Preparation of TRF-Targeted temozolomide Nano-micelles and their Anti-Glioma effect (#102197)

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Comment on strengths (as well as weaknesses) of the manuscript	*I commend the authors for their extensive data set, compiled over many years of detailed fieldwork. In addition, the manuscript is clearly written in professional, unambiguous language. If there is a weakness, it is in the statistical analysis (as I have noted above) which should be improved upon before Acceptance.*

Preparation of TRF-Targeted temozolomide Nano-micelles and their Anti-Glioma effect

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Objectives Using the biodegradable polymer material stearic acid as a carrier and selecting temozolomide (TMZ) as the model drug, brain-targeted temozolomide nanograins were prepared using targeted nanotechnology. The appearance, particle size, size distribution, drug loading capacity, in vitro release rate, stability, and other characteristics of the nanograins were studied. The anti-tumor effects were evaluated through *in vitro* experiments

Methods In the first part, we used Transmission Electron Microscopy and Malvern Size Analyzer to observe the stability of the temozolomide nanospheres via checking the morphological and particle size characterization at different points. Secondly, we evaluate the effects of temozolomide nanograins on the cell viability, apoptosis of glioma cells through MTT assay and flow cytometry.

Results TMZ targeted glioma nano-micelles were successfully synthesized. After loading and targeted modifications, the particle size of the nano-micelles increased from 50.7 nm to 190 nm, which indicates successful loading of TMZ. The average particle size of the nano-micelles remained around 145 ± 10nm at 1 day, 15 days, and 30 days after preparation. Furthermore, the release rate of the nano-micelles was monitored, with TMZ concentration measurements conducted at 2 hours, 12 hours, 24 hours, and 48 hours post-dialysis. The release rate gradually increased, ultimately reaching 95.8%. The toxicity is elevated after TMZ loading with polymer material stearic acid compared with TMZ itself accompanied with the enhancement of Glioma cells apoptosis. Mitochondrial Membrane Potential and ROS levels are increased as well after TMZ nano-micelles treatment.

Conclusions After successfully loaded with nano-micelles. TMZ achieved gradually release effects towards U251 cells compared with TMZ alone. Therefore, TMZ loaded nano-micelles has more significant inhibitory effects on U251 cells.

Preparation of TRF-Targeted Temozolomide Nano-micelles and their Anti-Glioma effects

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Abstract

Objectives

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lactic acid? please check. or PEG-PLA.
(TMZ) as the model drug, brain-targeted temozolomide nanograins were prepared using targeted
how was it brain targeted? not addressed clear.
nanotechnology. The appearance, particle size, size distribution, drug loading capacity, in vitro release rate, stability, and other characteristics of the nanograins were studied. The anti-tumor effects were evaluated through *in vitro* experiments

Methods

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TMZ
particle size ~~characterization~~ at different points. Secondly, we evaluate the effects of
features
~~temozolomide~~ nanograins on the cell viability, apoptosis of glioma cells through MTT assay,
TMZ
~~western blot,~~ flow cytometry.
western blot was not conducted in this work

Results

TMZ targeted glioma nano-micelles were successfully synthesized. After loading and targeted modifications, the particle size of the nano-micelles increased from 50.7 nm to 190 nm, which indicates successful loading of TMZ. The average particle size of the nano-micelles remained around 145 ± 10nm at 1 day, 15 days, and 30 days after preparation. Furthermore, the release rate of the nano-micelles was monitored, with TMZ concentration measurements conducted at 2 hours, 12 hours, 24 hours, and 48 hours post-dialysis. The release rate gradually increased, ultimately reaching 95.8%. The toxicity is elevated after TMZ loading with polymer material stearic acid compared with TMZ itself accompanied with the enhancement of Glioma cells apoptosis. Mitochondrial Membrane Potential and ROS levels are increased as well after TMZ nano-micelles treatment.

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human glioma U251, understandable without support of other main text.

Introduction

You defined the term of GBM, but it seems you used it seldom in the following context while used " Glioblastoma" or "human gliomas" or "glioma" instead. Are they the same thing?

~~Glioblastoma multiforme (GBM)~~ is a highly heterogeneous histological type of tumor in the central nervous system (CNS), accounting for approximately 81% of CNS malignancies[1]. It is regarded as the most common and malignant brain tumor, accompanied with 5.6% 5-year survival rate and 12 to 15 months overall survival ~~rate~~[2]. The main treatment options include surgical resection, radiotherapy, chemotherapy, targeted therapy, and symptomatic treatment[3]. Surgical resection is one of the main methods for treating glioblastoma[4]. Radiotherapy is a common treatment for glioblastoma, using radiation to kill cancer cells ~~and~~ to control or alleviate symptoms[5]. Chemotherapy aims to kill cancer cells with drugs and has a certain effect on treating glioblastoma[6]. Chemotherapy is often combined with surgery and radiotherapy to reduce tumor size and prolong survival. However, due to ~~their~~ strong invasive nature and high genetic heterogeneity, it is unsatisfied about ~~the treatment~~ outcomes[7]. Additionally, the blood-brain barrier limits the transport and diffusion of chemotherapy drugs from the blood to the brain, reducing the ability of chemotherapy drugs to reach tumor sites, leading to poor prognosis of human gliomas, with a median overall survival of 14.6 months[8,9].

Temozolomide (TMZ) is an orally administered alkylating agent that exerts ~~its~~ anti-tumor effects by inducing cell death through DNA methylation[10]. Due to its good tolerance, high permeability across the blood-brain barrier, and oral bioavailability of up to 100%, TMZ is a first-line drug for treating human glioma[11]. Although TMZ is a standard drug for glioma treatment, its efficacy is not significant and its effect on gliomas can only be maintained for ~~a~~ few months[12]. As a result, it

because of rapid elimination with much smaller half-life?

a few, positive; few, negative. For months effect, is quite long, isn't it?

is necessary to discover other feasible methods to further enhance the efficacy of TMZ.

In recent years, drug carriers such as microspheres, microcapsules, liposomes, and polymer nanoparticles have been playing an increasingly significant role as effective delivery tools in the development of novel cancer therapies[13,14]. On one hand, they can improve the solubility and stability of drugs, ~~extend the~~ extending circulation time of drugs in the body. On the other hand, they can target drug delivery to tumor tissues, improving the intra-body distribution of drugs, effectively reducing drug toxicity, and enhancing bioavailability. Among these, the development and application of liposomes and polymer nanoparticles are the most widespread[15]. Currently, a series of chemically synthesized polymers have been developed and applied in the preparation of drug carriers. Poly lactic acid (PLA), a polyester polymer material, is non-toxic and biodegradable. It has been approved by the United States Food and Drug Administration (FDA) for medical use, making it a highly promising polymer carrier material[16]. Poly ethylene glycol (PEG) is a polymer material obtained by ~~ring-opening polymerization~~ ring-opening polymerization (ROP) of ethylene oxide. It has good solubility ~~(soluble in water and conventional organic solvents)~~ not essentially need to address more., biocompatibility, modifiability, and can evade recognition by the human immune system, possessing a property of remaining "invisible" in the body[17]. The polymer material PEG-PLA, obtained by ~~ring-opening polymerization (~~ROP~~)~~ of PLA using PEG as an initiator, possesses amphiphilic structure, good biocompatibility, and biodegradability. This type of material serves as an excellent drug carrier ~~material~~[18].

Herein, we hypothesized that the encapsulation of TMZ by PEG-PLA allows for sustained release, enhancing the anti-tumor efficacy *in vitro*.

Materials and Methods

92 **Cell culture ~~and reagents~~**

93 Human GBM cells U251, obtained from American type culture collection (ATCC, Manassas,
94 VA, USA) ~~and cultured~~ with 10% fetal calf serum (Fisher Scientific, Waltham, MA, USA), 1%
, were cultured...
95 penifilin/streptomycin (Life Technologies, Grand Island, NY, USA) in Dulbecco's modified
96 Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA). Cells were incubated at 37℃
97 ~~condition~~ in a humidified incubator (SANYO, Moriguichi, Osaka, Japan) containing 5% CO_2.

98 **Synthesis of nanomedicines**
NH<2, subscript>, of this type errors, right?
99 Mix ~~NH2~~-PEG-PLA (3 mg/ml) and TMZ (2 mg/ml) in a 1:1 mass ratio, stir slowly at room
mg/mL, of this kind typo errors.
100 temperature in the dark for ~~2 h~~ Then the mixture was transferred to a 3.5 kDa dialysis bag for
2 h.
101 overnight dialysis in 18.2 MΩ deionized water to obtain nano particles loaded with TMZ. After
102 drug loading, the volume increased from 2 mL to 3.06 ml. The measured concentration is
103 152.825 μg/ml, and the drug loading capacity is calculated as follows: volume×
104 concentration/2000. After finishing the previous steps, ~~it is essential to check the characterization~~
, it is essential to characterize the loaded nanoparticles for size and stability.
105 ~~of loaded nanoparticles for size and stability~~. According to the instructions, TRF ? was conjugated
What is the instruction exactly? you may cite relevant work.
106 to the surface of nanoparticles via EDC ?, NHS ?, ~~and characterize the morphology and size of~~
107 ~~nanoparticles through TEM~~ and Malvern particle size analyzer.
..., thereafter the morphology and size of nanoparticles were characterized through transmission electron microscope (TEM)...

108 **Characterization of morphology, particle size, zeta potential and stability of nanomedicine**

109 In order to investigate the morphological characteristics of targeted nano carriers and targeted
110 nano drugs, prepared sample was dropped onto a copper grid for 2 min, ~~then solution~~ was
then the leftover solution
111 quickly blotted away with filter paper, ~~and then~~ copper grid was air-dried. Further procedure was
112 operated and observed under the guidance of ~~transmission electron microscope~~. Moreover, the
TEM
113 nanoparticle size and zeta potential were tested according to the Malvern Particle Size Analyzer
114 instructions. Besides, the stability of targeted nano carrier was evaluated by comparison with the

particle size in day 1, day 15 and day 30.

Drug loading and release performance of targeted nano carrier

Nano drug was placed in a dialysis bag which then was immersed in a PBS solution. 50 μl dialysate was collected at ~~the~~ 2 h, 12 h, 24 h and 48 h ~~of dialysis~~, then equal volume of acetonitrile was added into the collected dialysate. After that, the mixed solution was 5-fold diluted with 0.1 M hydrochloric acid. Release performance of nano carrier was calculated based on the concentration of TMZ at different time points.

Cell viability

Cell viability was conducted by MTT reagent under the guidance of manufacture's instructions.
please provide source info. of MTT reagent.
Briefly, U251 cells were seeded into a 96-well plate (1×10^5 cells/well) and were incubated for 24 h. Cells were then treated with PP, TMZ, PPT, TPPT for another 24 h, and 20 μl of MTT (5
what are these? what's the used dose for each?
mg/ml) was added to each well and continue to incubate for 4 hours. Formed formazan crystals were dissolved in DMSO, and cell viability was calculated according to absorbance at 570 nm as measured using a microplate reader (BioTek, Winooski, VT, USA).

Mitochondrial membrane potential

Mitochondrial membrane potential was assessed via the fluorescence intensity under the fluorescence microscope (Leica, Wetzlar, Germany). Briefly, U251 cells were seeded in 6-well plate (10^5 cells/well) and incubated overnight. Then cells were treated with TMZ, PPT and TPPT
see comments in 125, page 10.
for 24 h. Subsequently, 1 mL culture media and equal volume of JC-1 working solution (Beyotime, Shanghai, China) were added to incubate for 20 min after being washed with PBS twice. After ~~moving~~ removing the stained solution, cells were washed twice with buffer solute ion buffer solution? and then replaced with 1 mL culture medium. Finally, red and green fluorescence were observed

separately.

Detection of reactive oxygen species (ROS)

ROS levels were ~~confirmed~~ according to the instructions of ROS detection kit. Briefly, Logarithmic phase U251 cells were seeded ~~at a concentration of 1×10^5~~ in a 6-well plate. After overnight incubation, cells were further treated with TMZ, PPT and TPPT, respectively for 24 h. After discarding the culture medium, cells were washed twice with PBS. Then DCFH-DA probe was diluted 1000 times in serum-free medium. 1 ml of the diluted probe was added to each well and incubated for another 20 min. The plate was gently inverted every 3-5 min to ensure thorough contact between the probe and the cells. After incubation, cells were washed three times with serum-free medium to remove excess probe that did not enter cells. Finally, ROS levels ~~was~~ were evaluated with the detected optical density (OD) value ~~with~~ at an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

"determined" or other equivalent

please provide source info. for ROS detection kit

the expressed number is not in conc. unit.

see comments in 125, page 10.

Flow cytometry analysis

Logarithmic-phase human U251 cells were seeded at a concentration of 1×10^5 cells/well in a 6-well plate overnight. Then cells were treated with 1 μM TMZ, PPT and TPPT for 48 h. Cells were collected after discarding the supernatant through centrifuge at 1000 g for 5 min. Then 5,000 to 10,000 resuspended cells were taken ~~after centrifuge at 1,000 g for 5 min, and then supernatant were discarded and~~ ~~resuspended~~ in the mixture of 195 μL Annexin V-FITC binding buffer, 5 μl Annexin V-FITC and 10 μl propidium iodide staining solution~~. Then the cells were continued to incubate~~ in the dark at 20-25 ℃ for 20 min. Apoptosis status was finally analyzed using flow cytometry (BD Biosciences).

please refer to the comments in 125, page 10, and make your decisions to clarify while reducing repeates.

to remove the supernatant by centrifuge.

Resuspend cells

Incubate the cells

Statistical analysis

The data are presented as the means ± standard deviation. Mean differences were evaluated using post-hoc Duncan's test, or Student's t-test. A p value lower than 0.05 was considered statistically significant.

Results

Preparation and Characterization of TMZ-Incorporated PEG-PLA Micelles

~~In order to preparation of TMZ incorporated PEG-PLA micelles (Fig 1), accurately weigh 10mg of the synthesized copolymer, dissolve it in 1mL of DMF (or DMSO or THF), and slowly add the copolymer DMF solution into stirred pure water (or PBS buffer solution or saline). After stirring for 60 minutes, transfer the solution to a dialysis bag and dialyze with pure water for 48 hours, changing the water every 4 hours to remove DMF, thereby obtaining the self assembled conjugate.~~ To preliminarily confirm the success of the copolymer, the morphology and size of TPPT were captured through scanning electron microscopy. It was observed that ~~compared to PP~~, ~~TPPT enlarged~~, indicating the loading of TMZ drug component inside PEG-PLA (Fig 2A). To further verify the changes in nanoparticle size, this study utilized a nanoparticle size analyzer to measure the particle size changes of PP, PPT, and TPPT. As shown in Fig 2B, it can be seen that the nanoparticle size increased from 50.7nm to 141.7nm ~~before and~~ after drug loading, indicating the successful loading of TMZ into the nanoparticles. Furthermore, after targeted modification, the particle size increased to 190nm, confirming the success of the targeted modification. To further ~~understand and~~ demonstrate ~~the stability of~~ the copolymer under certain conditions, stability experiments were conducted ~~to assess its stability~~. As is shown in Fig 3, results demonstrate~~s~~ that there is no significant difference ~~about the~~ particle size of TPPT ~~in~~ different timepoints, which indicates that the TPPT is relatively stable. Meanwhile, we also checked the concentration of ~~TPPT in various timepoints, it shows that concentration increases from 0 to 95.8%~~

methodology details should go to Methods section.

see comments in 125, page 10. In Methods section, it was TEM, please check again.

TPPT micelles enlarged compared to PP,...

50.7 nm

pleas clarify

in

at

TMZ release from TPPT micelles over time, it shows that drug content increases from 0 to 95.8 % within 48 h,...

~~at 48 h~~, which indicates that ~~the~~ released ~~effect~~ of TPPT is achieved.
sustained/controled release of TMZ from TPPT is achieved.

Effect of Copolymers on the cell viability of U251

Further research on PEG-PLA nanomaterials was conducted to evaluate their potential therapeutic effects. MTT viability assay was employed to compare the cytotoxicity of drug-loaded micelles and free TMZ on U251 cells in vitro. The research results indicated that PEG-PLA itself did not significantly affect cell viability. However, after loading TMZ, it significantly inhibited the growth of U251 cells, and the activity was further enhanced after loading TRF (Fig 4).
italic, of this typo error.

Effect of PEG-PLA-TMZ nanomedicine on cell apoptosis

Previous studies have reported that TMZ alone increases mitochondrial membrane potential significantly[19], our ~~experiment~~ results demonstrate that PEG-PLA based TMZ nanomedicine further increases the mitochondrial membrane potential compared with TMZ itself (Fig 5A). Besides, many researches tell that dysfunction of mitochondria produces large amount of ~~reactive oxygen species (~~ROS~~)~~. Therefore, we compared with the ROS levels between TMZ and TMZ nanomedicines~~, the results~~ discover that nanomedicine significantly increased the ROS levels relative to TMZ itself. As a result, it induced more pronounced apoptosis (83.75% ~~apoptotic rate~~) compared with control group (2.82% ~~apoptotic rate~~) (Fig 5C).
, The results

Discussion

Glioblastoma is the most common malignant tumor of the central nervous system. Previous studies have shown that chemotherapy drugs ~~have~~ had been largely ineffective in treating glioblastoma, leading to suboptimal overall prognosis for patients. In the study by Stupp et al., the combination of TMZ with concurrent radiotherapy followed by six cycles of temozolomide
see comment for Line 51, page 7.

chemotherapy increased the median survival ~~period~~ to 14.6 months, raised the two-year survival rate to 27.2%, and achieved a five-year survival rate of 9.8%[20]. These results have led to increasing attention from clinical neuro-oncologists towards TMZ, establishing it as a frontline drug for treating patients with malignant gliomas. The current efficacy of temozolomide in the treatment of GBM remains below 45%, with the main limitations for clinical use including: 1) adverse reactions such as liver damage, gastrointestinal reactions, and bone marrow suppression after medication; 2) low effective drug concentration in the tumor area leading to insufficient anti-tumor effects[21].

see comment for Line 51, page 7.

Ensuring the efficient aggregation and release of chemotherapy drugs in the tumor area has been a research hotspot in the field of cancer treatment in recent years[22]. The low-toxicity drug delivery carriers prepared from ~~materials~~ can significantly reduce systemic adverse reactions of drugs while greatly improving treatment effectiveness[23]. Consequently, they can enhance post-chemotherapy survival quality for patients. The premise of targeted drug delivery is that polymer drug carriers exhibit strong stability in the bloodstream ~~circulation~~, effectively prolonging the circulation time of drugs in the body to achieve drug accumulation in tumor tissues. Early in vivo studies have shown that some PEG-polyester polymer nanomedicines experienced burst drug release in vitro release experiments, preventing complete drug delivery to tumor tissues, causing adverse reactions in normal tissues. Moreover, the accumulation of nanomedicines at tumor sites was mostly less than 5% of the injected dose per gram of tissue (ID·g−1)[24]. The possible reason for this outcome is that the structure of the prepared polymer nanoparticles is unstable, and the weak physical adsorption between the drug and the polymer is easily disrupted, leading to premature drug release.

polymers, PLA, PEG are polymers.

?

In recent years, many researchers have been devoted to designing and synthesizing more

structurally stable polymer nanocarriers through various modification methods, ~~significantly~~ to significantly enhance ~~enhancing~~ the therapeutic effects of anticancer drugs. Zhang et al. prepared PEG-PLA nanoparticles co-loaded with the anticancer drug paclitaxel (PTX) and the anti-angiogenic drug itraconazole (ITA) using a combination therapy approach. Compared to nanoparticles solely loaded with PTX, the introduction of ITA allowed for intermolecular interactions with PTX, ~~inhibiting~~ which inhibits the drug's crystallization ability within the nanoparticles and enhancing the stability of the polymer nanoparticles. The study demonstrated that these PTX/ITA co-loaded nanoparticles not only exhibited high anti-proliferative efficacy against non-small cell lung cancer cells but also improved the resistance of non-small cell lung cancer to PTX. Additionally, there was a significant enhancement in the accumulation of the nanoparticles in tumor tissues[25]. In order to achieve more structurally stable polymer nanoparticles, Lu et al. directly linked the topoisomerase I inhibitor 2-ethyl-10-hydroxy camptothecin SN38 to the polymer mPEG-PLA via ester bonds to obtain the polymeric macromolecular prodrug mPEG-PLA-SN38. The amphiphilic structure of mPEG-PLA-SN38 allows for self-assembly into polymer nanoparticles in aqueous solutions, overcoming the poor solubility issue of SN38. Furthermore, the compact structure of the polymer nanoparticles ensures their stability during transport in the body[26].

Cell apoptosis is a programmed cell death process that plays an important regulatory role in maintaining normal physiological states, developmental processes, and pathological conditions[27]. Reactive oxygen species (ROS) are a highly reactive group of oxygen-containing molecules, including superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (·OH), etc[28]. ROS are mainly generated from the mitochondrial respiratory chain, NADPH oxidase, cytochrome P450 enzymes, etc[29]. These enzyme systems maintain the redox balance within cells under normal circumstances. When the cellular environment changes, the activity of these

enzyme systems increases, leading to an increase in ROS production. On the other hand, there are antioxidant systems within cells, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione-S-transferase (GST), which can eliminate ROS, maintaining the redox balance within cells. When ROS production is excessive or the clearance system is inhibited, ROS accumulates in cells, triggering oxidative stress, which in turn promotes cell apoptosis[30]. In this study, we discovered that TPPT induced more production of ROS compared with TMZ alone, which is why intensity of apoptosis is increased.

~~As a results,~~ those results provide~~s~~ a feasible material (PEG-PLA) to be used as a carrier to improve the efficacy of TMZ, our experimental results indicate a significant enhancement in the therapeutic efficacy of TMZ after encapsulation in PEG-PLA, suggesting that this strategy provides a feasible approach to address the existing issues with TMZ. However, so far, only cell-based in vitro experiments have been conducted. Further animal studies and exploration in clinical stages are required to validate these findings.

Conclusions

This study aimed to improve the efficacy of TMZ in treating glioblastoma by using PEG-PLA nanocarriers. The encapsulation of TMZ in PEG-PLA significantly enhanced its therapeutic efficacy, as demonstrated by increased ROS production and cell apoptosis compared to TMZ alone. This suggests that PEG-PLA is a promising carrier for TMZ, addressing its current limitations of adverse reactions and low effective concentration in tumor areas.

see comment for Line 51, page 7.

Future studies should focus on validating these findings in animal models and exploring clinical applications. The potential of PEG-PLA to enhance drug delivery and reduce systemic toxicity warrants further investigation.

These results provide a promising strategy for improving TMZ-based therapy, contributing to

more effective treatment options for glioblastoma patients.

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Figure 1

Fig1

Illustration of conjugate preparation.

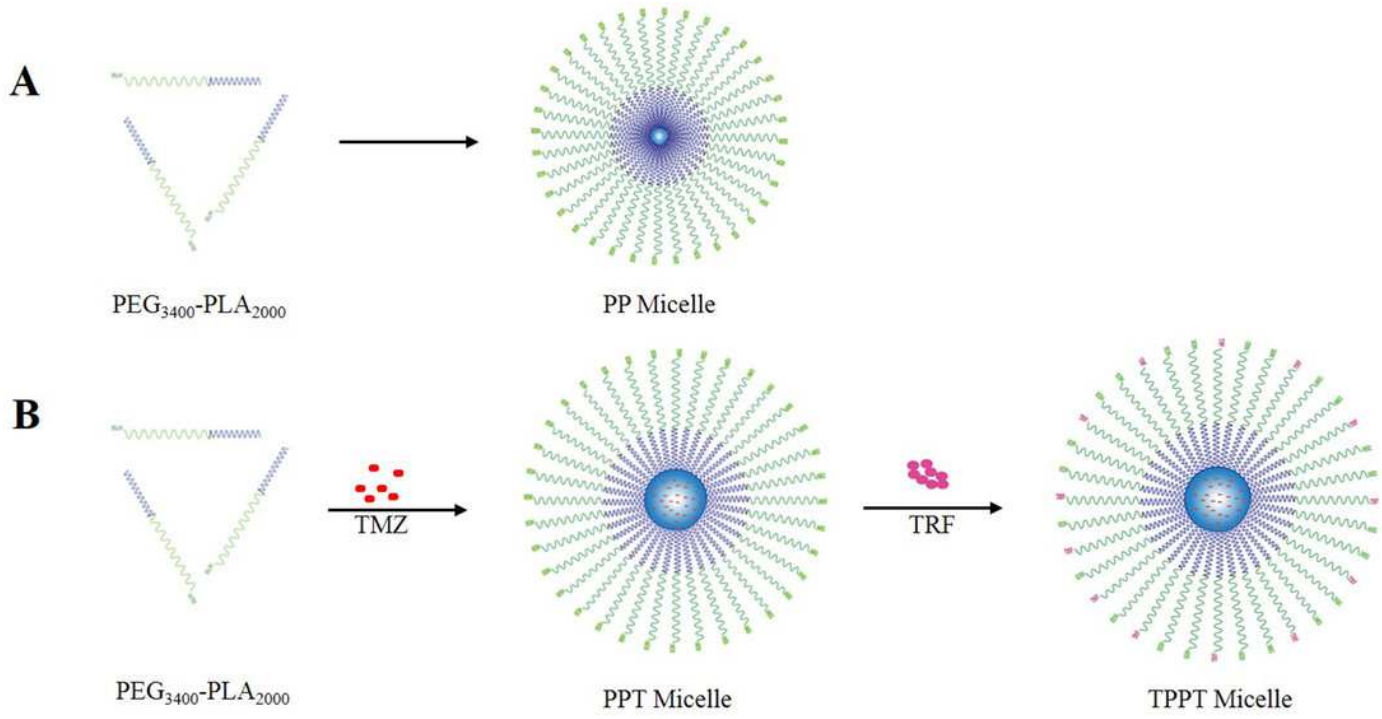


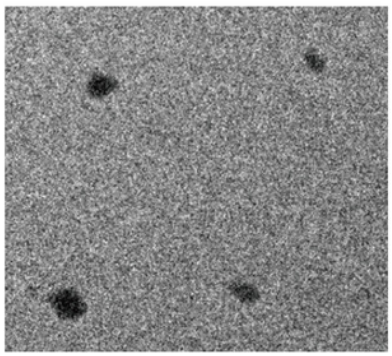
Figure 2

Fig2

Characterization of TMZ-Incorporated PEG-PLA Micelles. A, The morphology of the copolymer captured by scanning electron microscopy. B, The particle size of the copolymer measured by a nanoparticle size analyzer.

scanning electron microscopy, or TEM? please check carefully.`

A add a scale bar for panel A?



B

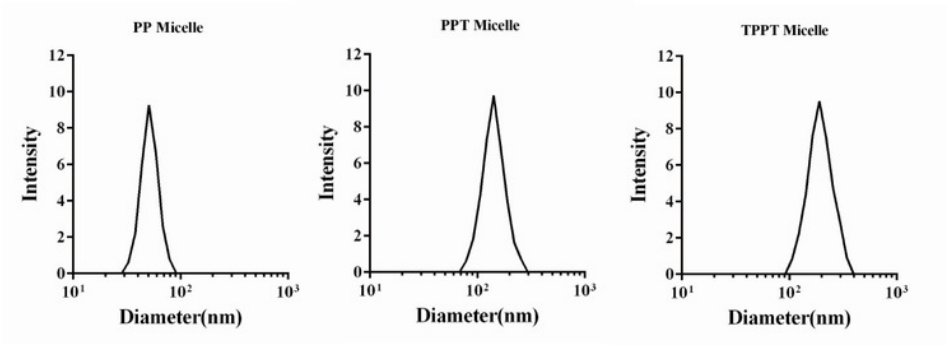


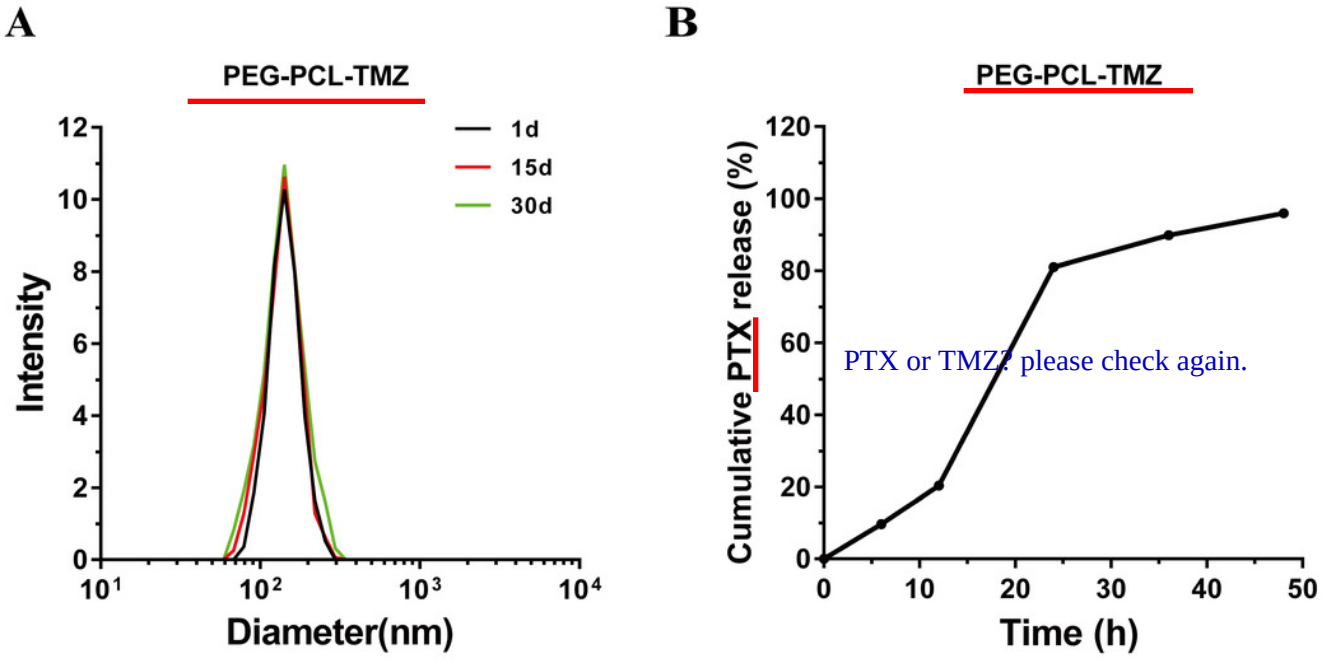
Figure 3

Fig3

Stability and sustained release ~~effects~~ of TPPT copolymers. A, The particle size of TPPT measured at different time points (1 d, 15 d, 30 d) using a nanoparticle size analyzer. B, Determination of drug concentration of TPPT at different time points.

TMZ release, right ?

PEG-PCL-TMZ or PEG-PLA-TMZ? In the context, it more like the latter, please check it.



PTX or TMZ? please check again.

Figure 4

Fig4

Analysis of the activity of nanoparticle micelles versus free TMZ on U251 cells. A, 1×10^5 cells/well ~~were~~ seeded in a 96-well plate were treated with PP, TMZ, PPT and TPPT for 48 h, ~~and an~~ MTT assay was used to assess cell viability. B, 1×105 cells/ well ~~were~~ seeded in a 96-well plate were treated with ~~various concentration of TMZ~~ and ~~survival rate~~ was quantified compared with control group. Values are presented as the means ± standard deviation (n=3).

superscript

TMZ at various concentrations

Dose response survival curve

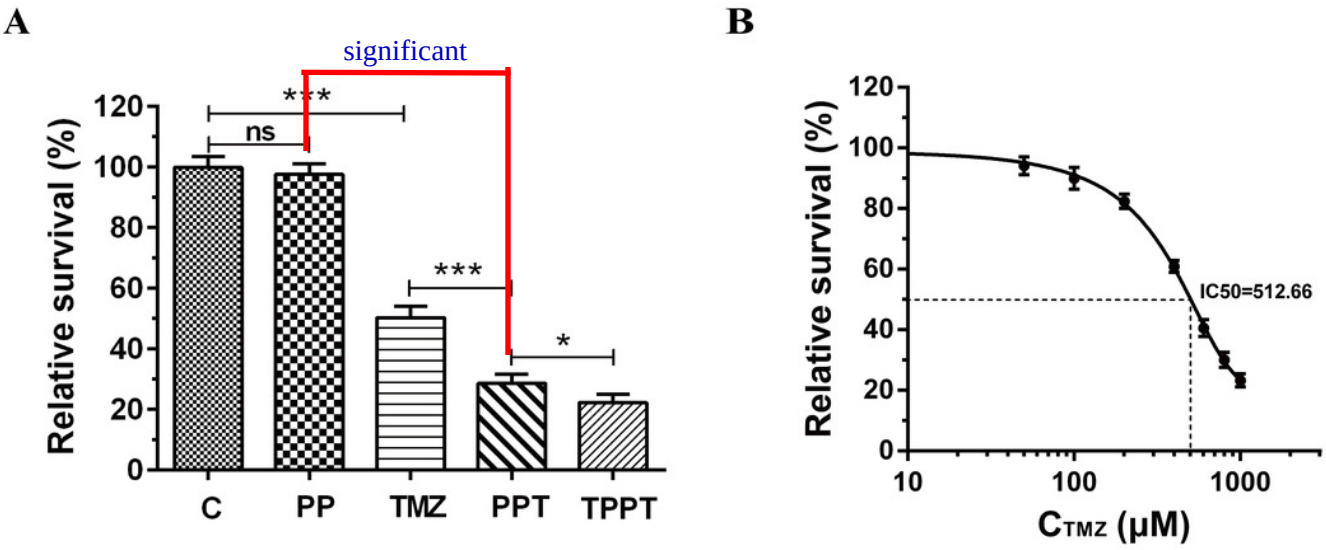


Figure 5

Fig5

Effect of TMZ nanomedicine on the cell survival related processes. A, Effect of nanomedicine on mitochondrial membrane potential. B, effect of nanomedicine on ROS levels. C, Effect of nanomedicine on apoptosis status by flow cytometry.