Reduction-responsive PEtOz-SS-PCL micelle with tailored size to overcome blood–brain barrier and enhance doxorubicin anti-glioma effect

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ABSTRACT
A series of novel reduction-responsive micelles with tailored size were designed and prepared to release doxorubicin (DOX) for treating glioma, which were developed based on amphiphilic block copolymer poly (2-ethyl-2-oxazoline)-b-poly (ε-caprolactone) (PEtOz-SS-PCL) and the micelle size could be regulated by designing the polymer structure. The DOX-loaded PEtOz-SS-PCL micelles had small size and rapid drug release in reductive intracellular environments. Biodistribution and in vivo imaging studies in C6 glioma mice tumor model showed that DOX loaded PEtOz-SS-PCL43 micelles with the smallest size had superior accumulation and fast drug release in tumor sites. In vivo antitumor activity demonstrated that DOX-loaded PEtOz-SS-PCL43 micelles improved antitumor efficacy in contrast to PEtOz-SS-PCL micelles with larger size toward the orthotopic C6-Luci cells-bearing mice. This study shows great potential in tailoring the micelle size and introducing the responsive bonds or compartment for intracellular drug delivery and release in glioma treatment by designing the architecture of the polymer.

1. Introduction
Malignant gliomas are the most common high-grade primary brain tumor with high morbidity, mortality and are poorly responsive to current treatments (Wei et al., 2014). The median survival of patients with glioblastoma seldom exceeds 14.6 months (Minniti et al., 2008). A major obstacle for glioma treatment is to deliver drugs effectively to the tumor cells. The blood–brain barrier (BBB) is a major obstacle to the delivery of drugs into brain tumors (Groothuis, 2000). Thus, most of small molecules with low lipid solubility including brain tumor chemotherapeutics rarely cross the BBB and do not enter into the brain (Partridge 2007a, b; Biddlestone-Thorpe et al., 2012). Developing some strategies for increasing drug in glioma is a great challenge in glioma therapy.

Over the past years, great efforts have been made to overcome these obstacles. Recently with the development of nanotechnology, nanoparticle therapeutic carriers have provided new opportunities to achieve effective therapeutic distribution at tumor sites (Brunetti et al., 2015; Kunjachan et al., 2015; Stylianopoulos, 2013; Liu et al., 2016). These drug carriers are passively targeted to tumors through the enhanced permeability and retention (EPR) effect, so they are ideally suited for the delivery of chemotherapeutics in cancer treatment. The complexity of glioma, especially the existence of the BBB, higher requirements to the control of nanoparticles size emerged. It has been reported that the vascular endothelial cells and associated pericytes are often abnormal in tumors and some BBB was destroyed in brain tumors (Schneider et al., 2004; Wohlfart et al., 2012). Based on this occasion, the glioma-targeted drug delivery systems are mainly divided into two categories. The drug delivery systems must deliver drugs across the BBB for glioma therapy. When the BBB is intact, the systems that must deliver drugs across the BBB for tumor-targeting are defined as cascade-targeting systems. Other drug delivery systems utilized in high-grade glioma are designated as glioma-targeting systems, which mainly accumulated in the glioma by EPR effect (Mager et al., 2017). Creating more safe and small size drug delivery systems is the key to the success of glioma therapy.

Micelle-based drug delivery system has been proven to be an attractive alternative for the delivery of chemotherapy drugs (Sun et al., 2009; Liang et al., 2014; Chen et al., 2015; Zhang et al., 2016). The particle size of micelles was controlled by adjusting the length and ratio of the hydrophilic and hydrophobic segments. However, to design stimuli-responsive
micelles would be more effective for tumor therapy, owing to anticancer drugs released exclusively in tumor tissue or inside tumor cell. The reduction responsibility is widely used stimuli in designing drug delivery system for triggered release (Felber et al., 2012; He et al., 2013). Poly(ethylene glycol) (PEG) currently is the most extensively used polymer in drug delivery as hydrophilic parts. Based on its low dispersity (D), biocompatibility and limited recognition by the immune system (stealth behavior), PEG remains the gold standard in polymer-based biomedical applications (Knop et al., 2010). However, formation of PEG-antibodies (Tagami et al., 2010), the accelerated blood clearance of PEG (Koide et al., 2010) and nonbiodegradability of PEG resulting in body accumulation of high-molar-mass PEGs limited the usefulness of PEGs in drug delivery in clinical trials (Pasut & Veronese, 2007). Poly(2-ethyl-2-oxazoline)s, abbreviated as PEtOz, provide higher stability, tunability, and functionalization than PEG, while retaining the requisite features of biocompatibility, stealth behavior and low dispersity (Hsiue et al., 2006; Hoogenboom 2009; Zhao et al., 2015). It is better replacements for PEG in drug delivery. A first PEtOz drug conjugate for treatment of Parkinson’s disease recently entered Phase II clinical trials (Moreadith et al., 2017). Therefore, in this study, PEtOz was chosen as hydrophilic part. Owing to biocompatibility and biodegradability nature of polycaprolactone (PCL), it is extensively studied for controlled drug delivery (Manavitehrani et al., 2016; Senevirathne et al., 2017). Thus, PCL was used as hydrophobic part in our study.

In this study, we synthesized reduction-responsive and size-controllable micelles based on amphiphilic polymer poly (2-ethyl-2-oxazoline) – polycaprolactone (denoted as PEtOz-SS-PCL) to encapsulate DOX for glioma therapy. Control over micelle size is therapeutically important for glioma, as it has been observed that particles with diameter between 20 and 100 nm are effective in penetrating into BBB (Crommelin et al., 2003; Duncan, 2003). In this experiment, PEtOz are hydrophilic parts with fixed polymerization degree and PCL are hydrophobic parts with three different degrees of polymerization (DPs) of 23, 33 and 43. We can obtain micelle with different size by a series of amphiphilic polymer with different DP of PCL block, denoted as PeOz-SS-PCL23, PeOz-SS-PCL33 and PeOz-SS-PCL43. As shown in Scheme 1, the introduction of a disulfide bond between PEtOz and PCL was beneficial to rapidly release DOX in glioma cells. The integration of above features makes PEtOz-SS-PCL an excellent carrier for delivery DOX for glioma therapy.

2. Materials and methods

2.1. Materials

2-ethyl-2-oxazoline (EtOz), ε-caprolactone (ε-CL) and bis (2-hydroxyethyl) disulfide) (HES) were dried over CaH₂ and purified by vacuum distillation. Toluene and tetrahydrofuran (THF) were dried by refluxing over sodium wire, benzophe none as indictor and distilled prior to use. Acetonitrile, N, N-dimethyl formamide (DMF), dichloromethane (DCM), chlorobenzene and methanol were dried by refluxing over CaH₂ and distilled under dry nitrogen. Methyl-p-toluenesulfonate, potassium thioacetate, 2, 2-dithiodipyridine, dithiothreitol (DTT) and 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was purchased from Alfa Aeser Co., Ltd. Doxorubicin (DOX) was got from Beijing Zhongshuo Pharmaceutical Technology Development Co., Ltd. All other chemicals were of analytical grade and used without further purification.

2.2. Characterization

The 1H NMR spectra were recorded on a Unity Inova 400 spectrometer operating at 400 MHz using chloroform-d. The molecular weight and polydispersity of copolymers were determined by a PL GPC 50 instrument equipped with Jordi GPC columns (10E4, 2 M) following a differential refractive-index detector (PL-RI). The measurements were performed.
using DMF as the eluent at 50 °C and a sense of narrow poly-
styrene standards for the calibration of the columns. The size
and zeta-potential of micelles was determined by a Zetasizer
Nano-ZS from Malvern Instruments. Transmission electron
microscopy (TEM) measurement was performed on a FEI
Tecai GT 12 operated with an accelerating voltage of 200 KV.
The absorbance of each well in MTT assays was measured
using a Synergy H4 Hybrid Multi-Mode Microplate Reader.

2.3. Synthesis of PEtOz-SS-PCL

The copolymers of poly(2-ethyl-2-oxazoline) pyridyl disulfide
(PETOz-SS-Py) were prepared following a procedure reported
by Ging-Ho Hsiue (Hsiue et al., 2006) and PCL-SH were syn-
thetized by ring-opened polymerization of ε-CL using HES as
initiator, then reacted with DTT, following a procedure
reported previously (Sun et al., 2009). The synthesis of amphi-
philic polymer PETOz-SS-PCL43 was used as an example in
the following procedure. The PCL-SH (150 mg, 0.0302 mmol)
was added into a DCM (6 mL) solution of PETOz-SS-Py
(195.93 mg, 0.0362 mmol) under a nitrogen atmosphere at
room temperature and adjusted the pH to 2.5 by adding acetic
acid. The reaction was allowed to proceed under stir-
ring for 48 h. The product PETOz-SS-PCL polymer was isolated
into cold diethyl ether, filtration and washing with cold
methanol several times to remove excess PETOz-SS-Py and
vacuum-dried to yield the product. The yield of PETOz-SS-PCL
was about 30%.

2.4. Preparation of PETOz-SS-PCL micelles and
physicochemical characterization

Micelles of PETOz-SS-PCL were prepared under stirring by
dropwise 1.2 mL double distilled water to 0.5 mL THF solution
of block copolymer (0.4 wt%) at room temperature, and
removed THF thoroughly by extensive dialysis against PB
(10 mM, pH 7.4) for 24 h.

Transmission electron microscopy (TEM) was used to
detect particle morphology. Before micelles were measured,
10 μL fresh micelles sample was placed onto carbon-coated
copper grids, and micelles were air-dried and then negatively
stained by a 2% sodium phosphotungstate solution.

Dynamic light scattering (DLS) measurements were per-
formed in aqueous solution using a Malvern Zetasizer Nano
ZS apparatus. To evaluate particle size, the intensity-weighted
Z-average of the particle diameter is reported in nm.

The critical micelle concentration (CMC) of polymer PETOz-
SS-PCL was determined by fluorescence spectrometer (FL
4600) using pyrene as a fluorescence probe. The concentra-
tion of PETOz-SS-PCL micelle was changed in a range from
1.0 × 10^{-3} to 0.1 mg/mL, and the pyrene concentration
was 0.6 μM. The excitation wavelength of fluorescence spectra
was fixed at 330 nm, and then, the emission fluorescence at
372 nm and 383 nm was recorded, and the CMC value was
obtained as the interaction point by extrapolating the intensity
ratio I372/I383 at above test concentrations.

2.5. In vitro cellular uptake

To evaluate the cellular uptake of DOX-loaded PETOz-SS-PCL
micelles, flow cytometric analysis was used. C6 cells at a
density of 5 × 10^4 were cultured in 12-well tissue culture
plates and cultured at 37 °C in a 5% CO2 humidified atmos-
phere with DMEM containing 10% FBS for 24 h. Later, old
medium was replaced with fresh medium. Then, the cells
were treated with DOX loaded PETOz-SS-PCL23, PETOz-SS-
PCL33, PETOz-SS-PCL43 nanoparticles and PBS at DOX con-
centration of 1 μg/mL for 2 h. The cells were trypsinized,
washed three times with cold PBS and resuspended in 500 μL
cold PBS for flow cytometric analysis using a BD FACSCalibur
flow cytometer.

2.6. In vitro drug release

The in vitro release of DOX from PETOz-SS-PCL micelles was
using a dialysis tube (MWCO 12000) incubated at 37 °C for
24 h in two different media, that is, PB (10 mM, pH 7.4) with
10 mM DTT and PB (pH 7.4, 10 mM). In order to acquire sink
conditions, drug release studies were performed at low drug-
loading contents (ca. 0.5 wt%), 0.7 mL DOX-loaded micelle
solution dialysis against 20 mL of the same medium. At
desired time intervals, 6 mL release media were taken out
and replenished with an equal volume of fresh media. The
concentration of DOX was determined by fluorescence
measurements (excitation at 480 nm). To determine
the amount of DOX released, calibration curves were run
with DOX/PB buffer solutions with different DOX concen-
trations at pH 7.4. The emission at 600 nm was recorded. The
release experiments were performed in triplicate. The results
are presented as the average ± SD.

2.7. Reduction-responsive size change of PETOz-SS-PCL
micelles

Reduction induced size change of PETOz-SS-PCL micelles was
measured by DLS, which was performed in response to PB
buffer (pH 7.4) with or without reductive agent (10 mM DTT)
at 37 °C. Typically, PETOz-SS-PCL43 micelles (1.5 mL) in PB
buffer (pH 7.4, 10 mM) were first removed oxygen by bub-
bling with nitrogen, then, DTT was added to obtain a final
concentration of 10 mM. The above mixture was placed in a
shaking bed with a speed of 200 rpm at 37 °C. The size was
measured by DLS at predetermined time points.

2.8. Cell viability assays

The cytotoxicity of PETOz-ss-PCL23, PETOz-ss-PCL33, PETOz-ss-
PCL43 and DOX was detected by the 3-(4, 5-dimethylthiazol-
2-y1)-2,5-diphenyltetrazolium bromide (MTT) assay. Three
thousand C6 cells were seeded in 96-well tissue culture plate
and cultured at 37 °C in a 5% CO2-humidified atmosphere in
150 μL DMEM containing 10% vol% FBS for 24 h. Then, free
DOX and DOX-loaded PEtOz-SS-PCL micelles at different DOX concentrations were added and incubated for another 48 h. After that 10 \( \mu L \) MTT reagent (5 mg mL\(^{-1} \)) was added to the each well and incubated for an additional 4 h. Medium was removed lightly and 150 \( \mu L \) DMSO was added to each well for 15 min to dissolve purple formazan. At last quantification, measurements (optical density) were obtained at a wavelength of 480 nm using a spectrophotometric analysis.

2.9. Mice

ICR mice (male, 18–20 g) were purchased from Beijing HFK Bioscience Co., Ltd. (Beijing, China). All animals received care in compliance with the guidelines in the Guide for the Care and Use of Laboratory Animals. All procedures were approved by Xuzhou Medical University of China Animal Care and Use committee.

Glioma-bearing ICR mice were prepared by intracranial injection (striatum, 1.8 mm right lateral to the bregma and 3 mm of depth) of \( 1 \times 10^6 \) C6-Luci cells suspended in 4 \( \mu L \) of L15 medium into male ICR mice (Li et al., 2014; An et al., 2015). After 7 days of xenograft glioma, nude mice were randomly divided into five groups (\( n = 10 \)) and housed in a controlled temperature room with regular alternating cycles of light and darkness.

2.10. In vivo distribution of DOX-loaded PEtOz-SS-PCL

Glioma-bearing ICR mice were first injected with freshly prepared luciferin substrate and imaged with the Xenogen IVIS Spectrum optical imaging device to prove to have similar volume tumors in the brain after 7 days of xenograft glioma. After that, glioma-bearing ICR mice were injected intravenously with free DOX, DOX loaded PEtOz-SS-PCL23, PEtOz-SS-PCL33 and PEtOz-SS-PCL43 micelles through the tail vein at DOX dose of 3 mg kg\(^{-1} \). The survival time and body weight of mice were measured. The survival time and body weight of mice were measured. After the last treatment, the mice were euthanized and the hearts were collected. The hearts were washed with saline and fixed in the 4% paraformaldehyde. The sections of hearts were stained with hematoxylin and eosin and observed under fluorescence microscopy and photographed (Olympus, Japan).

2.12. Statistical analysis

Statistical analysis was performed using the Student’s t-test with \( p < .05 \) as significant difference. The experimental results were given in the format of mean, mean ± SD in the figures.

3. Results and discussion

3.1. Synthesis of PEtOz-SS-PCL

A series of novel amphiphilic PEtOz-SS-PCL block copolymer was synthesized through exchange reaction between poly (2-ethyl-2-oxazoline) pyridyl disulfide (PEtOz-SS-Py) and mercap to PCL (PCL-SH) in dichloromethane with molar ratio of 1/2/1 (Figure S1, Table 1). Figure 1 showed both hydrophilic components of PEtOz (\( \delta \) 1.10, \( \delta \) 3.45) and hydrophobic components of PCL (\( \delta \) 1.37, \( \delta \) 1.65, \( \delta \) 2.30, \( \delta \) 4.05) in \(^{1}H\) NMR spectra. The integral ratio of signals at \( \delta \) 1.10 and \( \delta \) 4.05 manifested an equivalent coupling of PEtOz and PCL. The DP of PEtOz and PCL was calculated from the \(^{1}H\) NMR end group analysis (Figure S2) (Zhang et al., 2010; Zhu et al., 2011; Yang et al., 2012). Moreover, GPC results showed that the resultant copolymers PEtOz-SS-PCL had a narrow distribution of molecular weight after the exchange reaction between PEtOz-SS-Py and PCL-SH, with a PDI of 1.27, 1.17, 1.21, respectively (Figure S3). Therefore, these results showed we

| Table 1. Molecular characteristics of PEtOz-SS-PCL. |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Block copolymers | \( M_n \) (design) | \( M_n^{(\text{GPC})} \) | \( M_n^{(\text{HNM})} \) | PDI |
| PEtOz-SS-PCL23 | 4200–5100 | 5200–4900 | 4200–6400 | 1.27 |
| PEtOz-SS-PCL33 | 4200–4300 | 5200–3600 | 4200–4300 | 1.17 |
| PEtOz-SS-PCL43 | 4200–2900 | 5200–2400 | 4200–2500 | 1.21 |

\( M_n \) determined by \(^{1}H\) NMR. \( M_n^{(\text{GPC})} \) determined by GPC. \( M_n^{(\text{HNM})} \) was determined by GPC measurements in DMF (1.0 mL min\(^{-1} \), 30 °C, and polystyrene standards).

Figure 1. \(^{1}H\) NMR spectra (400 MHz, CDCl\(_3\)) of PEtOz-SS-PCL23 (A), PEtOz-SS-PCL33 (B) and PEtOz-SS-PCL43 (C).
have synthesized diblock copolymer PEtOz-SS-PCL successfully. These block copolymers were prepared by varying the length of PCL block, while the hydrophilic PEtOz block was fixed. The molar ratio of the initial monomer and initiator concentration were 43: 1, 33: 1, 23: 1 respectively, resulting in different lengths of PCL block. The molecular weight and a PDI of block copolymers PEtOz-SS-PCL were determined by GPC (Figure S4) and summarized in Table 1.

3.2. Characterization of PEtOz-SS-PCL micelles

TEM examination revealed that the PEtOz-SS-PCL43 micelles were dispersed as individual particles with a spherical shape (Figure 2(A)). DLS analysis showed that the average particle size of PEtOz-SS-PCL decreased with the increase of the polymerization degree of PCL, which might due to increasing the concentration of hydrophobic chain. From Table S1, it can be seen that the sizes of the PEtOz-SS-PCL23, PEtOz-SS-PCL33 and PEtOz-SS-PCL43 are 160.2 ± 1.4 nm, 140.6 ± 1.6 nm and 97.3 ± 1.8 nm, respectively. Thus, the loading of DOX in PEtOz-SS-PCL23 and PEtOz-SS-PCL33 do not increase the micelles size significantly. However, the size of DOX-loaded PEtOz-SS-PCL43 are smaller than these of corresponding blank micelles, it is 88.4 ± 2.7 nm. The strong attractive hydrophobic interactions between the encapsulated drug and inner core causes the reduced micelle size (Ding et al., 2013; Shi et al., 2015). The physical properties and loading capacity of PEtOz-SS-PCL23, PEtOz-SS-PCL33 and PEtOz-SS-PCL43 were listed in Table S1.

Cytotoxicity of PEtOz-SS-PCL micelles was studied at 37 °C in PB buffer (pH 7.4) with or without 10 mM DTT. The disulfide bond located between the hydrophilic compartment and hydrophobic compartment would break in the reductive conditions (10 mM DTT) which is mimicking the intracellular cytoplasm (Figure 2(C)). The reduction-responsive drug loaded PEtOz-SS-PCL43 micelle has great potential using as a drug delivery system for the treatment of tumor.

We also used different buffer and reducing reagent DTT to investigate the reduction sensitivity of PEtOz-SS-PCL micelle. The size change of PEtOz-SS-PCL43 micelle was tracked by DLS (Figure S7). Here, we used PB buffer (10 mM, pH 7.4) to simulate the intracellular condition, respectively. The DLS results showed that the micelle size fastly increased from about 100 nm to hundreds of nanometers after 4.0 h in the presence of 10 mM DTT, indicating that the PEtOz-SS-PCL micelle is sensitive to reductive condition. This phenomenon attributed to the reason that reduction sensitivity caused by the disulfide bond between hydrophilic and hydrophobic parts of polymer PEtOz-SS-PCL, which resulted in the disassembly of micelle.

3.3. Cell viability assays

The in vitro cytotoxicity of DOX-loaded PEtOz-SS-PCL micelles against C6 cells was evaluated by MTT assay. DOX-loaded

Figure 2. Characteristics of PEtOz-SS-PCL micelles. (A) TEM reveals the morphological structure of PEtOz-SS-PCL43 micelles. (B) Cellular uptake of DOX-loaded PEtOz-SS-PCL23, PEtOz-SS-PCL33 and PEtOz-SS-PCL43 micelles. (C) Reduction-triggered drug release from DOX-loaded PEtOz-SS-PCL43 micelles.

Table S1) (Sun et al., 2009). The lower CMC values also suggested the micelles self-assembled from PEtOz-SS-PCL were stable upon dilution under physiological conditions, which is beneficial for the drug delivery application in vivo. The CMCs of PEtOz-SS-PCL micelles decreased with an increasing amount of hydrophobic PCL block, because the micelle with longer hydrophobic PCL block which played an important role in the stability of the micelle were prone to form. We also studied the effects of size on the cellular uptake efficiency of PEtOz-SS-PCL23, PEtOz-SS-PCL33 and PEtOz-SS-PCL43 micelles by flow cytometry. As shown in Figure 2(B), PEtOz-SS-PCL43 with size around 80 nm were taken up by tumor cells far more than larger micelle of PEtOz-SS-PCL23 and PEtOz-SS-PCL33. The result indicated that the cellular uptake process of PEtOz-SS-PCL micelles highly depended on the size of micelles and small size micelles exhibited high uptake efficiency.

The in vitro release of DOX from DOX-loaded PEtOz-SS-PCL43 micelles was studied at 37 °C in PB buffer (pH 7.4) with or without 10 mM DTT. The disulfide bond located between the hydrophilic compartment and hydrophobic compartment would break in the reductive conditions (10 mM DTT) which is mimicking the intracellular cytoplasm (Figure 2(C)). The reduction-responsive drug loaded PEtOz-SS-PCL43 micelle has great potential using as a drug delivery system for the treatment of tumor.
PEToz-SS-PCL43 micelles presented the significantly enhanced cytotoxicity compared with DOX-loaded PEToz-SS-PCL23 and DOX-loaded PEToz-SS-PCL33 at all the DOX concentrations studied (Figure 3). **IC**\textsubscript{50} of DOX-loaded PEToz-SS-PCL43 micelles was 6.67 µg mL\textsuperscript{-1} on C6 cells, which was lower than that of DOX-loaded PEToz-SS-PCL23 and DOX-loaded PEToz-SS-PCL33 with IC\textsubscript{50} value of 45.68 and 16.16 µg mL\textsuperscript{-1}, respectively. It was suggested that higher uptake efficiency of DOX-loaded PEToz-SS-PCL43 compared with DOX-loaded PEToz-SS-PCL23 and DOX-loaded PEToz-SS-PCL33 provided higher cytotoxic activity toward the glioma cells. The difference of DOX-loaded PEToz-SS-PCL43 and DOX-loaded PEToz-SS-PCL23 and DOX-loaded PEToz-SS-PCL33 may be due to the size of drug-loaded micelle, which was also in line with the cellular uptake result of flow cytometry. This phenomenon also demonstrated that the micelle size is one of the key point for cell endocytosis, the smaller size is beneficial for the cell uptake in this procession, and further released cargo under reductive condition in plasma, resulting in different in vitro cytotoxicity. Obviously, in vitro cytotoxicity of DOX-loaded PEToz-SS-PCL micelles can be adjusted by tailoring the micelle size.

### 3.4. In vivo glioma distribution of PEToz-SS-PCL

The in vivo targeting of glioma and localization in various organs of DOX-loaded PEToz-SS-PCL were observed in orthotopic C6-Luci cells-bearing mice by the IVIS kinetic imaging system (Caliper Life Sciences, Hopkinton, MA). First, the brain tumor model was developed in ICR mice using stereotactic intracranial injection of \(\sim 1 \times 10^5\) C6-Luci cells into the primary somatosensory cortex. After 7 days post implantation, the bioluminescence signals were analyzed by in vivo bioluminescence imaging. As shown in Figure 4(A), the in vivo image finding was confirmed the existence of brain glioma. And then, DOX-loaded PEToz-SS-PCL and free DOX were administered intravenously to orthotopic implantation model of glioma cells in ICR mice, and was examined after 4h by an in vivo imaging system and fluorescence microscopy, respectively. The fluorescence intensity of free DOX in the brain was negligible, indicating that free DOX barely crossed the BBB in the mice. DOX loaded PEToz-SS-PCL micelles with different PD of PCL had stronger DOX fluorescence than free DOX (Figure 4(B,C)). Compared to DOX-loaded PEToz-SS-PCL23, PEToz-SS-PCL33 treated mice, the strongest DOX fluorescence was found at the mice’s tumor region treated with DOX-loaded PEToz-SS-PCL43. These results indicated that PEToz-SS-PCL43, which has the smallest nanosize could effectively enter into glioma by EPR effect. The tumor distribution of PEToz-SS-PCL and free DOX was also confirmed by frozen tumor tissue section observed by fluorescence microscope (Figure 4(D)). Brain tumor tissue was identified by areas of hypercellularity as evident from DAPI-stained cell nuclei shown in blue (Figure 4(C)). The C6-Luci bearing mice treated with PEToz-SS-PCL43 had more DOX fluorescence in brain tumor than treated with other groups, indicating that PEToz-SS-PCL43 could effectively deliver DOX to brain tumor. After that, C6-Luci bearing brains and major organs were excised for ex vivo imaging to reveal the tissue distribution. A clearer result was found in the ex-brain imaging (Figure 4(E)). Besides, the amount of PEToz-SS-PCL was less in liver compared to free DOX group, suggesting a lower toxicity to liver. While the DOX fluorescence in heart, spleen, lung and kidney was similar in DOX-loaded PEToz-SS-PCL23, PEToz-SS-PCL33 and PEToz-SS-PCL43 groups. These findings demonstrated that PEToz-SS-PCL43 group with the smallest nanosize could effectively transport the DOX across the BBB and could result in the highest cellular uptake of DOX by glioma.

We next evaluated the therapeutic efficacy of locally administered free DOX, DOX-loaded PEToz-SS-PCL23, DOX-loaded PEToz-SS-PCL33 and DOX-loaded PEToz-SS-PCL43 (the concentration of DOX 3 mg kg\textsuperscript{-1}) by intravenous injection at 11 days established in ICR mice by stereotactic intracranial injection of \(\sim 1 \times 10^5\) C6-GFP-Luci human glioma cells into the cortex (Figure 5(A)). Tumor growth was measured in vivo using bioluminescence imaging. Tumors in animals in the control group (PBS) grew rapidly (Figure 5(B)). By day 24, the tumor loaded in PBS group, as reflected by bioluminescence measurements, was 12.11-fold higher than at day 10. The tumor growth rate in mice that treated with free DOX, DOX-loaded PEToz-SS-PCL23, DOX-loaded PEToz-SS-PCL33 and DOX-loaded PEToz-SS-PCL43 were 1.18-fold, 2.85-fold, 3.13-fold and 0.99-fold higher than at day 10, respectively. From this result, we found that DOX-loaded PEToz-SS-PCL43 had the highest antiglioma activity, due to DOX-loaded PEToz-SS-PCL43 delivered more DOX to glioma than DOX-loaded PEToz-SS-PCL23 and DOX-loaded PEToz-SS-PCL33, which was confirmed by the distribution of DOX fluorescence in glioma.

To further estimate the antitumor efficacy, the body weight and overall survival of the glioma-bearing mice were assessed (Figure 5(C,D)). As shown in Figure 6(C), treatments with DOX-loaded PEToz-SS-PCL23 and DOX-loaded PEToz-SS-PCL33 did little in improving mice survival, registering a median survival of 29 days and 27.5 days versus 23.5 days for the PBS-treated group. Although remarkable tumor inhibition was observed in DOX-treated group, no benefit of median survival time emerged. Compared to the DOX-treated group (median survival, 31.5 days), DOX-loaded PEToz-SS-PCL43-
treated groups possessed prolonged survival times. The median survival time was 45 days for DOX-loaded PETOz-SS-PCL43 treated group. The dominance of DOX-loaded PETOz-SS-PCL43 was also reflected on the body weight change. The body weight showed a slow decrease of DOX-loaded PETOz-SS-PCL43, while other groups had a rapid decrease (Figure 5(D)). All the above results demonstrated DOX-loaded PETOz-SS-PCL43 had a predominant therapeutic efficacy for glioma, due to its the smallest nanosize.

3.5. H and E staining analysis

DOX is a highly effective and widely used chemotherapeutic drug to cure various types of cancer; however, its effectiveness is limited by its cardiac toxicity (Cho et al., 2012; Guan et al., 2013; Subburaman et al., 2014). In order to further evaluate the cardiotoxicity of DOX and DOX-loaded PETOz-SS-PCL treatment, the histological analysis of the cardiac tissues was tested. As shown in Figure S8, histological
examinations did not show any myocardial lesions in the group of mice treated with free DOX, DOX-loaded PEtOz-SS-PCL23, DOX-loaded PEtOz-SS-PCL33 and DOX-loaded PEtOz-SS-PCL43, suggesting that the treatment with free DOX, DOX-loaded PEtOz-SS-PCL23, DOX-loaded PEtOz-SS-PCL33 and DOX-loaded PEtOz-SS-PCL43 at the experimental dosage did not damage the heart of the mice during the experimental period.

4. Conclusions

In summary, we have successfully synthesized reduction-responsive and controlling size DOX-loaded PEtOz-SS-PCL micelles to encapsulate DOX for glioma therapy. The DOX-loaded PEtOz-SS-PCL43 micelles showed the smallest size distribution and effectively entered into glioma in vivo. Moreover, treatment with DOX-loaded PEtOz-SS-PCL43 micelles inhibited significantly brain tumor growth in ICR mice orthotopic glioma model compared to other groups. Therefore, PEtOz-SS-PCL43 micelles can be potentially applied as a safe and efficient drug delivery for glioma treatment.

Disclosure statement

No potential conflict of interest was reported by the authors.

Figure 5. In vivo efficacy in C6-Luci glioma model in mice. C6-Luci-bearing mice received three injections of PBS, Free DOX, DOX-loaded PEtOz-SS-PCL23, DOX-loaded PEtOz-SS-PCL33 and DOX-loaded PEtOz-SS-PCL43 at a dose of 3 mgkg⁻¹ DOX. (A) Bioluminescent signal change correlating to tumor growth over time following inoculation. (B) Quantification of the tumor bioluminescence signal (n = 5 mice per group). (C) Kaplan–Meier survival curve of the mice. Data are presented as Mean ± SD (n = 8, **p < 0.01). (D) Body weight change.

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