Biomaterials Science

Accepted Manuscript

This article can be cited before page numbers have been issued, to do this please use: Z. Zhao, J. Shen, L. Zhang, L. Wang, H. Xu, Y. Han, J. Jia, Y. Lu, R. Yu and H. Liu, *Biomater. Sci.*, 2020, DOI: 10.1039/D0BM00338G.

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DOI: 10.1039/D0BM00338G

Injectable postoperative enzyme-responsive hydrogels for reversing temozolomide resistance and reducing local recurrence after gliomas operation

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Abstract

Glioma is the most aggressive primary malignant brain tumor. It is failed to eradicate the gliomas by performing neurosurgery due to the diffuse nature of malignant gliomas. Temozolomide (TMZ) is the first-line agent in treating gliomas after surgery, and its therapeutic efficacy is limited mainly due to high activity levels of the DNA repair protein O^6-methylguanine-DNA methyltransferase (MGMT) in glioma cells. Herein,

we used an injectable matrix metalloproteinase (MMPs) enzymes responsive hydrogel that loaded TMZ and O^6-benzylamine (BG) (MGMT inhibitor) for eradicating residual TMZ-resistant gliomas after surgery. The hydrogels contained three features: 1) TMZ and BG could be encapsulated within the hydrophobic lamellae of the hydrogel to form Tm (TMZ + BG) hydrogel; 2) The hydrogels could release the TMZ and BG in response to the high concentration of MMPs enzymes after glioma surgery; 3) The hydrogels could increase local TMZ concentration and reduce side effects of BG. *In vivo*, Tm (TMZ + BG) hydrogel inhibited the MGMT expression and sensitized TMZ-resistant glioma cells to TMZ. Moreover, Tm (TMZ + BG) hydrogel effectively reduced the recurrence of TMZ-resistant glioma after surgery and significantly enhanced the efficiency of TMZ to inhibit glioma growth. Together, these data suggest that MMPs-responsive hydrogel is a promising localized drug delivery method to inhibit TMZ-resistant glioma recurrence after surgery.

Key words: Glioma; Hydrogel; Matrix metalloproteinases (MMPs); Temozolomide (TMZ); O^6-benzylguanine (BG).

Introduction

Glioma has been regarded as a highly malignant brain tumor, which can infiltrate into normal brain parenchyma and form a satellite tumor [1]. Surgical operation is the preferred clinical protocol for treating glioma. However, its effect is a limited clinical therapeutic outcome, due to post-surgical frequent recurrence [2-4]. Although considerable efforts on decreasing recurrence of glioma have been made in recent years, the average survival time of glioma patients is less than 14.6 months, resulting from its infiltrative nature. Decreasing the recurrence rate of glioma has become important.

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TMZ is still a first-line agent in treating gliomas after surgery [5]. However, the clinical outcomes showed poor therapeutic efficacy of TMZ. The efficacy of TMZ is hampered by chemoresistance induced by MGMT, a DNA repair protein that repairs damaged DNA following methylation [6-8]. BG inactivates MGMT by alkyl group transfer to Cys145 to improve the efficiency of TMZ in glioma cells [9]. The combination of BG and TMZ in treating glioma effectively reduces the maximum tolerated dose (MTD) of TMZ by 50% in clinical trials [7]. While, BG is not widely used in clinical anti-glioma therapies, mainly due to the accumulation in bone marrow producing significant myelosuppression, hepatic toxicity, pulmonary fibrosis and it is difficult to cross the blood-brain barrier (BBB) [10, 11]. Therefore, it is urgent to find more effective methods to transport BG diffusion through BBB and to decrease its side effects on healthy tissues. If we solve the two major problems of glioma recurrence and TMZ resistance, the prognosis and therapeutic effect of glioma patients after surgery will be significantly improved.

On the other hand, clinical studies have found that 80%-90% of the recurrence of III~IV gliomas are within 2 cm of the original tumor [2, 12, 13]. The drug concentration in the tumor tissue is increased by 3-4 times, the lethality of chemotherapy drugs on tumor cells will increase by about 10 times [14]. So, it is essential for high-efficiency chemotherapy of glioma to increase the drug concentration of glioma cells [15]. In recent years, the local administration of hydrogel-encapsulated chemotherapeutic drugs has skillfully solved such problems, and its greatest advantage can bypass the BBB and directly act on the central nervous system to increase local drug concentration [14, 15].

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The hydrogels mainly form a three-dimensional network structure by cross-linking hydrophilic macromolecules, and the unique three-dimensional spatial structure can absorb a large amount of tissue fluid and water [15]. Different types of hydrogels can accelerate their own decomposition under conditions of specific temperature [16], pH [17], light [18, 19] and other stimuli, and promote the release of internal load substances [20, 21]. At the same time, the good biocompatibility, non-cytotoxicity and low price of hydrogels lead to a broad prospect in the treatment of malignant tumors. The hydrogel itself can be used as a carrier to load various drugs, and the drug utilization efficiency can be improved while prolonging the action time of the drug by injecting it in situ or directly on the surface of the tissue [22]. What's more, hydrogel-loaded drugs can also be delivered directly to the deep surface of the tumor without the blood circulation. Studies have found that percutaneous administration of hydrogel can treat melanoma, which not only enhances the penetration ability of the drug itself, but also significantly prolongs the action time of the drug, and plays a role in the application of hydrogel in the tumor [23, 24].

Hence, in this study, we have used injectable hydrogel loaded with TMZ and BG, through direct administration into the operation cavity of gliomas, which releases the TMZ and BG in response to the high concentration of MMPs enzymes after glioma surgery. Firstly, we studied hydrogel loaded with TMZ and BG, and investigated drug release in response to MMPs enzyme activities *in vitro*. Secondly, we used TMZ-resistant cell line C6 to make situ glioma operation nude mice model and subcutaneous transplanted glioma nude mice model to demonstrate the feasibility, safety and

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therapeutic effect of local delivery via a response MMPs enzymes hydrogel on glioma growth. Our study presented a unique approach as a next-generation chemotherapy strategy for post-surgical residual glioma treatment, which permitted to further exploration.

2. Materials and method

2.1 Materials

Triglycerol monostearate, Tm was bought from Co., Ltd (Dalian, China). D-Luciferin potassium salt and temozolomide (TMZ) were obtained from Dalian Meilun Biotech Co., Ltd (Dalian, China). O^6-benzylamine (BG) and 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were bought from Shanghai Macklin reagent Co., Lt (Shanghai, China) and Beijing Zhongshuo Pharmaceutical Technology Development Co., Ltd (Beijing, China), respectively. Live-Dead Cell Staining Kit and Anti-MGMT Rabbit monoclonal antibody [EPR4397] (ab108630) were got from Jiangsu Keygen Biotech Co., Ltd (Jiangsu, China) and Abcam Co., Ltd (Shanghai, China), respectively. Beta-actin mAb was purchased from Proteintech Antibodies People Trust (Chicago, IL, USA). Recombinant human MMP9 were obtained from Dalian Meilun Biotech Co., Ltd (Dalian, China). MMP9 Elisa kit were obtained from Jianglai biotech (Shanghai, China).

2.2 Hydrogel preparation

According to the pieces of literature, (10% w/v) blank hydrogel was prepared [25, 26]. 200 mg of Tm was put into a glass vial, and then 2 mL dimethylsulfoxide

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(DMSO)/water mixture (2: 8 volume ratio) was added into a glass vial with Tm. Tm was completely dissolved under 70 ℃. Then the vial was cooled to room temperature for about 20-30 minutes, resulting in hydrogel formation. When the vials are inverted and no gravity flow is observed, the gelation is complete. The resulting hydrogel is easily injected with a 1 mL syringe. The weight ration of TMZ and BG with 9: 1 were mixed with Tm to form Tm (TMZ + BG) in the vial for a final TMZ concentration of 18 mg mL^{-1} and BG concentration of 2 mg mL^{-1}.

2.3 Physicochemical properties of hydrogels

2.3.1 Field emission scanning electron microscopy (SEM) analysis

The hydrogel was imaged using a SU8010 UHR FESEM by field emission scanning high resolution scanning electron microscopy (Hitachi High-Technologies Corporation, Tokyo, Japan, acceleration voltage, 5 kV). Insert the hydrogel into the holes of the sample platform for cryogenic transmission. And then, the samples were quickly frozen to -210 ℃ in liquid nitrogen. The samples were transferred to sample preparation room with a transmission rod. The temperature of the sample preparation indoor raised from -140 ℃ to 90 ℃ and sublimated for 3 minutes. The surface of the sample was sprayed with platinum for 50 seconds [27]. At last, get observation and take photos with the SEM.

2.3.2 In Vitro enzyme-responsive drug release

To evaluate the sustained release of TMZ and BG from Tm hydrogel, the hydrogel (1 mL) samples were placed in a 15 mL centrifugal tube and suspended in 10 mL PBS. PBS (control), MMPs (MMP9)(100 ng mL^{-1}), MMPs (MMP9)+ inhibitor (100 ng mL^{-}

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[1])， glioma post-operative patient's CSF and CSF + MMPs (MMP9) + inhibitor were used to assay the TMZ and BG release. The tubes were closed and incubated at 37 ℃ with a shaking speed of 150 rpm. An aliquot (200 μL) from the PBS reservoir and the concentration of TMZ and BG were analyzed by HPLC. The incubation medium was supplemented with PBS after withdrawing each aliquot. At multiple time points, the supernatants (200 μL) were sampled and then were replaced using a fresh release medium at each time point. The amount of TMZ and BG were analyzed using HPLC (Shimadzu, LC-10AT/SPD-10A, chromatographic column: Hyersil ODS C18 4.6 × 200 mm, 5 μm particle size), according to the protocol and the release profiles of were plotted with time [28].

The standard curve of TMZ. $y = 0.1158x-0.1756$ $R^2 = 0.9963$

The standard curve of BG. $y = 0.2063x-0.0891$ $R^2 = 0.9967$

2.3.3 Disassembly of DiR-loaded hydrogels *in vivo*

The glioma model and post-operative model of male C57BL/6 mice were constructed, 10 μL Tm/DiR hydrogels were injected into the resection cavity (n = 3). The fluorescence signal was quantified on day 0, 1, 3, 5 by the Xenogen IVIS Spectrum optical imaging device.

2.4 Cell experiments

2.4.1 Cell culture

The glioma resistant-TMZ cell line C6 were purchased from the Shanghai Cell Bank, Chinese Academy of Sciences. C6-GFP-Luci cells were transfected with the luciferase gene. Culture media containing Dulbecco's modified Eagle's medium

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(DMEM) (Gibco, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS, Gibco) were used for all the cells culture at 37 °C in 5% CO_2.

2.4.2 In Vitro hydrogel cytotoxicity assay

6×10^3 C6 cells were plated in 96-well culture plates for 24 h. Then different time points (1, 2, 3, 4, 5 day) aliquots from the Tm hydrogel immersion solution were replaced the media and incubated for another 24 h. After that MTT reagent was used to detect the cytotoxicity of Tm hydrogel. The result was measured the absorbance at 570 nm.

2.4.3 In vitro anti-glioma activity

The Live-Dead Cell Staining Kit was used to assay anti-glioma activity efficacy. Glioma cells C6 were plated on 24-well plates with transwell inserts at 5×10^4 cells/well overnight. And then, the medium was removed and replaced with fresh medium. 100 μL blank hydrogel, 100 μL TMZ-loaded immersion solution, 100 μL TMZ + BG-loaded hydrogel immersion solution, TMZ + BG-loaded hydrogel immersion with MMP9 (100 ng mL^{-1}) and MMP9 (100 ng mL^{-1}) immersion solution was entered into the upper chamber. The cells were stained with a Live-Dead staining kit after incubation for 72 h. Cells were imaged by fluorescence microscopy (Olympus, Takachiho, Japan).

2.5 Animal care and maintenance

5-6 weeks old BALB/c nude male mice were got from Beijing HFK Bioscience Co., Ltd. (Beijing, China). All animal experiments were approved by Xuzhou Medical University of China Animal Care and Used committee.

2.6 Orthotopic glioma Anti-TMZ-resistant glioma study

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The orthotropic glioma model was constructed according to our previously described method [29-31]. Briefly, a 1 cm vertical small incision was incised along the previous surgical scar at the midline and a 2.0 mm diameter circular cranial window was drilled at the previous hole using a high-speed drill to expose the brain tissue. The 2 mm-diameter biopsy punch was inserted and depth is 3 mm. Next rotated the biopsy punch to cut glioma tissue for 15s. Once withdrawal, tumor and brain tissue were suctioned by the vacuum pump. And hydrogels were injected into the postoperative tumor cavity after hemostasis. The wound was closed with sutures. Luciferase assay was tested to ensure the existence of glioma with a 95% success rate in day 7 post-transplantation, and then nude mice were randomly divided into 5 groups. The glioma growth rate and location of the tumors were determined by the IVIS kinetic imaging system. The glioma growth rate was assessed using the fluorescence imaging analysis. On 8-day post-glioma injection, the glioma surgically removed and had intracavitary residual tumors. 50 μL Tm (blank hydrogel), intragastric administration of TMZ, Tm (TMZ) (TMZ-loaded hydrogel), Tm (TMZ + BG) (TMZ and BG-loaded hydrogel) were individually injected into glioma resection cavity after post-operation. The intensity of bioluminescence signals was tested to evaluate the inhibition recurrence of glioma at 14 and 24 day after tumor implantation by Xenogen IVIS Spectrum optical imaging device (Caliper Life Sciences). 26 days after implantation, three mice in each group were euthanized for tumor H & E, immunofluorescence histochemical analysis (TUNEL, Ki67, and MGMT expression) and organ toxicity evaluation. During the entire study, the weight of the rest mice was measured regularly and the survival time

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was calculated (n = 6). Mice were divided into 3 groups: Normal group (normal brain tissues), Sham group (tissues with sham treatment), Resection group (brain tissues with residue glioma tissues). All samples were collected and detected the MMP9 concentrtions according to the instructions of MMP9 Elisa kit.

2.7 Anti-glioma experiments in subcutaneous transplantation tumors mice

5×10^5 C6 cells were subcutaneously transplanted in the right flank of male nude mice. The C6-bearing mice were randomly divided into five groups (n = 5 per group). 100 μL PBS, Tm, blank hydrogel and intragastric administration of TMZ, Tm (TMZ, Tm (TMZ + BG) hydrogels were uniformly injected around tumors. Tumor volume was measured using the formula: $V = 0.5 \times a \times b^2$, where a and b represent the major and minor axes of a tumor, respectively. The body and tumor weight were measured. After 14 days of transplantation, all the mice were euthanized for tumor H & E, immunofluorescence histochemical analysis (TUNEL, Ki67, and MGMT expression) and organ toxicity evaluation.

The dose of TMZ and BG administered were performed as previously reported and made some changes [6, 7, 32, 33]. The TMZ dose of intragastric administration was the same as that within loaded in hydrogels.

2.8 Liver and kidney toxicity evaluation

The blood samples from healthy tumor-free mice were administrated with PBS, Tm, TMZ (*i. g*), Tm (TMZ), Tm (TMZ + BG) at a same dose of TMZ and BG were measured the levels of ALT, AST, BUN, CREA by an automated chemical analyzer (Cobas 8000 modular analyzer series, Roche Diagnostics USA) to evaluate the potential

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liver and kidney toxicity of Tm (TMZ + BG) hydrogel.

2.9 Statistical analysis

Data are reported as mean ± SEM. Statistical analysis was performed using SPSS version 13.0 and student T-tests with $P < 0.05$ were recognized as significant ($*P < 0.05$, $**P < 0.01$).

3. Results

3.1 Preparation of hydrogels and encapsulation of drugs.

According to the previous reports, Tm, which encapsulated TMZ and BG, were prepared (Figure 1Aa, b) [35, 36]. In the self-assembly process, the TMZ and BG could be embedded into the hydrophobic core of the Tm hydrophobic gel layered fiber to form Tm (TMZ + BG) hydrogel. The Tm (TMZ + BG) hydrogel could be injected in the post-operative cavity (Figure 1Ba, b). The surface of Tm (TMZ + BG) hydrogel with different drugs and Tm hydrogel ratios were imaged by SEM (Figure S1). Based on the complete surface structure of Tm (TMZ + BG) hydrogel, we chose to use TMZ and BG (9: 1, w/w) in Tm hydrogel (10% w/v) with loading efficiency up to 30% (w/w) for all subsequent studies (Figure 1C). The result of rheology indicated that Tm (TMZ + BG) was in a semi-solid state at room temperature (Figure S2).

MMPs are present in both acute and chronic wounds [37, 38]. Surgical wounds are the acute wounds that are created by glioma surgical procedures. Therefore, MMPs are active after glioma surgery. Also, Matrix metalloproteinases (MMPs) in the tumor microenvironment plays a major role in tumor progression and metastasis. In

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glioblastoma, there is an overexpression of MMP-2 and MMP-9 [39, 40]. Tm had been reported that could be degraded in MMPs condition [35]. Thus, we evaluated the ability of Tm to release the encapsulated drug in response to MMPs. Tm (TMZ + BG) hydrogel was immersed in PBS, MMPs (100 ng mL-1) and MMPs inhibitor to assay the release profiles of TMZ and BG. Plotting cumulative release of TMZ or BG (%) versus time revealed that MMPs triggered hydrogels degradation to release the encapsulated TMZ and BG were 62.55 ± 2.38% and 59.58 ± 5.14%, respectively, whereas Tm (TMZ + BG) hydrogel in PBS and MMPs inhibitor did not release significant amounts of the TMZ and BG, with less than 25.27 ± 4.13%, 31.68 ± 4.05%, 22.67 ± 4.04%, and 34.01 ± 2.94%, respectively at 15 days (Figure 1D, E). These results indicated that the presence of MMPs was required for hydrogel disassembly to release TMZ and BG drugs. In order to closely resemble the glioma postoperative milieu, cerebrospinal fluid (CSF) from post-operative glioma patients were got to investigate the release of TMZ and BG from Tm (TMZ + BG) hydrogel. Tm (TMZ + BG) hydrogel was immersed in PBS without or with the addition of CSF from post-operative glioma patients (Neurosurgery, Affiliated Hospital of Xuzhou Medical University. Hospitalization Number: 1782969). At beginning, CSF was added similar to the studies with MMPs activities, CSF from post-operative glioma patients significantly increased the cumulative TMZ and BG release 77.70 ± 4.53% and 68.37 ± 3.11% at 15 days, respectively (Figure 1D, E). The cumulative TMZ and BG release were decreased under MMPs inhibitor + CSF condition with 35.67 ± 4.66% and 43.67 ± 3.92%, respectively. The release of drugs from Tm (TMZ + BG) hydrogel had a same trend under MMPs

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and CSF conditions. These above results suggested that the presence of MMPs in CSF, which were identical with some previous papers reported [35, 36]. The MMP9 enzyme concentration was measured in post-operation glioma mice, suggesting that Tm (TMZ + BG) hydrogel could response to the postsurgical environment to release drugs (Figure S3). In order to test how long the drug lasts, Tm was loaded with 1, 1'-dioctadecyl-3, 3, 3', 3'-tetramethylindotricarbocyanine iodide (DiR) to form the Tm/DiR hydrogel. And then, Tm/DiR hydrogel was injected into in situ postoperative gliomas to measure the fluorescence of DiR using the Xenogen IVIS Spectrum optical imaging device *in vivo*. The DiR fluorescence signal of Tm/DiR hydrogel decreased over 5 days. Compared with the 0 day, about 90% DiR fluorescence signal was decayed. These results demonstrate that the Tm hydrogel is responsive to the postsurgical environment *in vivo* and releases the encapsulated drugs and lasts more than 5 days.

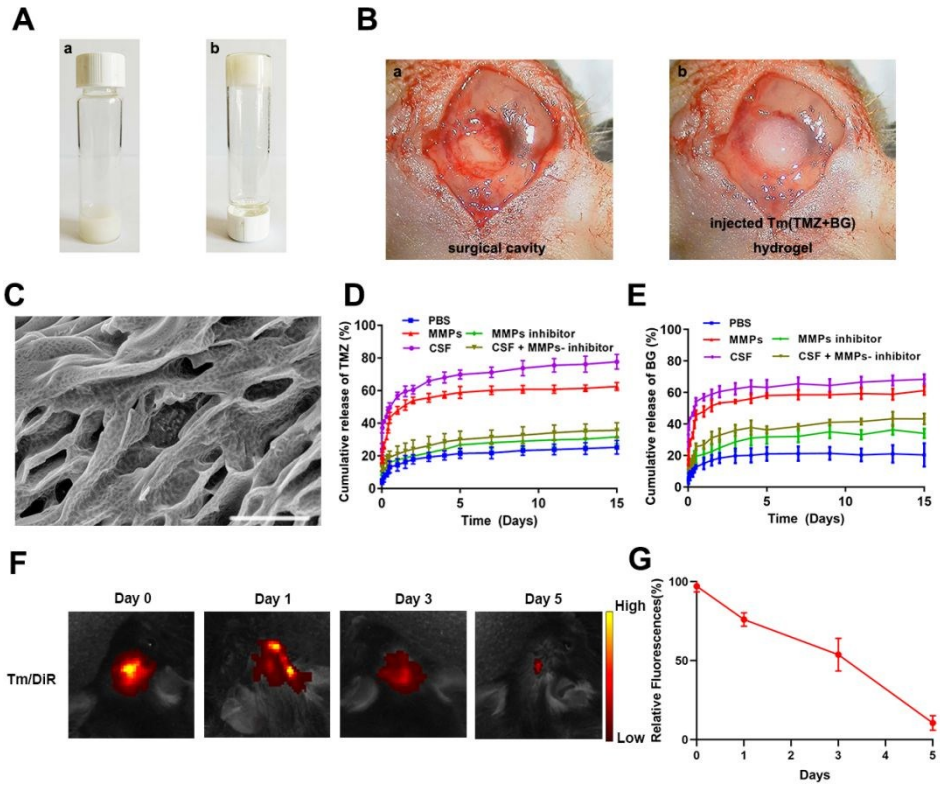

Figure 1. Preparation and characteristics of Tm (TMZ + BG) hydrogel. (A) Gelation of Tm (TMZ

+ BG) hydrogel before (a) and after cooling (b). (B) a) Make an incision in the midline and create a cranial window to expose the brain, and cut the brain-tumor tissue. b) Tm (TMZ + BG) hydrogel is placed into the operation cavity before sealing the cranial window. (C) SEM of Tm (TMZ + BG) hydrogels. The scale bar is 10 μm. (D)-(E) The TMZ and BG release profile of Tm (TMZ + BG) hydrogel in PBS at 37 ℃ with gentle stirring under MMPs, MMPs + inhibitor, CSF and CSF + inhibitor conditions, data are presented at as mean ± SEM, n = 4. (F) Fluorescence signals of Tm/DiR hydrogels after injection at 0, 1, 3, 5 days. (G) Quantitative analysis of DiR fluorescence signals of mice at 0, 1, 3, 5 days (n = 3).

3.2 In vitro biocompatibility and anti-glioma of Tm (TMZ + BG) hydrogel

The safety of materials is the primary problem in clinical application. Tm is a food additive approved by U. S. Food and Drug Administration (FDA), which has good biological safety [35]. In this study, the cytotoxicity of Tm hydrogel was determined by MTT assay. As shown in figure 2A, the survival rate of C6 cells was higher than 90% incubated with 1, 2, 3, 4, 5-day Tm hydrogel immersion solution, indicating that Tm had high favorable security. The Live-Dead staining was used to evaluate the cell viability treatment with PBS, Tm, Tm (TMZ) and Tm (TMZ + BG) hydrogels. The experimental results were shown in Figure 2B, C, no obvious red fluorescence was observed in PBS group and Tm group, which was consistent with the cytotoxicity assay. All these results implied that the good biocompatibility of Tm hydrogel. In the Tm (TMZ) group and Tm (TMZ + BG) group, the intensity of red fluorescence increased gradually. As shown in Figure 2C, compared with Tm (TMZ) group, the intensity of red fluorescence in Tm (TMZ + BG) group increased significantly ($*P = 0.03 < 0.05$). These results suggested that BG reversed TMZ resistance and enhanced the TMZ curative effect of TMZ-resistant glioma.

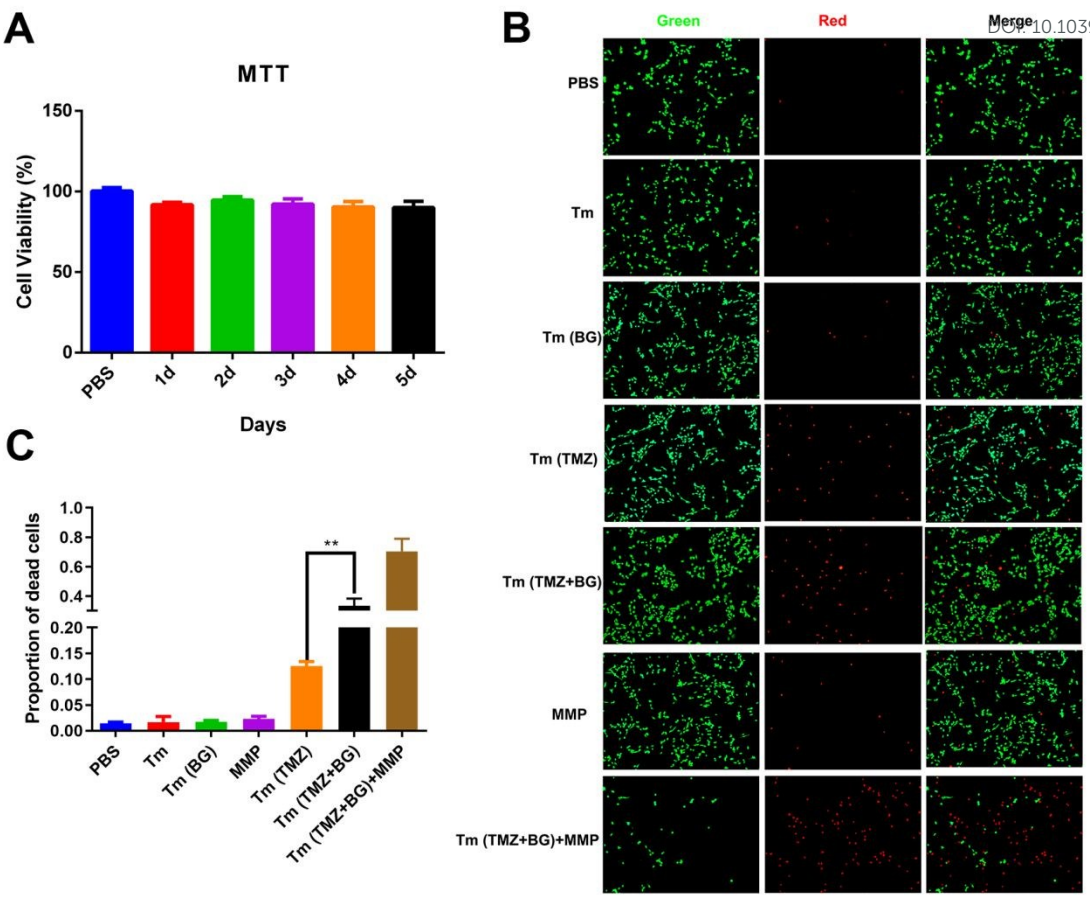

Figure 2. (A) C6 glioma cells were incubated in a 96-well plate in medium. PBS or in medium with 100 μL blank hydrogel 1, 2, 3, 4, 5-day immersion solution added to the 96-well plate. After 24 h of incubation, metabolic activity was determined by MTT assay. Data are presented at as Means ± SEM. (n = 4). (B) C6 glioma cells for 72 h in medium or medium with blank hydrogel, TMZ-loaded hydrogel or TMZ + BG-loaded hydrogel added to the upper chamber of the transwell, the scale bar is 200 μm. Viable cells stain green with calcein-AM, whereas dead cells stain red with ethidium homodimer-1. (C) Quantitative analysis of dead cells. (mean ± SEM, n = 4, $^{**}P < 0.01$).

3.3 In vivo anti-tumor of Tm (TMZ + BG) hydrogel for glioma by postoperative

Motivated by the above results, mice orthotopically engrafted with TMZ-resistant C6-Luci glioma cells were developed to determine if the Tm (TMZ + BG) hydrogel would sensitize gliomas to TMZ. After 8 days of implantation, the gliomas had an incomplete tumor operation and the Tm (TMZ + BG) hydrogels were injected into the glioma operation cavity to validate the therapeutic effects of Tm (TMZ + BG) hydrogel. The bioluminescence signals from C6-Luci glioma cells were monitored to evaluate the

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glioma growth (Figure 3A, B). Compared with Tm group (after surgery injection blank hydrogel), gliomas treated with PBS group (without surgery) grew rapidly, suggested that surgery delayed the recurrence of glioma in Tm-treated group, due to Tm hydrogel no side effects. Tm (TMZ) group showed better antitumor efficacy than TMZ (intragastric administration, *i. g*) in terms of reducing the recurrence rate of glioma, suggesting that in situ injections of TMZ embedding hydrogel increased therapeutic effect of TMZ in residual glioma cells. Furthermore, Tm (TMZ + BG) group exhibited an anti-glioma efficacy superior to Tm (TMZ) and TMZ (*i. g*) groups, indicating that BG enhanced the cytotoxic effect of TMZ on TMZ-resistant glioma cells (Figure 3A and B). Not surprisingly, the Tm (TMZ + BG) hydrogel-based therapy showed the best therapeutic performance in extending the median survival times (54.5 days) (Figure 3C). The median survival times for mice treated with PBS, Tm, TMZ (*i. g*), Tm (TMZ) were 25.5, 33.5, 36.5 and 39.5 days, respectively. The dominance of Tm (TMZ + BG) hydrogel was also reflected by the changes in body weight. The body weights of mice treated with Tm (TMZ + BG) hydrogel slowly decreased, while all other groups lost weight rapidly (Figure 3D). After 26 days of implantation, the mice treated with different groups were sacrificed after anesthetized and the brains were collected for H ＆E staining. As shown in Figure 3E, the mice treated with Tm (TMZ + BG) hydrogel group reduced the postoperative recurrence of glioma. We also observed that immunohistochemical MGMT expression had a significant reduction in Tm (TMZ + BG) hydrogel treatment mice (Figure 3F and F'). The Tm (TMZ + BG) hydrogel significantly decreased proliferation (Figure 3G, G') and increased apoptosis (Figure

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3H, H') as indicated by staining Ki67 and TUNEL, respectively. Collectively, these results suggested that the combination of TMZ and BG in Tm (TMZ + BG) hydrogel-treated mice effectively decreased the TMZ-resistant glioma cells postoperative recurrence rate.

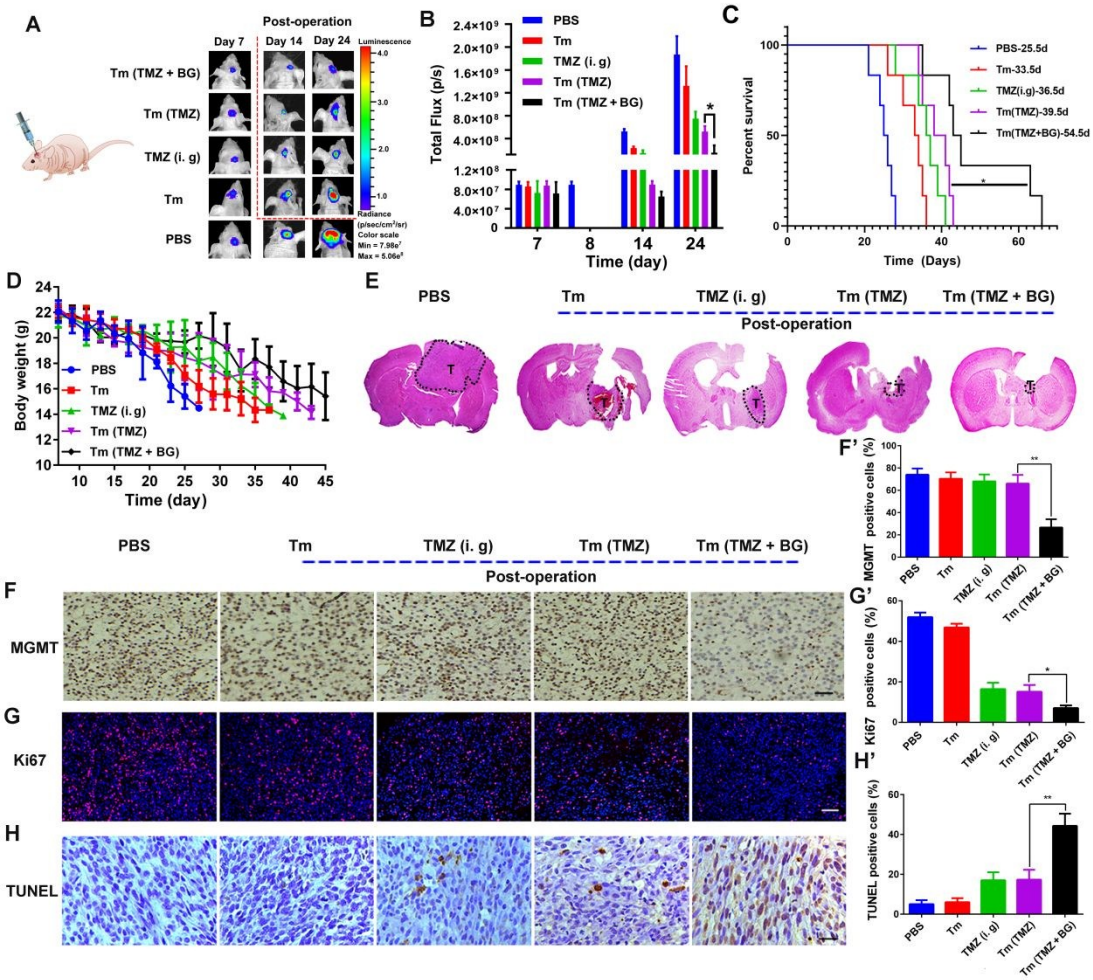

Figure 3. Efficacy of Tm (TMZ + BG) hydrogel in postoperative glioma mice model. (A, B) Bioluminescence images (A) and quantification (B) of the glioma-bearing mice 7, 14, and 24 days after treating with PBS, Tm, TMZ (*i. g*), Tm (TMZ), Tm (TMZ + BG) hydrogel. $*P < 0.05$. (C) Survival rate of the glioma-bearing mice after treating with PBS, Tm, TMZ (*i. g*), Tm (TMZ), Tm (TMZ + BG) hydrogel. (D) The body weight of the mice treated with various drug formulations was also measured. (E) Images of the HE-stained full-brain sections (the normal tissue and tumor tissue are separated by the dashed line. T denotes tumor). F) MGMT immunohistochemistry staining of the tumor tissues dissected at day 26 after tumor implantation and treated with PBS, Tm, TMZ (*i. g*), Tm (TMZ), Tm (TMZ + BG). Scale bar = 50 μm. (G) Ki67 and (H) TUNEL immunochemistry of glioma tissues. Scale bar = 20 μm. In Ki67, the nuclei were stained blue, and the positive cells

were stained red. In TUNEL and MGMT analyses, the positive cells were stained brown. Quantitative analysis of (F') MGMT positive cells (G') Cell proliferation and (H') Cell apoptosis (mean ± SEM, n = 3, $*P < 0.05$, $**P < 0.01$).

To further evaluate the antitumor efficacy of Tm (TMZ + BG) hydrogel, the Tm (TMZ + BG) hydrogel was injected subcutaneously around the tumors. By that time, the average tumor sizes had reached 50 mm^3, which were treated PBS, Tm, TMZ, Tm (TMZ), and Tm (TMZ + BG) hydrogels by injection around the tumors. TMZ-resistant gliomas in mice treated with Tm (TMZ + BG) hydrogel showed remarkable tumor suppression (Figure 4A, B, C). The photo of excised tumors treatment with Tm (TMZ + BG) hydrogel showed the smallest tumor size among the five groups (Figure 4A). Tm (TMZ + BG) hydrogel treated mice exhibited the lowest weight (Figure 4D). No significant fluctuated in the body weights among all groups was observed during the process of experiment, suggesting that no treatment-induced toxicity (Figure 4E). The large area of tumor tissue necrosis for the Tm (TMZ + BG) groups confirmed that Tm (TMZ + BG) hydrogel could efficiently inhibit tumor growth, due to BG enhancing the sensitivity of TMZ treatment (Figure 4F). The results of MGMT staining were further validated the effect of BG inhibiting MGMT expressing (Figure 4G, G'). After treatment with the Tm (TMZ + BG) hydrogel, the cell proliferation was decreased significantly, and cell apoptosis was significantly increased (Figure 4H, H', I, I'). These results proved that Tm (TMZ + BG) hydrogel enhanced chemosensitivity to TMZ-resistant gliomas and possessed prominent therapeutic effect on TMZ-resistant glioma with no significant systemic toxicity.

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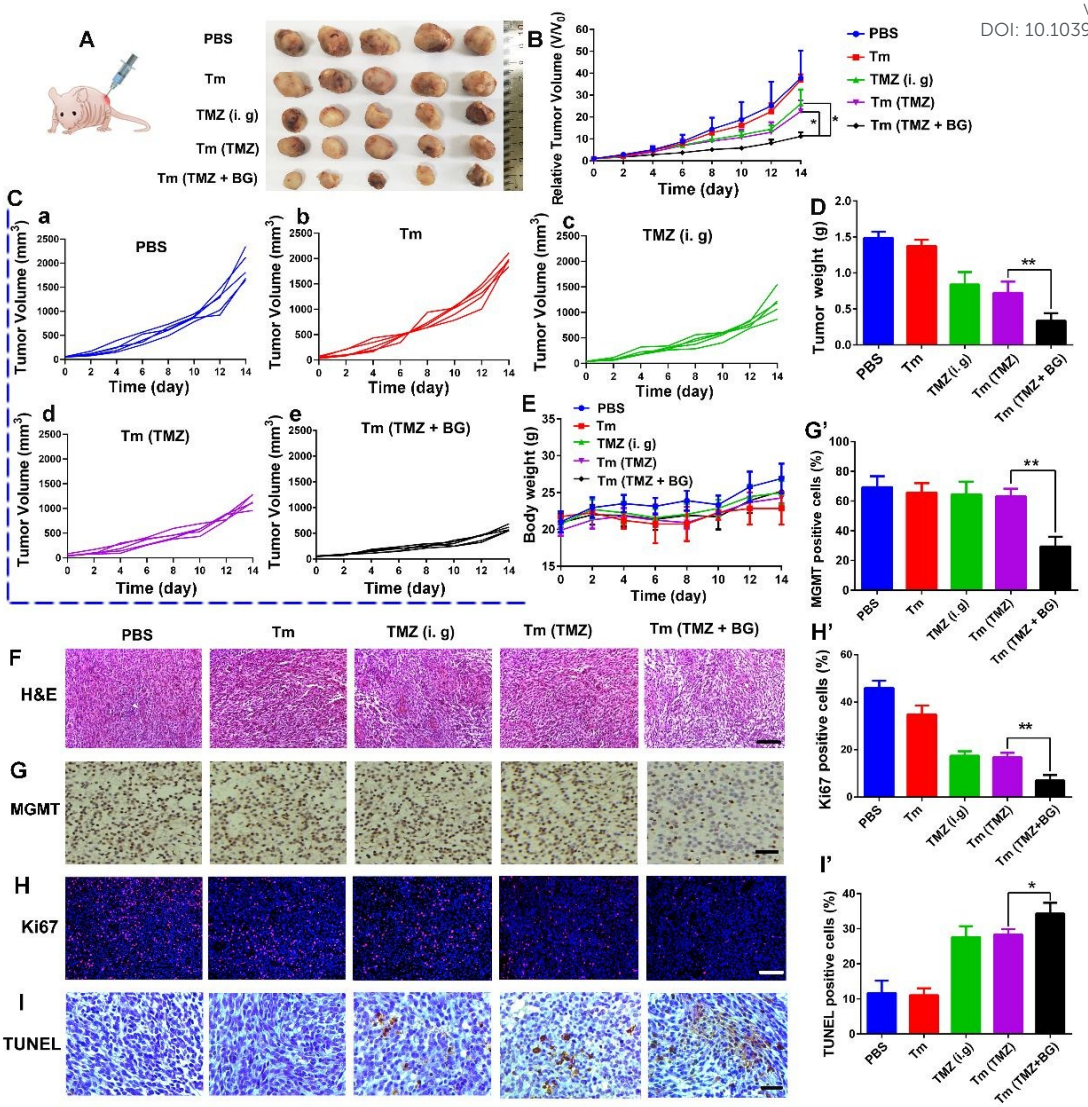

Figure 4. The therapeutic efficacy of Tm (TMZ + BG) hydrogel in subcutaneous transplantation tumors. (A) The photographs of the solid tumor. (B) Relative tumor volume. (C) Tumor volume of the animals treated with various drug formulations. (D) The tumor weight of the different groups was obtained on day 14. (n = 5, **P < 0.01). (E) The body weight of the tumor-bearing mice treated with various drug formulations. The values are the mean ± SEM, n = 5. (F) Images of the HE-stained tumor of different groups. Scale bar = 50 μm. (G) MGMT staining of subcutaneous tumors. Scale bar = 50 μm. (G') Quantitative analysis of MGMT positive cells. (H) Ki67 staining of subcutaneous tumors. Scale bar = 50 μm. (H') Quantitative analysis of Ki67 positive cells. (I) TUNEL staining of subcutaneous tumors. Scale bar = 20 μm. (I') Quantitative analysis of TUNEL positive cells.

3.5 In vivo biocompatibility

The systemic biosafety of Tm (TMZ + BG) hydrogel was assessed *in vivo*. Compared with PBS group, the H & E staining of the main organs treated with Tm

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(TMZ), Tm (TMZ + BG) hydrogels had no noticeable tissue damage and changes in morphology, indicating that the formations above had no obvious biological toxicity (Figure 5A). The biochemical analysis was measured to further evaluate the potential toxicity of hydrogels. Liver function (AST, ALT) and renal function (CREA, BUN) had no significant differences for the Tm, Tm (TMZ), Tm (TMZ + BG) groups compared with PBS, demonstrating that Tm (TMZ + BG) hydrogel did not induce damage to the liver or kidneys (Figure 5B). Taken together, these data demonstrated that Tm (TMZ + BG) hydrogel induce negligible systemic toxicity and had potential value for clinical use.

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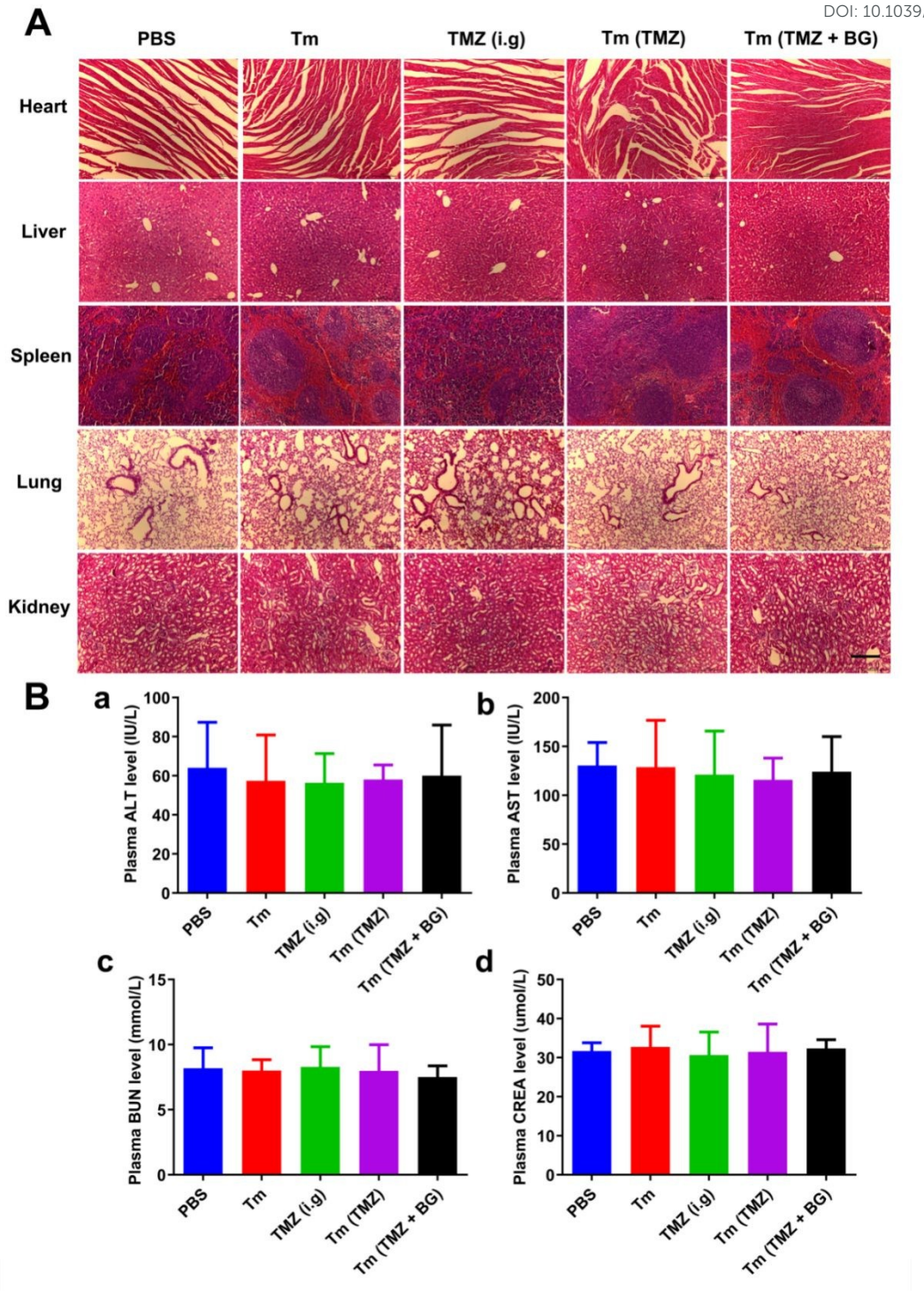

Figure 5. (A) H&E staining of heart, liver, spleen, lung and kidney treated with PBS, Tm, TMZ (*i. g*), Tm (TMZ) and Tm (TMZ + BG). Scale bar = 200 μm. (B) Determination of the liver and kidney toxicity of Tm (TMZ + BG) hydrogel. a) alanine aminotransferase (ALT), b) aspartate aminotransferase, c) blood urea nitrogen (BUN) and d) creatinine (CREA) levels recorded for tumor ICR mice 24 h after administration of PBS, Tm, TMZ (*i. g*), Tm (TMZ) and Tm (TMZ + BG) hydrogel.

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4. Conclusion

Taken together, we successfully used an injectable Tm hydrogel to embed TMZ and BG, which formed Tm (TMZ + BG) hydrogel, for reversing TMZ-resistance. The Tm (TMZ + BG) hydrogel could overcome the two main challenges of clinical glioma treatments, which reversed the marked treatment TMZ-resistant of malignant glioma and reduced a local glioma recurrence rate after surgery. *In vitro* drug release evaluation showed that Tm (TMZ + BG) hydrogel could be disintegrated in the presence of MMP-2 or CSF. The results of *in vivo* anti-glioma effect on incomplete tumor operation model indicated that Tm (TMZ + BG) hydrogel effectively inhibited the recurrence of TMZ-resistant glioma. Therefore, it suggested that Tm (TMZ + BG) hydrogel may be utilized as a potential formulation strategy to reduce the resistance of TMZ-resistant glioma cells to TMZ, and significantly improve the efficiency of TMZ to inhibit the recurrence of residual glioma after surgery.

Conclusion

Taken together, we successfully used injectable Tm hydrogels to embed TMZ and BG, which formed Tm (TMZ + BG) hydrogels, for reversing TMZ-resistance. The Tm (TMZ + BG) hydrogels could overcome the two main challenges of clinical glioma treatments, which reversed the marked treatment TMZ-resistant of malignant glioma and inhibited residual tumor proliferation after surgery. *In vitro* drug release evaluation showed that Tm (TMZ + BG) hydrogels could be disintegrated in the presence of MMP-2 or CSF. The results of *in vivo* anti-glioma effect on incomplete tumor operation model

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Acknowledgments

This work was supported by a grant from the National Natural Science Foundation of China [No. 81772665]. This paper was also financed from Social Development Project of Jiangsu Department of Science and Technology [BE2016646], Jiangsu Provincial Commission of Health and Family Planning [No. Q201608]. Research was supported by Six Talents Peak Foundation of Jiangsu Province [No. 2018-WSW-071], This work was supported by a grant from Postgraduate Research & Practice Innovation Program of Jiangsu Province [No. KYCX19-2236].

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Supporting information

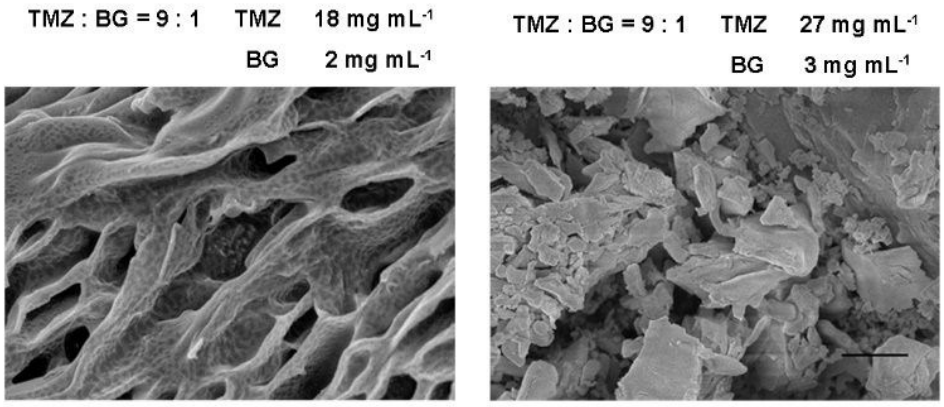

Figure S1. SEM images show a clear 3D porous network of different drug concentrations in the Tm hydrogel. Scale bar = 4 μm.

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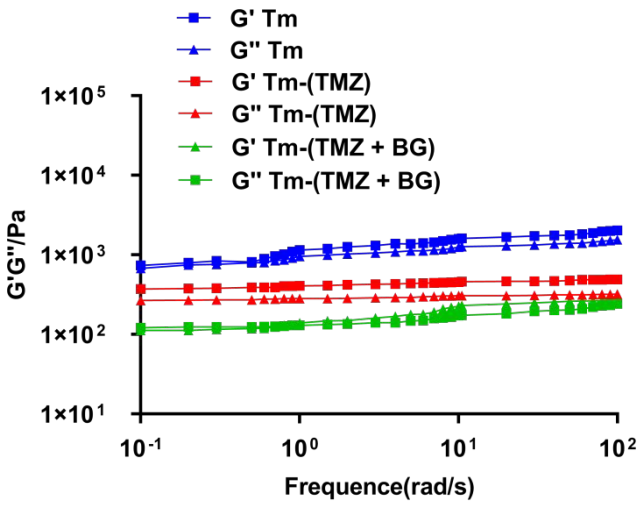
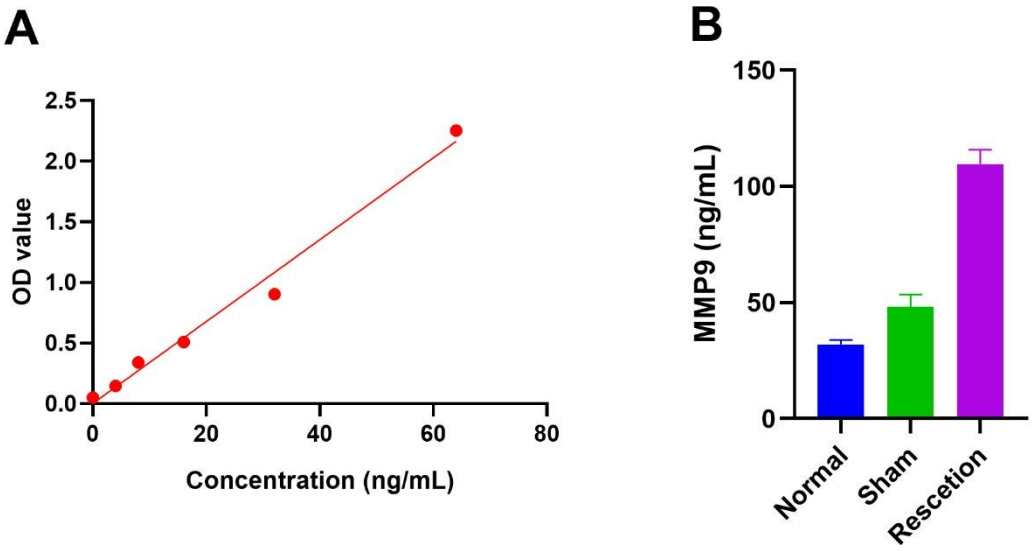


Figure S2. Rheological behaviors of the Tm, Tm (TMZ) and Tm (TMZ + BG).



(A) standard curve. (B) MMP9 expressions in Normal group, Glioma group, Sham group, Resection group *in vivo*.