugs were prepared to the same volume, and the weight of dry samples was recorded as W_d . These scaffolds were placed into EP tubes filled with deionized water, and the tubes were placed in a shaker at 110 r/min (37 °C). After 24 h, the wet scaffolds were weighed and recorded as W_w . The water absorption rate (WA) of the samples is calculated as follows:

$$WA\% = \frac{W_w - W_d}{W_d} \times 100\% \quad (1)$$

Differential Scanning Calorimetry (DSC)

Thermal analysis of the dried PTX-PDC-scaffolds was performed

by DSC (TA, DSC250, USA). During the procedure, the temperature of the sample first dropped to −80 °C, equilibrated for 5 min, and then increased up to 150 °C at a rate of 10 °C/min. The mass of all samples was about 10 mg, and the analysis was performed under nitrogen protection.

Fourier Transform Infrared (FTIR) Spectroscopy

The infrared analysis of the PTX-PDC-scaffolds was performed using a Nicolet-6700 spectrometer with an attenuated total reflectance (ATR) objective equipped with a zinc selenide crystal (Spectra Tech.). The spectra were collected at a spectral resolution of 4 cm^{−1} by accumulating 32 scans.

Mechanical Performance Test

The compression profiles of the PTX-PDC-scaffolds were evaluated using a commercial universal tensile tester (HZ-1004 Dongguan Lixian instrument Scientific Co., Ltd.). The mechanical properties of the scaffolds in both dry and wet state were tested. The compression speed was 10 mm/min, and a mechanical sensor of 1 kg was selected. All scaffolds were square in shape (5 mm × 10 mm × 10 mm). In the compression test, the compression process stopped when the maximum compression deformation reaches 70%. All mechanical performance tests were performed at 25 °C. All of the values were expressed as mean±standard deviation ($n \geq 3$).

In Vitro Degradation Test of Scaffolds

The blank- and PTX-PDC-scaffolds with uniform size (5 mm × 10 mm × 10 mm) were weighed and put into 10 mL of phosphate buffer solution (PBS, 0.01 mol/L, pH=7.3). Then, all samples were sealed and placed into a shaker (100 r/min, 37 °C). The scaffolds were taken out every 3 days. After being washed 2–3 times with deionized water, all scaffolds were dried by freeze-drying, then the scaffolds were weighed. All of the values were expressed as mean±standard deviation ($n \geq 3$).

In Vitro Drug Release Profiles of PTX

The release profiles of PTX from PTX-PDCs NPs were studied by dialysis. Briefly, a certain amount of fresh PTX-PDCs NPs was transferred into a dialysis bag (MWCO: 1000 Da) and then immersed in 30 mL of releasing media on a gentle shaker (100 r/min) at 37 °C. The releasing media was composed of PBS buffer (pH 7.3), PBS buffer (pH 7.3) with 10 mmol/L GSH, HAc-NaAc buffer (pH 5.0), or HAc-NaAc buffer (pH 5.0) with 10 mmol/L GSH. The releasing solution (1 mL) was taken at specified time points and an equal amount of fresh releasing media was added. PTX content in the releasing media was analyzed using a high-performance liquid chromatography (HPLC) equipped with a symmetry C18 column. The mobile phase was acetonitrile/water (V/V=8/2). PTX was detected with a UV detector at 227 nm. All of the values were expressed as mean±standard deviation ($n \geq 3$).

Cell Culture

GL261, L929, BV2, and HUVEC cells were cultivated in Dulbecco's modified Eagle's medium (DMEM, Gibco Life, USA) supplemented with 10% fetal bovine serum (FBS, Gibco Life, USA) and 1% penicillin-streptomycin (Gibco Life, USA). The cell cultures were maintained in a 37 °C incubator (HeraCell, Thermo Scientific) with a humidified 5% CO₂ atmosphere.

In Vitro Cytotoxicity Assay

The *in vitro* cytotoxicity of free PTX and PTX-PDCs NPs was

evaluated on GL261, L929, BV2, and HUVEC cells by MTT method. Briefly, cells in the logarithmic phase were seeded in 96-well plates at 3×10^3 cells per well and incubated overnight. The next day, the culture media was removed and replaced with 100 μ L of medium containing various concentrations of all PTX formulations. After 24 h and 72 h of incubation, the cells were exposed to 20 μ L of MTT solution (5 mg/mL) and incubated for 4 h at 37 °C. Then, the MTT solution was removed and the insoluble formazan crystals were dissolved in 100 μ L of dimethyl sulfoxide (DMSO). The plates were shaken for 10 min, and the absorbance of formazan product was measured at 490 nm on a microplate reader (DNM-9602, Nanjing Perlove Medical Equipment Co., Ltd., China). The cell viability was normalized to that of untreated cells.

The *in vitro* cytotoxicity of PTX-PDC-scaffolds was carried out by cultivating GL261, L929, BV2 and HUVEC cells with the extract of each drug-scaffolds. The cell viability was investigated using the similar MTT assay.

Cell Growth on the Scaffold

Cells in the logarithmic phase were seeded on sterilized blank and PTX-PDC-scaffolds (1×10^4 cells per scaffold) in 96-well plates. Each well was filled with 100 μ L of full cell medium and incubated for 1 day, 2 days, and 3 days. At each time point, the scaffolds with cells were fixed in 4% paraformaldehyde (PFA) in DPBS for 1 h, permeabilized with 0.2% Triton-X (Sigma, USA) in PBS for 2 min and blocked with 1% BSA (bovine serum albumin, Gibco) and 5% FBS for 20 min at room temperature before phalloidin staining. The samples were incubated for 30 min at room temperature with rhodamine-labeled phalloidin. The nuclei were counterstained with DAPI for 8 min, and the samples were immersed in antifade mounting medium. Confocal images were obtained with a Nikon A1R MP⁺ laser scanning microscope (Nikon Microsystems, Japan).

In Vivo Anti-GBM Efficacy

Anticancer efficacy of PTX-PDC-scaffolds was investigated using a postoperative model of GBM in C57BL/6 mice. The animal studies were carried out with approval from the Ethics Committee of Sichuan University and in compliance with the Principles of Laboratory Animal Care of the National Institutes of Health, China. The mice were housed at a controlled temperature of 20–22 °C and relative humidity of 50%–60% with 12 h light-dark cycles and provided with standard laboratory chow and tap water *ad libitum*. All animals were quarantined for a week before the experiment. Tumor inoculation was done by subcutaneously injecting 5×10^5 GL261 cells in the right flank region. The tumors were allowed to grow to a volume of 100 μ L. Then the mice were anesthetized with 2% isoflurane and randomly assigned to seven groups ($n=3$). All mice underwent tumor resection with residual tissue about 2.5 mm $\times$ 2.5 mm $\times$ 2.5 mm, the scaffolds (diameter 4.5 mm, thickness 2.0 mm) were implanted at the residual tumor site, and mice without any implant were set as a shame group. Lastly, the incision was closed with a continuous suture. After the surgery, ceftriaxone (20 mg per kg BW) was administered, and the surgical site was treated with povidone-iodine. The tumor volumes of the mice were measured every two days with a caliper for a period of 19 days. Tumor volume was estimated using the equation: Volume = $\pi/6 \times \text{length} \times \text{width}^2$. After 19 days, all the mice were sacrificed and tumor tissues were

collected for IHC assay.

Statistical Analysis

All data are presented as the mean and standard deviation. Statistical analysis was performed with a one-way ANOVA followed by Tukey's multiple comparisons test using Prism 7.0a (GraphPad Software, Inc.). A value of $P < 0.05$ was considered statistically significant: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$; ns: $P > 0.05$.

RESULTS AND DISCUSSION

Preparation and Drug Delivery Mechanism of PTX-PDC-Loaded WBPU Scaffolds

As shown in Fig. 1, modified PTX and PEG reacted to produce PTX-PEG with disulfide linkage (PTX-ss-PEG, PDP). As a control, redox non-sensitive PEG-PTX conjugate was synthesized using similar method without a disulfide bond (PTX-cc-PEG, PAP). RGD was then introduced into PDP or PAP to obtain RGD-decorated PTX-PDCs [PDP-RGD (PDPR) and PAP-RGD (PAPR)]. Meanwhile, a well-designed WBPU emulsion was synthesized according to our previous work.^[22] The resultant PTX-PDCs (PDP, PDPR, PAP, and PAPR) could self-assemble into NPs in water and were blended into WBPU emulsion to fabricate different formulations of PTX-loaded scaffolds. PTX-PDC-loaded scaffolds could be implanted at residual tumor site directly, the released PTX-PDCs might be endocytosed by GBM cells (with/without RGD-mediated pathways), and degraded by intracellular environment to release PTX (with/without redox reaction).

Chemical Composition and Structure

The chemical composition of PTX-PDCs was characterized by ¹H-NMR spectroscopy, as shown in Figs. S1 and S2 (in the electronic supplementary information, ESI). The resonance of b'-CH proton of PTX-PA (PAP) and PTX-DTDPA (PDP) was shifted from 6.16 ppm to 5.58 ppm compared with that of free PTX (Fig. S1 in ESI). Meanwhile, the characteristic peaks of DTDPA methylene proton at 2.57 and 2.83 ppm were clearly observed, demonstrating the successful conjugation of disulfide bond to PTX. According to the characteristic resonances of PAP, PAPR, PDP, and PDPR, the PTX-ss or PTX-cc was successfully conjugated to the PEG (Fig. S2 in ESI). The characteristic peaks of PEG methylene proton and maleimide proton at 3.51 and 7.0 ppm were observed for both PAP and PDP. Nevertheless, the disappeared signals of maleimide proton at 7.0 ppm for both PAPR and PDPR verified the conjugation of RGD with PAP and PDP.

Morphological structures of PTX-PDCs, WBPU emulsion, and blended solution were observed by TEM, as shown in Fig. 2(a). The results demonstrated that the average size of PTX-PDCs was nearly 30 nm, smaller than that of 60 nm for emulsion NPs. After blending, the PTX-PDCs and emulsion NPs existed independently and could be distinguished clearly at the TEM field. The average size and zeta potential of PTX-PDCs were measured by DLS (Table 1). After the RGD conjugation, the size of PAPR and PDPR changed slightly compared with that of PAP and PDP. The zeta potentials increase from −13.2 mV (PAP) to −9.91 mV (PAPR) and −18.1 mV (PDP) to −7.38 mV (PDPR), respectively, attributed to the drop of particle charges after conjugation with RGD. After conjugation, the hydrodynamic size and PDI of complex NPs decrease slightly with increasing proportion for all PTX-PDCs in

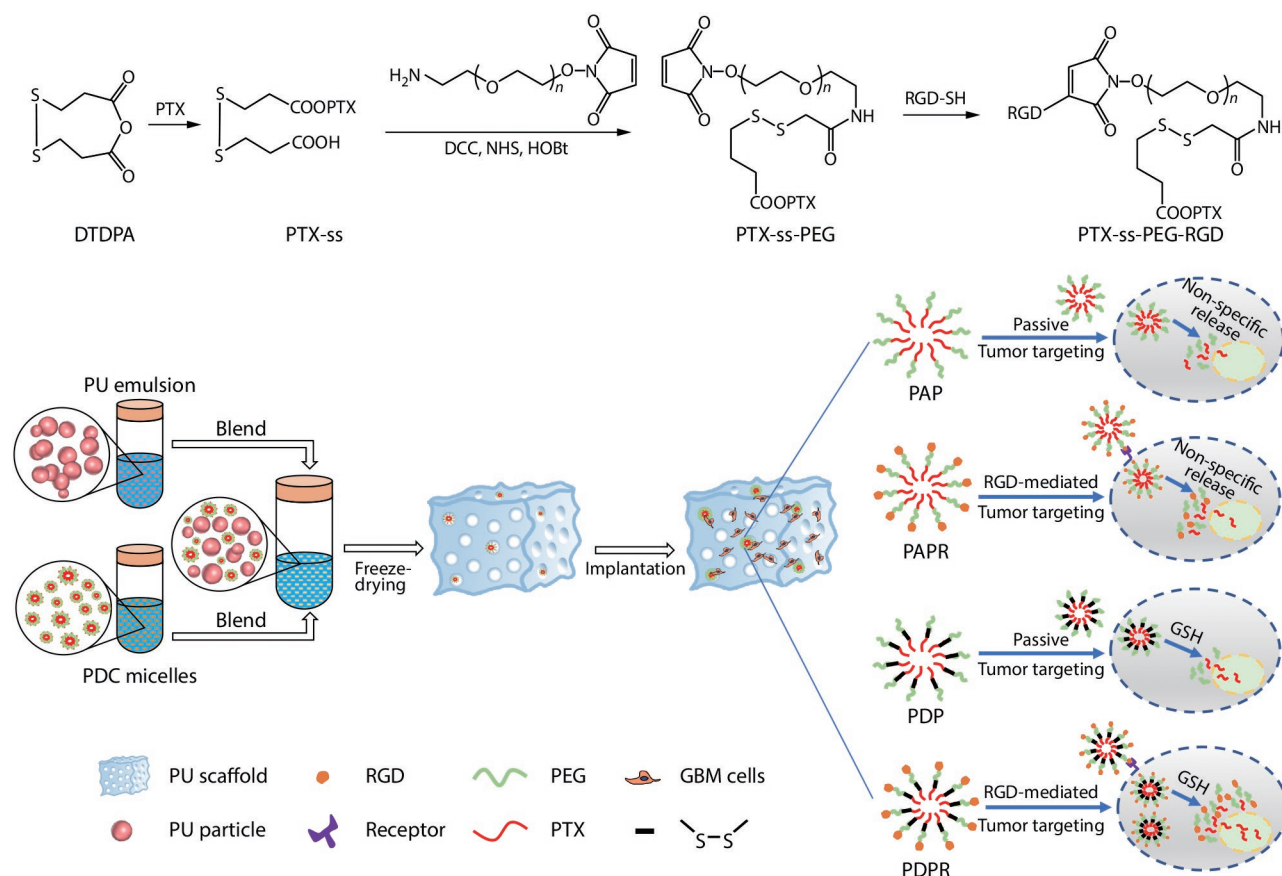


Fig. 1 Schematic illustration of scaffold-loading, RGD-mediated, and redox-responsive PTX-PDCs for GBM therapy.

the WBPU emulsion (Fig. 2c, Table 2).

The morphology and porosity of scaffolds fabricated from complex emulsion were observed by SEM, as shown in Fig. 2(b). The SEM images indicated typical 3D porous structures of PTX-PDC-loaded scaffolds as well as blank scaffolds. The pore size and porosity of the scaffolds have no obvious change after incorporation with PTX-PDCs (Table 3). It was calculated that the porosity of both blank scaffolds and PTX-PDC-loaded scaffolds was almost 80% with a water absorption rate over 300% (data not shown), ensuring homogeneous release of each drug formulation and well exchange of nutrients and metabolic substances. In addition, high magnification of SEM image also indicates granular deposition of PTX-PDCs on the pore-wall of drug-loaded scaffolds. The form of PTX-PDCs which exist on scaffolds would facilitate the release of drugs into residual GBM once they are implanted in it.

To explore the phase structure of the PTX-PDC-loaded scaffolds, thermodynamic properties of the scaffolds were tested by DSC (Fig. S3 in ESI). The DSC results show that the glass transition temperature (T_g) of all the samples was $-55\text{ }^{\circ}\text{C}$, indicating that the thermodynamic characteristics of WBPU scaffolds were stable after PTX-PDCs incorporation. The microphase separation structures of the WBPU scaffolds with various proportions of PTX-PDCs were further demonstrated by ATR-FTIR spectra (Fig. S4 in ESI). The highly similar curves from PTX-PDC-loaded and blank scaffolds indicated that the incorporation with PTX-PDCs had no impact on the micro-

phase separation structures of WBPU. The stable thermodynamic parameters and consistent chemical structures of blank- and PTX-PDC-scaffolds suggested that PTX-PDCs might have a homogeneous distribution in drug-scaffolds.

It is imperative to examine the mechanical property of the PTX-PDC-loaded scaffolds as implants to be used *in vivo*. According to the compression curve and modulus of blank and drug-loaded scaffolds shown in Fig. 3, scaffolds that were incorporated with PTX-PDCs showed higher compression modulus than blank scaffold in dry state, modulus of drug-loaded scaffolds did not change significantly with increasing concentration of all drug formulations. When in wet state, however, both blank and PTX-PDC-loaded scaffolds showed a similarly low compression modulus, meeting the *in vivo* application of soft tissue repair (e.g., brain regeneration).

In addition, these WBPU scaffolds will degrade with time in water environment. To investigate the degradation rate of drug-loaded scaffolds, scaffolds incorporated with various concentrations of PTX-PDCs were immersed in PBS (pH 7.3, $37\text{ }^{\circ}\text{C}$) for several days. The degradation curve was shown in Fig. S5 (in ESI). All scaffolds degraded steadily and reached almost 10% of degradation within 15 days. Interestingly, PTX-PDC-loaded scaffolds show faster degradation rate with increasing concentrations of drug formulation. Due to the highly hydrophilic properties of PTX prodrugs, the degradation rate of PTX-PDC-scaffolds could be accelerated with the redissolving of PTX prodrugs under aqueous circumstances. The degrada-

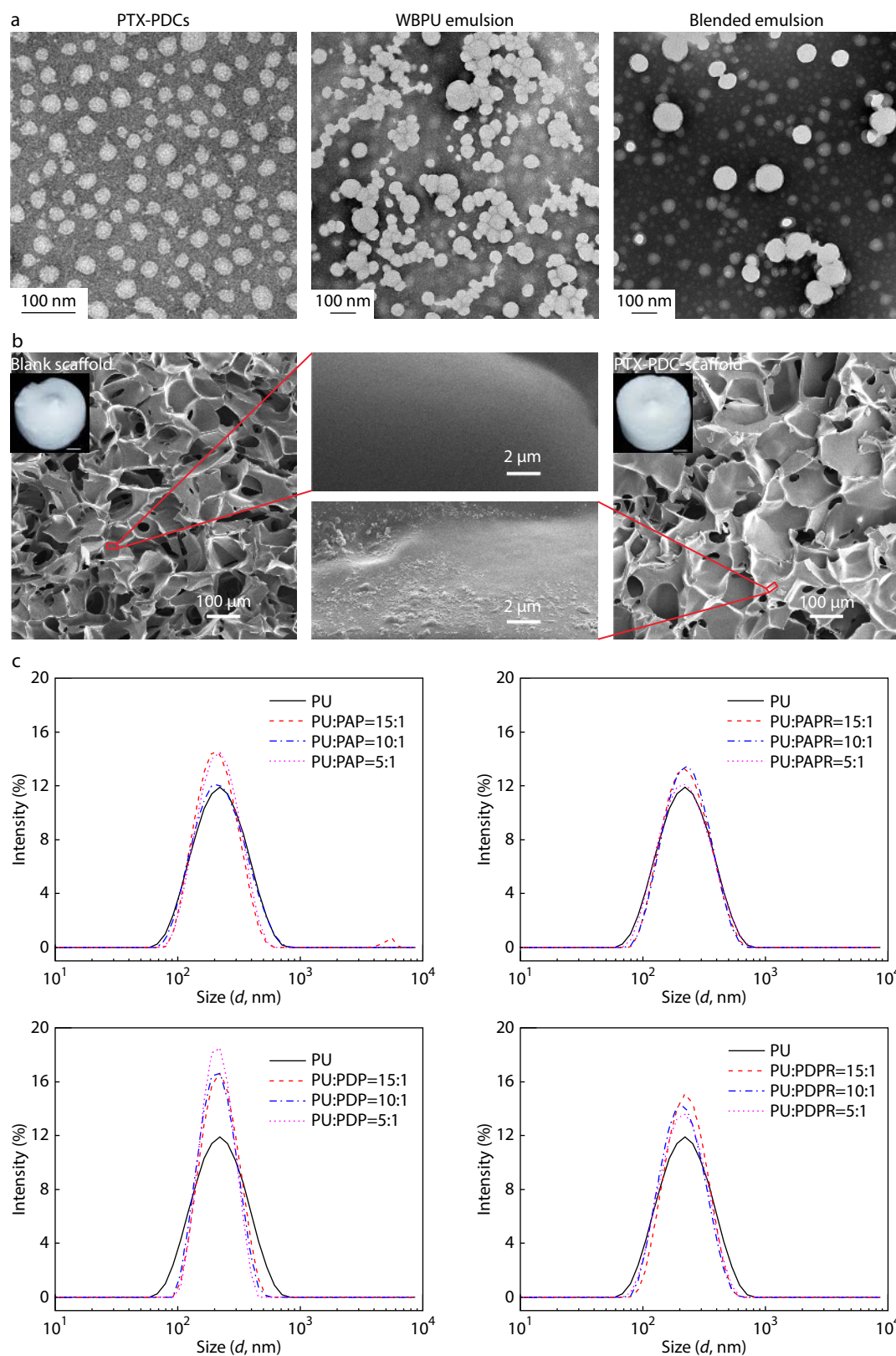


Fig. 2 (a) TEM images of representative PTX-PDCs (left), WBPU emulsion (middle), and blended emulsion (right); (b) Physical photos and SEM images of blank scaffolds and representative PTX-PDC-scaffolds. Scale bars 1 mm for white field, 100 μm for low-magnification field, and 2 μm for high-magnification field; (c) Size distributions of mixtures after various incorporation ratios of PTX-PDCs into WBPU emulsion.

Table 1 Micellar characteristics of PDP, PDPR, PAP, and PAPR.

Samples	Size (nm)	PDI	Zeta potential (mV)
PAP	109.43±0.94	0.103±0.037	−13.2±0.6
PAPR	117.93±5.57	0.110±0.002	−9.91±0.3
PDP	129.07±1.76	0.166±0.003	−18.1±0.8
PDPR	114.63±1.53	0.065±0.012	−7.38±0.8

Table 2 Particle size and PDI of blending emulsion.

Ratio	PU:PAP		PU:PAPR	
	Size (nm)	PDI	Size (nm)	PDI
1:0	243.5±5.8	0.263±0.03	243.5±5.8	0.263±0.03
15:1	199.6±1.4	0.19±0.02	207.5±0.9	0.13±0.01
10:1	197.6±4.1	0.17±0.03	204.2±2.2	0.16±0.02
5:1	195.8±1.1	0.13±0.01	207.5±1.1	0.13±0.02

Ratio	PU:PDP		PU:PDPR	
	Size (nm)	PDI	Size (nm)	PDI
1:0	243.5±5.8	0.263±0.03	243.5±5.8	0.263±0.03
15:1	194.9±2.0	0.11±0.04	201.9±5.0	0.14±0.01
10:1	197.5±2.0	0.10±0.02	198.2±3.6	0.15±0.02
5:1	194.9±3.5	0.10±0.01	196.5±1.5	0.14±0.01

tion characteristics of drug-scaffolds also met the delivery and release requirements of PTX-PDCs in residual tumor tissues.

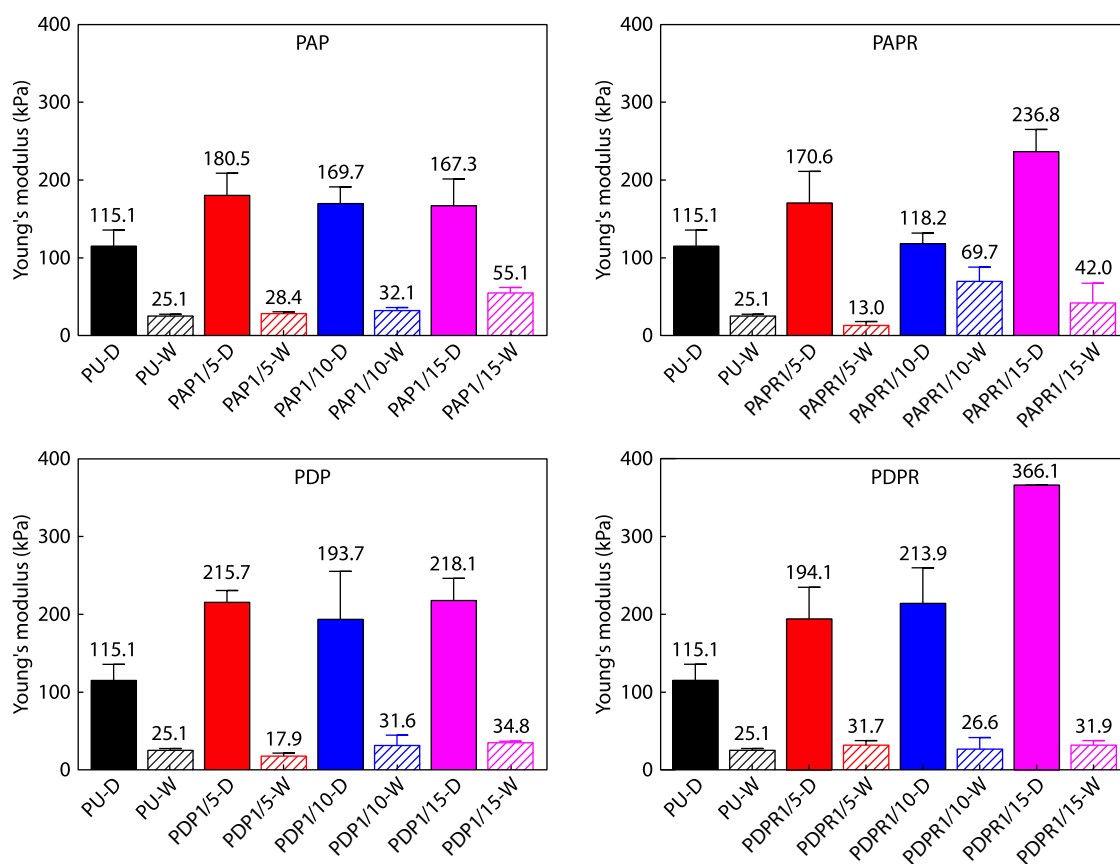
In Vitro Drug Release

It is well known that disulfide bonds are unstable and easily broken under redox circumstances.^[25] To characterize the stability of redox sensitive PTX-PDCs under redox environment,

Table 3 Average pore sizes and porosity of PU scaffolds with or without PTX-PDCs.

Sample	Average pore size (μm)	Porosity (%)
PU	68.2±28.5	84.0±3.7
5PU+1PAP	71.8±18.9	82.2±6.7
5PU+1PAPR	74.4±18.8	79.5±0.9
5PU+1PDP	63.8±24.0	81.1±1.5
5PU+1PDPR	71.0±25.0	81.5±1.1

the hydrodynamic size changes of PDP and PDPR after incubation with GSH were investigated by DLS. The size changes of the PTX-PDCs with time in the presence of GSH (10 mmol/L, pH 7.3, 37 °C) are shown in Fig. 4(a). The average diameter of the PAP and PAPR changed slightly after incubation with GSH for a period of time, indicating stability of such PDCs under redox environment. In the PDP and PDPR group, by contrast, the diameter of the micelles increased with incubation time, to over 800 nm at 4 h with a broad distribution. Due to the reductive GSH, the disulfide bonds in PDP and PDPR were broken, and the PDCs were decomposed and aggregated to extend the particle size and release PTX.^[26] On the other hand, the long-term stability of the PTX-PDCs under physiological environments was evaluated by investigating the size changes of the PDCs after incubation in PBS over a period of 7 days (Fig. S6 in ESI). The hydrodynamic size and PDI of PDCs in PBS exhibit a lower increase compared with those in GSH solution, indicating that the PDCs are relatively stable under physiological

**Fig. 3** Compression modulus of blank- and PTX-PDC-scaffolds under dry and wet conditions.

conditions. To evaluate the drug release properties of the PTX-PDCs, the release profiles of PTX from the PDCs were examined. The cumulative release of PTX from PAP, PAPER, PDP, and PDPR in the presence of GSH (10 mmol/L, pH 5.0/pH 7.3, 37 °C) is shown in Fig. 4(b). At pH 7.3, more than 90% of the PTX was released from PDP and PDPR after incubation for 120 h. When the pH was 5.0, a slight reduce in the release of PTX (80%) was observed within the same period. The release profiles of PTX-ss-PDCs under neutral and slightly acidic environment also met the tumor characteristics.

To examine the drug release properties of the PTX-PDC-

scaffolds, the release profiles of PTX from the scaffolds were investigated. The drug release profile of free PTX-loaded scaffolds obtained with the same method was used as a control. As shown in Fig. 5, PTX was released at a higher rate from free PTX-loaded scaffolds either at pH 7.3 or pH 5.0. By contrast, the release rate of PTX from PTX-PDC-scaffolds was even lower in comparison with that of free PTX-PDCs. In the presence of GSH (10 mmol/L), only 15% of the PTX was released after the PDP/PDPR-scaffolds were incubated for 192 h, and pH values seemed to have little effect on the release of PTX from PDC-scaffolds. However, less than 10% of the PTX was

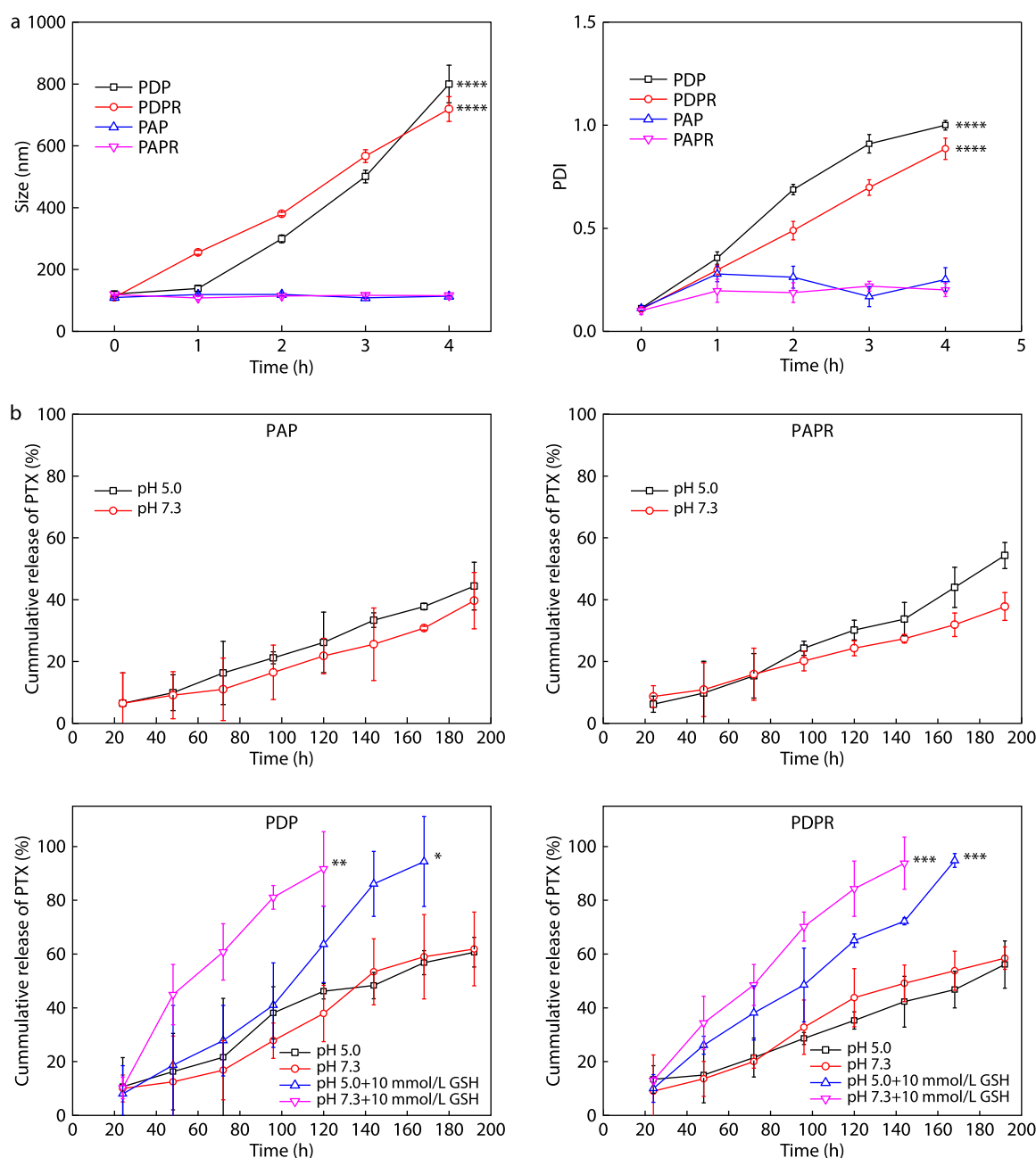


Fig. 4 Release profiles of PTX from PTX-PDCs in PBS buffer (pH 7.3) or HAc-NaAc buffer (pH 5.0) with 10 mmol/L GSH. (a) Size changes and PDI of the PTX-PDCs with time in the presence of GSH (pH 7.3). **** $P < 0.0001$ versus PAP/PAPER. (b) Release profiles of PTX from PTX-PDCs. * $P < 0.05$ versus pH 5.0; ** $P < 0.01$ versus pH 7.3; *** $P < 0.001$ versus corresponding pH value.

released from PAP/PAPR-scaffolds within the same period. On the other hand, the remanent PTX in free PTX/PDP/PDPR-scaffolds was less than 20% for a long period of incubation (50 days) (Fig. S7 in ESI). By contrast, the remanent PTX in PAP/PAPR-scaffolds was more than 30% within the same period. In general, these results indicated that the complex scaffolds slowed down the release of PTX, exhibiting a steady-release characteristics. The reductive environment of tumor tissues may promote the release of PTX from redox-sensitive PTX-PDC-scaffolds such as PDP- or PDPR-scaffolds.

In Vitro Anti-GBM Effects

To evaluate the antitumor efficacy of the PTX-PDCs on the inhibition of GBM cells, the GL261 cells were incubated with each PTX formulation at different concentrations for 1 day and 3 days, cells without any treatment were set as control. As can be seen in Fig. 6(a), the cytotoxicity of GL261 cells elevate along with the increasing concentration for all PTX formulations. Moreover, the redox-sensitive PDCs (PDP), RGD-decorated PDCs (PAPR), and dual-targeting PDCs (PDPR) exhibit higher toxicity on GL261 cells than non-targeting PDCs (PAP), which became more visible with the increasing concentrations of PTX-PDCs. In comparison with other PTX-PDCs, PDPR exhibits the highest inhibition on the proliferation of GL261 cells at the half maximal inhibitory concentration (IC₅₀) (Fig. 6b), as more PTX was endocytosed by the cells through RGD-mediated pathway^[27] and more PTX was released via click release properties of disulfide bonds inside the cells. The cytotoxicity of the PTX-PDCs to normal cells was also investigated by incubating L929, HUVEC, and BV2 cells with all PTX formulations for 1 day and 3 days, respectively. The cell viability results are shown in Fig. S8 (in ESI), all PTX-PDCs exhibit lower inhibition on the growth of normal cells than free PTX. Specifically, the IC₅₀ of each PTX-PDC in normal cells is considerably higher than those in GL261

cells (Fig. 6b). These results indicated that PTX-PDCs had a higher affinity for GBM cells than normal cells and could be safe for the tissue repair after antitumor therapy.

To examine the effects of the PTX-PDC-scaffolds as *in situ* anticancer devices on the inhibition of GBM, the GL261 cells were incubated with scaffold extract for 1 day and 3 days, and the cell viability was measured by MTT assay. As revealed in Fig. S9(a) (in ESI), the cytotoxicity of GL261 cells elevates with increasing blending proportion in scaffolds for all drug formulations. At the same proportion, the extract from PDP/PDPR-scaffolds lead to higher cytotoxicity of GL261 cells with time than that from PAP/PAPR-scaffolds, as more PTX was released from PDP/PDPR-scaffolds. Due to lower expression of $\alpha\beta 3$ integrin and GSH in normal tissue than GBM, the cytotoxicity of the PDC-scaffold extract to normal cells (L929, HUVEC, and BV2) are lower than that to GL261 cells (Figs. S9b–S9d in ESI), indicating the normal tissue protection of implanted PTX-PDC-scaffolds. By contrast, the extract from free PTX-scaffolds shows a significantly higher toxicity to both tumor and normal cells, as free PTX often undergoes a burst release from the complex scaffolds. To further explore the *in situ* anticancer effects of PTX-PDC-scaffolds, GL261 cells and HUVEC cells were also seeded on the scaffolds and incubated for 1 day, 2 days, and 3 days, and the morphology of the cells was examined by CLSM. As shown in Fig. 6(c), PDPR-scaffolds show a larger extent of inhibition on tumor cell proliferation than other PTX-PDC-scaffolds. All drug-loaded scaffolds exhibit low toxicity to normal cells except for free PTX-scaffolds due to a burst release of drug (Fig. S10 in ESI). Taken together, all formulations of PTX-PDCs exhibit a steady-release inhibition on GBM cell proliferation and a concurrent protection to normal cells after incorporation into WBPU scaffolds. Both redox-sensitive and RGD-decorated PTX-PDCs (PDPR) show a better potential to

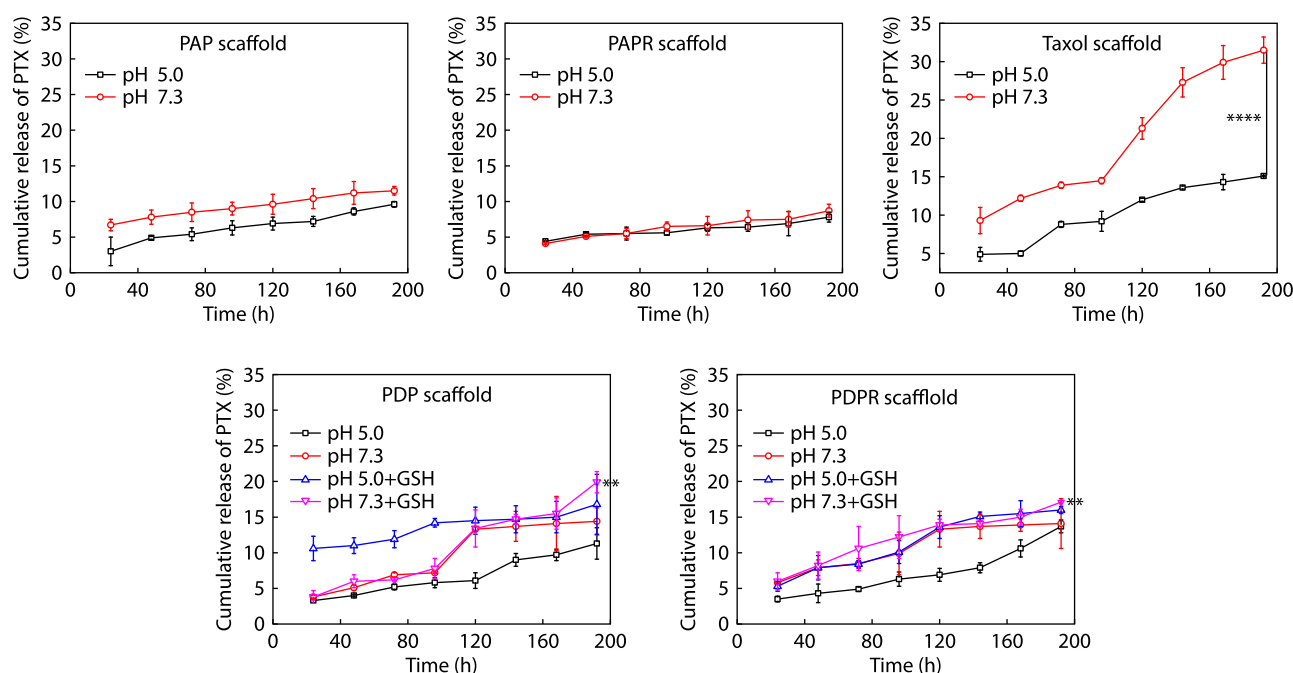


Fig. 5 Release profiles of PTX from PTX-PDC-scaffolds in PBS buffer (pH 7.3) or HAc-NaAc buffer (pH 5.0) with 10 mmol/L GSH. ** $P < 0.01$ versus pH 7.3; **** $P < 0.0001$.

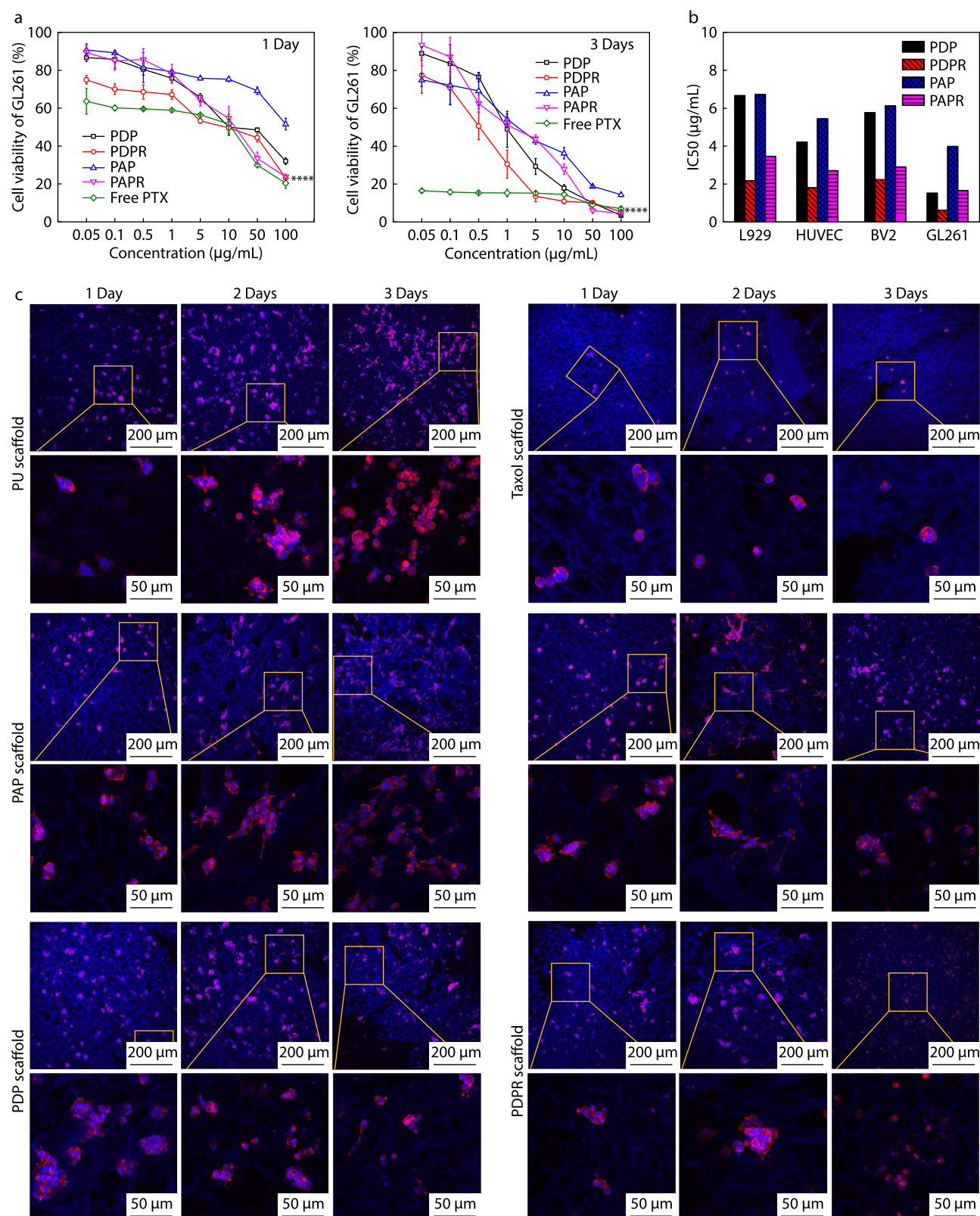


Fig. 6 *In vitro* anti-GBM effects of PTX-PDC-scaffolds. (a) Cytotoxicity of free PTX and PTX-PDCs at varied equivalent PTX concentrations to GL261 cells after 1 day and 3 days of incubation. **** $P < 0.0001$ versus PAP. (b) IC₅₀ of PTX-PDCs in GL261 cells and normal cells. (c) Growth morphology of GL261 cells seeded on blank-, free PTX-, and PTX-PDC-scaffolds for 1 day, 2 days, and 3 days. Cytoskeleton was labeled with red fluorescence (rhodamine-phalloidin), nuclei were labeled with blue fluorescence (DAPI), and the pore walls of the scaffolds were nonspecifically stained by DAPI. Scale bars 200 μm for low-magnification field and 50 μm for high-magnification field.

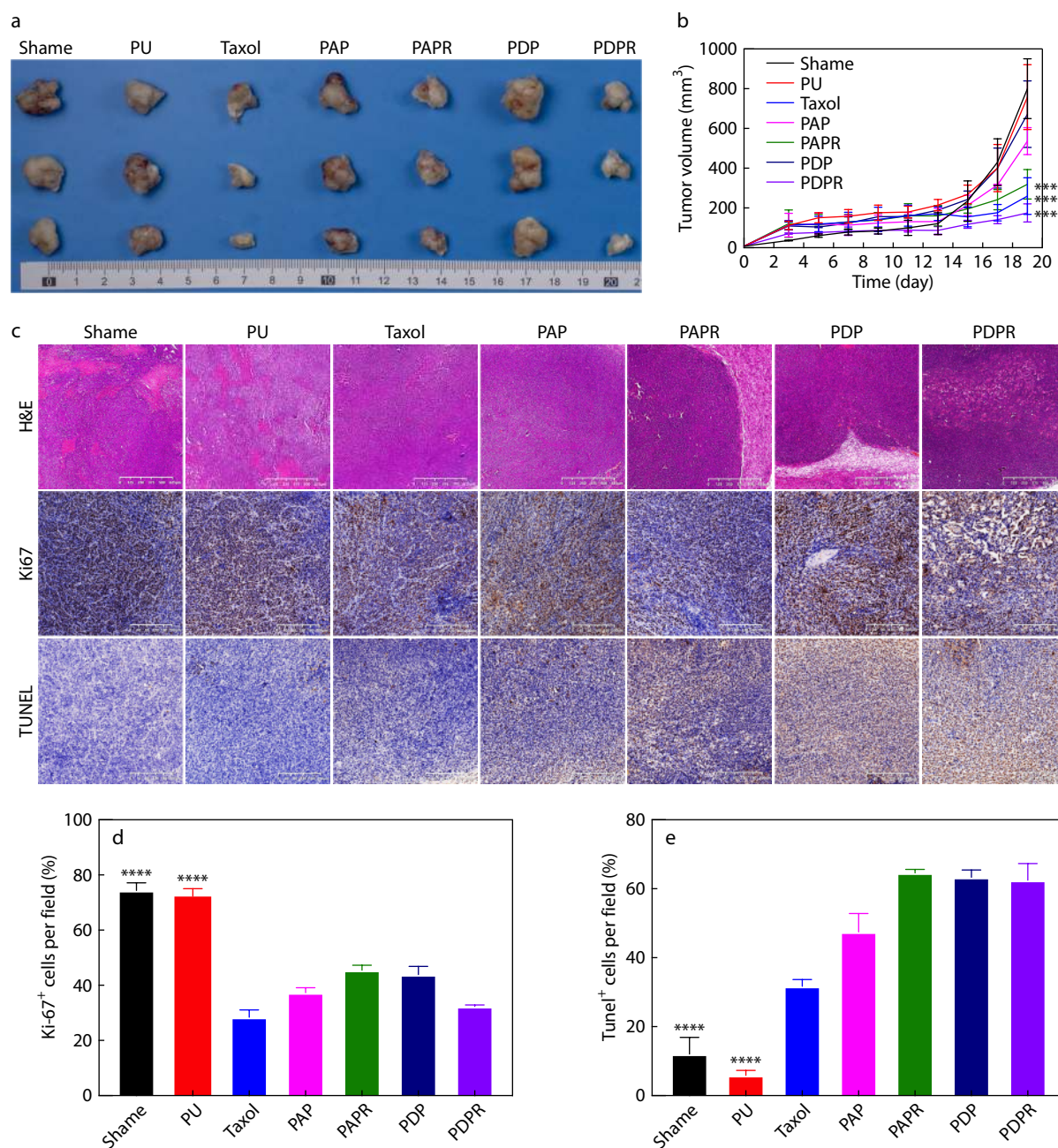


Fig. 7 *In vivo* anti-GBM efficacy of PTX-PDC-scaffolds. (a) Photos of the excised GBM on the 19th day; (b) Increase in the tumor volume of GBM-bearing mice after implantation with blank-, free PTX-, and PTX-PDC-scaffolds at residual tumor site for a period of 19 days, mice underwent tumor resection only were set as control. * $P < 0.05$, ** $P < 0.01$ versus PU; (c) Representative H&E and IHC staining of excised GBM tissues. Scale bars 625 μm for H&E images and 200 μm for Ki67 and TUNEL IHC images. Semi-quantification analysis of the Ki67 (d) and TUNEL (e) slices. **** $P < 0.0001$ versus Shame or PU group.

satisfy the goal as scaffold-delivery drugs for *in situ* GBM treatment.

***In Vivo* Anti-GBM Efficacy**

To investigate the *in vivo* antitumor efficacy of the PTX-PDC-loaded WBPU scaffolds on GBM, scaffolds carrying various PTX formulations, or blank scaffold as a control were implanted at residual GL261 tumor site after tumor resection. The tumor volume of each treated mice was measured every two days for a

period of 19 days. As shown in Fig. 7(a), the tumor volumes of the mice treated with PTX-PDC- and free PTX-loaded scaffolds are smaller than that treated with blank scaffolds and shame surgery at the end of the treatment (the 19th day). The tumor volume of the mice treated with PDPR-incorporated scaffolds shows a larger extent of suppression throughout the period (Fig. 7b), indicating the best GBM inhibition efficacy of the redox-sensitive and RGD-decorated PDPR-scaffolds. It is necessary to uncover the proliferation and apoptosis properties

of the GBM tumor cells treated with PTX-PDC-scaffolds. The tumor tissues were collected at the 19th day to evaluate the microstructure, the expression of Ki67, and the staining of TUNEL by H&E and immunohistochemistry assay (IHC). As can be seen in Fig. 7(c), the H&E slices of the tumor treated with drug scaffolds show less extracellular matrix than those treated with blank scaffolds and sham surgery. Meanwhile, drug groups reveal relatively lower expression of Ki67 and more positive staining of TUNEL than that in control groups (Figs. 7c–7e), indicating a growth inhibitory pattern in GL261 tumors treated with PTX-PDC-scaffolds. Certainly, PDPR-scaffolds show the highest extent of GBM inhibition with a very low expression of Ki67 and strongly positive TUNEL staining in the microscopic field.

CONCLUSIONS

In general, we developed WBPU scaffolds incorporation with redox-sensitive and RGD-decorated PTX-PDCs for targeted GBM therapy *in situ*. The dual-targeting PTX-PDCs were obtained by step conjugation of PTX and RGD with PEG. RGD conjugation endowed specific receptor-mediated uptake of the PTX-PDCs by GBM cells. The disulfide linkage between PTX and PEG gave the click release property of PTX inside GBM cells. The precise and efficient release of PTX in GBM cells will be an effective strategy to kill GBM cells and protect normal cells simultaneously. After incorporation into implantable WBPU scaffolds, the PTX-PDCs could act on GBM cells directly at residual tumor site. Since the dual-targeting PTX-PDC-scaffolds exhibited significant therapeutic effects on GBM, the WBPU scaffolds incorporation with PTX-PDCs may be a promising local strategy for GBM therapy.

NOTES

The authors declare no competing financial interest.

Electronic Supplementary Information

Electronic supplementary information (ESI) is available free of charge in the online version of this article at <http://doi.org/10.1007/s10118-022-2695-3>.

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