Injectable peptide hydrogel as intraperitoneal triptolide depot for the treatment of orthotopic hepatocellular carcinoma

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KEY WORDS

Chemotherapy is among the limited choices approved for the treatment of hepatocellular carcinoma (HCC) at intermediate and advanced stages. Preferential and prolonged drug exposure in diseased

Abbreviations: Akt, protein kinase B; ANOVA, analysis of variance; AST, aspartate transaminase; ATL, alanine transaminase; AUC0–13, areas under the curve; AURKA, aurora A kinase; Bel-7402/Luc, luciferase transfected human HCC cell line Bel-7402; BUN, blood urea nitrogen; CAS, Chinese Academy of Sciences; CD, circular dichroism; CKS2, cyclin kinase subunit-2; CRE, creatinine; C16-N, C16-GNNQQNYKD-OH; C16-N/DiI, DiI-labeled C16-N; C16-N/DiR, DiR-labeled C16-N; C16-N/T, triptolide-loaded peptide amphiphile-based hydrogel; DL, drug loading; D-Luciferin, (S)-4,5-dihydro-2-(6-hydroxy-2-benzothiazolyl)-4-thiazolecarboxylic acid potassium; DSPE-PEG, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000]; DSPE-PEG/DiI, DiI-labeled DSPE-PEG; DSPE-PEG/DiR, DiR-labeled DSPE-PEG micelle; DSPE-PEG/T, drug-loaded DSPE-PEG micelles; EE, encapsulation efficiency; FBS, fetal bovine serum; FI, fluorescence intensity; FI range, fluorescence intensity range; TACE, transarterial chemoembolization; TEM, transmission electron microscopy; TIR, tumor inhibition rate; Tmax, time to reach highest fluorescence intensity.

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1. Introduction

Liver cancer is the second leading cause of cancer-related death worldwide, resulting in more than 810,000 deaths in 2015, and the mortality was continuously growing in the past 25 years. Hepatocellular carcinoma (HCC) accounts for about 70%—90% of all primary liver cancer. HCC of early stage is eligible for curative therapies such as resection, transplantation and local ablation, with median survival time over 60 months. Unfortunately, most HCC patients have already developed local (intermediate stage) or portal vein (advanced stage) invasion at their first time of diagnosis, with 5-year survival rates of only 11% and 3%, respectively. Transarterial chemoembolization (TACE) using drug-eluting beads is now a standard treatment for intermediate stage HCC patients, and profoundly increases 5-year survival rate to 22.5%. While significantly alleviates the systemic side effects of chemotherapy, TACE requires imaging-guided surgery performed by an experienced doctor, and the subsequent blood vessel blockage hampers potential combination therapy with substantial damages to local liver tissues. For patients with advanced HCC, sorafenib and pembroliuzumab are two available treatments in the clinic. Sorafenib prolonged the median time to radiologic progression to 5.5 months from 2.8 months of non-treated patients, with no complete and 2% partial response recorded, while patients receiving pembrolizumab showed 1% complete and 16% partial responses. Systemic chemotherapy with gemcitabine and oxalipatin (GEMOX) has also been evaluated in the clinic on advanced HCC patients with a response rate of 22%, and tumor shrinkage was observed in some of the patient. However, more than 50% of the patients did not respond to the treatments, and these systemically administrated drugs all elicited grade 3 or 4 toxicity in ~10%—44% patients due to off-target drug exposure. In addition to these drugs that are either approved or in clinical trials, other compounds are also under intensive investigation at the preclinical stage. For instance, triptolide is a potent anti-tumor agent which kills cancer cells through heat shock gene (e.g., heat shock protein 70) expression inhibition, protein kinase B (Akt)/mammalian target of Rapamycin/p70S6K pathway inactivation, or cyclin kinase subunit-2 (CKS2) and aurora A kinase (AURKA) down-regulation. Triptolide was found to be more potent than doxorubicin and sorafenib against a panel of HCC cells in vitro, but it only stabilized the disease in vivo, probably due to limited drug exposure and severe systemic toxicity. Therefore, new drug delivery systems that can realize prolonged and preferential drug accumulation in HCC tumor are still of urgent need.

Nanomedicine can significantly modify the pharmacokinetics of encapsulated drugs, and it has been found that majority of intravenously injected nanoparticles accumulated in the liver. However, detailed investigations showed that these particles are mainly captured by Kupffer cells, B cells and endothelial cells. An extensive screen is necessary to identify ligands that could direct cargos specifically to HCC cells rather than hepatic cell and immune cells. A stimuli-responsive drug release in the liver was also found to be essential. However, the efficiency of these nanomedicines may be compromised by the formation of protein corona in vivo. Therefore, it is still challenging to maintain a steady and optimized drug exposure in the liver. Intraperitoneal chemotherapy is an alternative strategy to treat cancers that are confined to the peritoneal cavity. Intraperitoneal chemotherapy may be also advantageous in treating portal-vein-perfused micrometastatic cancer in the liver which was characteristic for intermediate and advanced HCC. To prolong drug retention in the peritoneal cavity, the use of drug-loaded nanoparticles was explored. Intraperitoneal injection of bioadhesive nanoparticles has been further explored by Zhao et al., in the aim of slowing nanoparticle clearance through lymphatic draining and realizing in situ release of encapsulated drug for localized therapy. Nevertheless, readily drug release from nanoparticles is routinely observed because of their high specific surface area, resulting in faster clearance of the drugs prior to their nanocarriers.

Hydrogel is able to retard drug release by physically limiting material exchange between immobilized content with outside environment. For instance, tunable drug release from liposomes has been achieved by varying the extent of crosslinking of the hybrid system. When the hybrid system was used, the peritoneal cavity was observed when the hybrid system was used in vivo, probably due to limited drug exposure and severe systemic toxicity. Therefore, new drug delivery systems that can realize prolonged and preferential drug accumulation in HCC tumor are still of urgent need.
supramolecular peptide hydrogels in controlling drug release is not compromised. These hydrogels could be successfully prepared with peptides of different molecular weights ranging from >100 kDa elastin-like peptides to small dipeptide and tripeptide. Peptides of different secondary structures including coiled coil, hairpin, and β-sheet, as well as enzyme-responsive groups, are all able to form hydrogels. Regardless of their difference in composition and secondary structure, most of these peptide hydrogelators self-assembled into one-dimensional fibrils, which further entangled with each other through salt bridge, hydrogen bond, and electrostatic attraction to form three-dimensional networks and therefore hydrogels. Additional functional groups such as targeting ligands, enzyme-responsive groups, drugs, fatty acids, DNA, chelator, fluorophores, etc. can be integrated into peptide gelators without disturbing their capability in hydrogel formation. These peptide-based hydrogels have found broad biomedical applications in areas, such as tissue engineering, drug delivery, and imaging.

In our previous study, an injectable peptide amphiphile-based hydrogel, namely C16-GNNQQNYKD-OH (C16-N), has been identified, and the losartan-loaded hydrogel showed sustained drug release after intratumoral injection with potent activity in cancer-associated fibroblast inhibition. Given its biocompatibility and sustained drug release in the site of injection, herein, we describe the influence of triptolide encapsulation on the physicochemical properties of the hydrogel, which was determined, and sustained drug release from the hydrogel was monitored in vitro and in vivo. The anti-tumor activity and toxicity were also evaluated on HCC cells and normal hepatic cells, and on orthotopic mice model. In vivo, C16-N/T significantly retarded the growth of the tumor and prolonged the survival of mice during the experiment. Our findings highlight the importance of spatiotemporal control of drug exposure in maximizing efficacy and minimizing side effects, and indicate that supramolecular peptide hydrogel could be a feasible drug delivery system for drugs against HCC.

2. Materials and methods

2.1. Materials and animals

Fmoc-Asp(OtBu)-Wang resin and Fmoc-protected amino acids were all obtained from GL Biochem (Shanghai, China). Other reagents used in solid-phase peptide synthesis such as N,N-diisopropylethylamine, O-benzotriazole-N,N,N,N-tetramethyluronium trifluoroacetic acid, hexafluorophosphate, 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) and trisopropylsilane were purchased from J&K (Shanghai, China). Triptolide (>98%) was purchased from Desite Biotechnology Co., Ltd. (Chengdu, China). 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG) was acquired from A.V.T (Shanghai) Pharmaceutical Co., Ltd. Alexa Fluor 594-conjugate AffiniPure Donkey Anti-Rabbit IgG was purchased from Jackson ImmunoResearch Inc. (West Grove, USA). F4/80 (D4C8V) XP® Rabbit mAb was bought from Cell Signaling Technology Inc. (Boston, USA). DiR and DiI dyes were obtained from Meilun Biotech (Dalian, China). EPIGLuS (OB glue) were obtained from MEYER-HAAKE GmbH (Germany). (S)-4,4-Dihydro-2-(6-hydroxy-2-benzothiazolyl)-4-thiazolecarboxylic acid potassium (o-Luciferin) was bought from Yeasen (Shanghai, China). Unless noted otherwise, all other reagents were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Luciferase transfected HCC cell line Bel-7402/Luc and human fetal hepatocyte cell line L-02 were provided by Shanghai Cell Resource Center of Shanghai Institute for Biological Sciences, Chinese Academy of Sciences (CAS, China). Both cell lines were cultured with RPMI 1640 Medium (Invitrogen, Carlsbad, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, USA) and 1% Penicillin–Streptomycin solution (Meilun Biotech, Dalian, China). Cells were kept in a humidified incubator containing 5% CO2 at 37 °C.

Female Balb/c nude mice (18–20 g) were purchased from Shanghai Experimental Animal Center (Shanghai, China). All animal experiments were approved by the Institutional Animal Care and Use Committee of the Shanghai Institute of Materia Medica, Chinese Academy of Sciences (China).

2.2. Peptide synthesis

Peptide amphiphile C16-N was synthesized and purified according to our reported procedures. Mass spectrometry and analytical high-performance liquid chromatography (HPLC) were used to confirm the molecule mass and purity of the obtained material, respectively.

2.3. Triptolide encapsulation

A co-assembly method was used to encapsulate triptolide into the hydrogel. Briefly, C16-N (5 mmol/L) was mixed with triptolide in HFIP at different molar ratios (2:1, 5:1, 10:1, 25:1, and 50:1). HFIP was then removed under vacuum, and the remaining was rehydrated with deionized water (~1 mmol/L in peptide amphiphile, pH 7.4) under sonication. The solutions
were allowed to age overnight before centrifugation (12,000×g, 5 min, 4 °C), and the supernatants were transferred into pre-weighted vials for lyophilization. The amounts of encapsulated triptolide in the reconstituted lyophylisates were determined by HPLC (Waters, Milford, USA) equipped with an XBridge™ BEH130 C18 column (5 μm, 150 mm × 4.6 mm), which was eluted by a mixture of water and acetonitrile (flow rate: 1 mL/min; acetonitrile gradient: from 10% to 90% over a period of 17 min; wavelength: 220 nm). The drug loading (DL) and encapsulation efficiency (EE) was calculated with Eq. (1) and Eq. (2):

\[
DL (\%) = \frac{W_{\text{recovered drug}}}{W_{\text{recovered lyophilisate}}} \times 100
\]

(1)

\[
EE (\%) = \frac{W_{\text{recovered drug}}}{W_{\text{added drug}}} \times 100
\]

(2)

2.4. Transmission electron microscopy (TEM)

C16-N or C16-N/T hydrogel (C16-N: 10 mmol/L) was instantly diluted to 500 μmol/L before sample preparation. Seven microliters of C16-N or C16-N/T solution (500 μmol/L in water) was deposited onto a carbon-film-coated copper grid. Excess solution was removed using filter paper to form a thin layer, and the sample was negatively stained using uranyl acetate (2% w/v). After being air dried at room temperature for 3 h, the samples were imaged on an FEI Tecnai 12 TWIN electron microscope (120 kV) accompanied with an SIS Megaview III wide-angle CCD camera.

2.5. Circular dichroism (CD)

To understand whether the secondary structure of C16-N was affected by drug encapsulation, CD spectra (190–400 nm) of C16-N/T (500 μmol/L in water, 50:1 C16-N-to-triptolide), C16-N (500 μmol/L in water) and free triptolide (10 μmol/L in methanol) were recorded on a JASCO J-810 spectropolarimeter (Japan), using water or methanol as references, respectively. The corrected signal was then converted from ellipticity (mdeg) to mean molar ellipticity (deg cm²/dmol).

2.6. Hydrogel formation

Hydrogel was prepared by mixing 9 parts of C16-N or C16-N/T (>10 mmol/L in peptide amphiphile in water, pH 7.4) with one part of 10 × PBS through vortex. The hydrogel formation and shear-thinning property of the mixture were determined using inverted-vial test. The effects of salt, drug encapsulation and shear force on the viscosity of the hydrogels were investigated with Anton Paar MCR 101 rheometer (Anton PaarTrading Co., Ltd., Shanghai, China) equipped with a stainless-steel parallel plate measuring system (50-mm plate diameter).

2.7. Drug release

Release profiles of triptolide from C16-N/T hydrogel and drug-loaded DSPE-PEG micelles (DSPE-PEG/T) were determined using a transwell-based protocol, as we reported previously. C16-N/T (50:1 or 25:1 C16-N-to-triptolide, pH 7.4, 2.5% w/v in PBS) was prepared as described above, and DSPE-PEG micelles with the same drug loading were prepared and used as control. Then, 500 μL of each sample was loaded into the donor inserted cells (n = 3 for each group), and allowed to release the encapsulated drug into 1 mL incomplete 1640 medium in the receptor cells (37 °C). The medium in the receptor cells was refreshed at 1, 2, 4, 8, 12 and 24 h in the first day, and the refreshed every day until day 14. The amount of released triptolide in the medium was monitored using HPLC, and the molecular weight of the released triptolide was confirmed with liquid chromatography—mass spectrometry (LC—MS, 6545 Q-TOF, Agilent, Santa Clara, USA). The cumulative triptolide release was plotted against the release time.

To test whether the hydrogel could also control the release of macromolecules, 25 ng Alexa Fluor® 594-labeled IgG was mixed with 50 mg C16-N to form antibody-loaded hydrogel (pH 7.4, 2.5% w/v in PBS containing 0.025% sodium azide, w/v). The transwell-based protocol described above was used, and 12.5 ng/mL antibody solution was used as a control. Half milliliter of each sample was placed into FBS pre-soaked donor inserted cells (n = 3), and the released antibody was collected in receptor cell containing 1 mL 1640 medium (1% FBS, v/v). The release medium was refreshed at 1, 2, 4, 8, 24 h on the first day, and every two days after that until day 14. The amount of antibody was determined via a multimode plate reader (Enspire PerkinElmer, Fremont, USA). The cumulative antibody release was plotted against time of release.

2.8. In vitro cytotoxicity and uptake

We first investigated the cytotoxicity of free triptolide on Bel-7402/Luc and L-02 cells, respectively. Briefly, Bel-7402/Luc or L-02 cells were seeded at 6 × 10³ cells/well in 96-well plates for 24 h, respectively. Cells were then incubated with fresh medium containing various concentrations of triptolide (0.0001, 0.001, 0.01, 0.02, 0.1, 0.2, 1 and 2 μmol/L) for 72 h. The cytotoxicity of the drug was determined using an MTT-based method, according to the manufacturer’s protocol.

To test the efficacy of triptolide released from either C16-N/T or DSPE-PEG/T, Bel-7402/Luc or L-02 cells were incubated with diluted (2000 ×) release medium collected at 2 h, day 1, 3 and 11 during release experiment. Dilution was conducted because more drugs were used in the release study than in efficacy study, in order to be quantified using HPLC. The cytotoxicity of triptolide was determined using an MTT-based method 72 h after incubation.

To explore the cellular uptake of the released drug by Bel-7402/Luc or L-02 cells, DiI-labeled C16-N hydrogel and DSPE-PEG micelles were prepared, and an in vitro release experiment was conducted under similar conditions as described above. Samples collected at 2 h, day 1, 3 and 11 were incubated 12 h with either Bel-7402/Luc or L-02 cells that had been seeded at 1.5 × 10⁵/well in 12-well plates 24 h before the experiment. Intracellular accumulation of DiI was qualitatively observed with an inverted fluorescence microscope attached to a monochromatic CCD camera (Olympus, Tokyo, Japan), and quantitatively determined using Flow cytometry (Calibur, BD Biosciences, Franklin lake, USA).

2.9. Abdominal retention and biodistribution of the hydrogel

Nude mice were assigned into two groups (n = 3), receiving either DiR-labeled C16-N hydrogel (C16-N/DiR, 19 mmol/L) or DSPE-PEG micelle of similar concentration (DSPE-PEG/DiR, 2 mg/kg DiR) through intraperitoneal injection (i.p.). The abdominal retention of either hydrogel or micelle was monitored and analyzed using an IVIS Spectrum imaging system.
RFItreatment

post-operation. With medical OB glue. After 1 min, the cut was closed with 5-0 swab, and then one tumor block was glued onto the rubbed surface. Length was made on the abdominal wall of the mice to expose the abdomen was disinfected with 75% ethanol, and a cut of 1 cm in sterile table with surgical film in supine position. The skin of the mice was anesthetized with sodium pentobarbital sulfate (i.p., 50 mg/kg), and then fixed on a agar system. The DiR in these organs was then extracted using methanol and determined with a fluorospectrometer (PerkinElmer, Fremont, USA). The fluorescence intensity was then plotted against the concentration of DiR in the tissues.

2.10. Orthotopic HCC mice model

Bel-7402/Luc cells (10⁷ cells in 100 µL) were injected subcutaneously into nude mice, and the tumors were harvested when the diameters reached 0.5 cm. The tumors were cut into small blocks (1 mm × 1 mm × 1 mm) in sterilized PBS containing 1% penicillin–streptomycin. The tumor blocks were kept on the ice and used within 1 h. Healthy nude mice were anesthetized with sodium pentobarbital sulfate (i.p., 50 mg/kg), and then fixed on a sterile table with surgical film in supine position. The skin of the abdomen was disinfected with 75% ethanol, and a cut of 1 cm in length was made on the abdominal wall of the mice to expose the left lobe of the liver. The liver was washed softly with a cotton swab, and then one tumor block was glued onto the rubbed surface with medical OB glue. After 1 min, the cut was closed with 5-0 suture line and disinfected with iodophor with an additional penicillin injection (i.m.). The mice were kept warm and monitored closely until conscious. Routine sterile feeding was taken post-operation.

To monitor the growth of tumors, α-Luciferin was injected through tail vein (15 mg/mL, 200 µL, i.v.), and the bioluminescence from the tumor was monitored with IVIS Spectrum imaging system at day 13 and day 45 after implantation. The livers were then dissected for further imaging and histological examination.

2.11. Anti-tumor efficacy

To determine the growth profiles of orthotopic HCC after different treatments, animals developed orthotopic HCC (confirmed with IVIS Spectrum imaging system 10 days after implantation) were divided into 5 groups (n = 3): C16-N/T (i.p.), DSPE-PEG/T (i.v.), DSPE-PEG/T (i.p.), C₁₀-N (i.p., 180 mg/kg), and PBS (i.v.), and then dissected for further imaging and histological examination. The tumor inhibition rate (TIR) of treatment at the end of the experiment was calculated based on the following Eq. (3):

\[
\text{TIR} \% = \left(1 - \frac{R_{\text{TIR}}}{R_{\text{PBS}}} \right) \times 100
\]

In a separate experiment, the survivals of mice bearing orthotopic HCC were also monitored after a single injection with C₁₀-N/T (i.p.), DSPE-PEG/T (i.v.), DSPE-PEG/T (i.p.), C₁₀-N (i.p., 180 mg/kg), or PBS (i.v.) at 1 mg/kg triptolide (n = 8 for each group). The body weights of the mice were monitored for 70 days.

2.12. Serum biochemical parameters and histological study

To evaluate the safety of C₁₀-N/T and other formulations, healthy nude mice receiving C₁₀-N/T (i.p.), DSPE-PEG/T (i.v.), DSPE-PEG/T (i.p.), C₁₀-N (i.p.), or PBS (i.v.) were killed to harvest orthotopic HCC were also monitored after a single injection with C₁₀-N/T (i.p.), DSPE-PEG/T (i.v.), DSPE-PEG/T (i.p.), C₁₀-N (i.p., 180 mg/kg), or PBS (i.v.) at 1 mg/kg triptolide (n = 8 for each group). The activities of alanine transaminase (ALT) and aspartate transaminase (AST) and the concentration of blood urea nitrogen (BUN) and creatinine (CRE) in the serum were determined and compared. The major organs were fixed, dehydrated, embedded in paraffin blocks, sectioned, and stained with hematoxylin and eosin (H&E) for histological analysis. Images were captured using an inverted fluorescence microscope attached to a monochromatic CCD camera (IX83, Olympus, Japan).

2.13. Statistical analysis

Each experiment was conducted at least in triplicate, and the data were given as mean ± SD. Data were analyzed using either two-tailed student’ t-test or one-way analysis of variance (ANOVA) using GraphPad Prism 6.0 software to assess the significance of the difference. Statistical differences were defined as significant when P < 0.05.

3. Results and discussion

3.1. Co-assembly and characterization of C₁₀-N/T

C₁₀-N was synthesized, purified and characterized according to our previously reported method. The obtained material was of high purity (>95%) with a correct molecular weight (Supporting Information Fig. S1). The co-assembly strategy was used for triptolide encapsulation, and the influence of C₁₀-N-to-triptolide ratio on the DL and EE of C₁₀-N/T was explored. The DL of C₁₀-N/T increased with increasing triptolide feeding (up to 11%), but the EE dropped significantly meanwhile (Fig. 2A). Quantitative encapsulation was achieved at C₁₀-N-to-triptolide ratio 50:1 (mol/mol) with DL of 1.19 ± 0.07% (in weight, n = 3). This C₁₀-N/T was chosen for further experiments, as we have previously observed that high DL could alter the morphology of self-assembled nanofiber and possibly disturb gelation. The C₁₀-N/T nanofiber solution was similar to that of C₁₀-N, showing α-sheet conformation as those in C₁₀-N, and triptolide co-assembled in PBS into nanofibers of 11.0 ± 1.5 nm (n = 80) in diameter (Fig. 2B and Supporting Information Fig. S2A), without significant differences in neither the morphology nor diameter when compared with the nanostructures formed by C₁₀-N alone (Fig. S2B). The CD spectrum of C₁₀-N/T nanofiber solution was similar to that of C₁₀-N, showing a negative signal in 216 nm and a positive signal between 285 and 300 nm (derived from tyrosine residues) (Fig. 2C). This result suggested that the peptide segments in C₁₀-N/T nanofibers adopted β-sheet conformation as those in C₁₀-N, and triptolide encapsulation did not affect the molecular packing of C₁₀-N molecules significantly.
Given that C16-N/T adopted filamentous morphology, they also formed a hydrogel at high concentration only at the presence of salts with a viscosity at $w_0 \approx 0.2$ Pa·s (Supporting Information Fig. S3). A significant drop in the viscosity was observed after a 5 min stirring (Fig. S3), confirming the shear-thinning properties of the hydrogel we observed previously. The release profile of triptolide from C16-N/T was determined using a Transwell-based methodology. The result showed that the encapsulated triptolide released completely after a 14-day incubation period without obvious burst release, but the rate of drug release declined as prolonged incubation (Fig. 2D). In sharp contrast, DSPE-PEG encapsulated triptolide released in 1 day (Fig. 2D). The effect of DL on drug release was also explored, and similar release profiles were observed from different C16-N/T (DL $= 1.22\%$ and $2.15\%$) (Supporting Information Fig. S4). To verify the stability of released triptolide after prolonged incubation, the molecular mass of released drug at day 6 and day 14 was further confirmed with LC-MS. The peak associated with the released drug showed similar retention time as triptolide with correct molecular mass (Supporting Information Fig. S5), indicating that the triptolide was intact through a 14-day period. In addition to hydrophobic molecules, the supramolecular nature of the C16-N/T allowed feasible entrapment of biomacromolecules within the hydrophilic network formed by C16-N nanofibers through simple mixing procedure. Sustained release of model antibody, Alexa Fluor 594-labeled IgG, from C16-N-based hydrogel was achieved over a period of 14 days with a burst release phase in the first 8 h, while the complete release was achieved in 1 day for free antibody (Supporting Information Fig. S6). In both cases, most hydrogels retained in the donor chamber at the end of experiments, suggesting that the encapsulated triptolide and entrapped antibody were released mainly through a diffusion-dependent mechanism rather than an erosion-dependent one. The concentration of the encapsulated drug in the nanofibers and its aqueous solubility determined the rate of drug diffusion out of the nanofibers, which explained the decelerated triptolide release as prolonged incubation and faster release of losartan than triptolide from the same nanofibers. Regardless of the exact mechanism of drug release, the results here clearly demonstrated that C16-N-based hydrogel could be an injectable depot for both hydrophobic drugs and biomacromolecules.

3.2. In vitro study of hydrogel

We presumed that sustained drug release could allow prolonged drug exposure at tolerable concentration. To test this hypothesis, we monitored the drug uptake and viability of human HCC cell Bel-7402/Luc and human normal liver cell L-02 after their incubation with the release mediums collected at different time points during in vitro release experiments using DiI-labeled C16-N (C16-N/DiI) or C16-N/T. Significant and consistent red fluorescence was observed in Bel-7402/Luc cells that were incubated with the release mediums collected at 2 h, day 1, 3 and 11 from C16-N/DiI, while noticeable red fluorescence was only recorded in the cells exposed to the release mediums collected at 2 h and day 1 from DiI-labeled DSPE-PEG (DSPE-PEG/DiI) (Fig. 3A). A similar trend was observed in L-02 cells after incubation with different release mediums, but the fluorescence signals were lower (Fig. 3A). These results were further confirmed quantitatively by flow cytometry analysis (Fig. 3B). The viabilities of cells treated

![Figure 2](image1.png)

**Figure 2** Characterization of C16-N/T. (A) DL and EE of C16-N/T prepared at different C16-N-to-triptolide ratios. (B) Representative TEM image of C16-N/T. (C) CD and UV–VIS spectra of C16-N (500 μmol/L in PBS), C16-N/T (500 μmol/L C16-N and 10 μmol/L triptolide in PBS), and triptolide (10 μmol/L in methanol). (D) Cumulative release of triptolide from C16-N/T and DSPE-PEG/T over 14 days. Data were presented as the mean ± SD ($n = 3$).

![Figure 3](image2.png)

**Figure 3** Cell uptake and cytotoxicity of C16-N/T in Bel-7402/Luc and L-02 cells. Fluorescence images (A) and flow cytometry analysis (B) of Bel-7402/Luc and L-02 cells after 12 h incubation with release mediums collected from C16-N/DiI or DSPE-PEG/DiI (Red) at 2 h, day 1, day 3, and day 11. The cell nucleus were stained with Hoechst 33342 (Blue). The scale bars represent a distance of 50 μm. Data were presented as the mean ± SD ($n = 3$).
with diluted release mediums for 72 h were then determined using an MTT-based method. The growth of Bel-7402/Luc cells was inhibited by ~50% after treated with diluted release mediums (2 h, day 1, 3 or 11) from C16-N/T, while the viabilities of L-02 cells were much less affected (Fig. 3C). On the contrary, the release medium collected at 2 h from DSPE-PEG/DiR was toxic to both Bel-7402/Luc and L-02 cells, and those collected at day 1, 3 or 11 were of no activity on both cells (Fig. 3C). In both cases, the activity of released triptolide (20–60 nmol/L) was comparable to that of free triptolide at the same concentration (Supporting Information Fig. S7), confirming that the encapsulated triptolide was released in vivo using IVIS spectrum imaging system, and DSPE-PEG/DiR was used as control. The shear-thinning property of the hydrogel was first examined, and fast regelation after vortex and needle injection was observed (Fig. 4A and Supporting Information Fig. S8). After intraperitoneal injection, prolonged retention of C16-N/DiR in the abdomen of the mice was observed, but no significant signal was observed in mice receiving DSPE-PEG/DiR 3 days after the injection (Fig. 4B). Relative fluorescence intensity (RFI) change was further semi-quantified, and the results showed that ~40% of injected DiR remained in the C16-N/DiR treated mice in sharp contrast with <10% in DSPE-PEG/DiR treated ones at the end of experiment (Fig. 4C). The low fluorescence intensity in C16-N/DiR treated mice at day 0 and day 1 was resulted from fluorescence quenching (Fig. S8).

### 3.3. Peritoneal retention and distribution of C16-N/T hydrogel

The behavior of the hydrogel after intraperitoneal injection may be different from that under in vitro conditions, due to the movement of animals and internal organs. We, therefore, monitored the intraperitoneal retention and distribution of C16-N/DiR in vivo using IVIS spectrum imaging system, and DSPE-PEG/DiR was used as control. The shear-thinning property of the hydrogel was first examined, and fast regelation after vortex and needle injection was observed (Fig. 4A and Supporting Information Fig. S8). After intraperitoneal injection, prolonged retention of C16-N/DiR in the abdomen of the mice was observed, but no significant signal was observed in mice receiving DSPE-PEG/DiR 3 days after the injection (Fig. 4B). Relative fluorescence intensity (RFI) change was further semi-quantified, and the results showed that ~40% of injected DiR remained in the C16-N/DiR treated mice in sharp contrast with <10% in DSPE-PEG/DiR treated ones at the end of experiment (Fig. 4C). The low fluorescence intensity in C16-N/DiR treated mice at day 0 and day 1 was resulted from fluorescence quenching (Fig. S8).

**Figure 4** Retention of C16-N hydrogel in vivo. (A) Shear-thinning property of C16-N/T. Representative fluorescence images (B) and semi-quantitative analysis of RFI changes (C) of mice receiving either C16-N/DiR or DSPE-PEG/DiR (2 mg/kg DiR) captured at day 0, 1, 3, 5, 11 and 13 after intraperitoneal injection. The total fluorescence intensity was normalized to the highest fluorescence intensity while plotted against time. Data were presented as the mean ± SD (n = 3).

The results confirmed that the injectable hydrogel could also prolong drug exposure in vivo. Longer drug retention was observed in vivo than in vitro, probably due to the limited fluid volume in the intraperitoneal cavity and high affinity of release drugs for the tissues.

We then explored whether prolonged drug exposure after hydrogel injection (i.p.) could lead to higher tissue selectivity of the drug. Therefore, the accumulation of DiR in the major organs and blood of C16-N/DiR-treated mice was monitored and semi-quantified using IVIS spectrum imaging system, and mice receiving DSPE-PEG/DiR via intravenous or intraperitoneal injection were used as controls. We found that DiR mainly accumulated in the liver and maintained a stable concentration in mice treated with C16-N/DiR (Supporting Information Fig. S9). The distribution of DSPE-PEG/DiR, on the contrary, showed no obvious tissue specificity and a relative fast clearance when administrated through either intravenous or intraperitoneal injection (Fig. S9). However, the maximal drug exposure in the liver increased and the time-to-peak elongated when DSPE-PEG/DiR was delivered via intraperitoneal injection compared with intravenous injection (Fig. S9). The average fluorescence intensities in different organs were semi-quantified and analyzed using organs with known DiR concentration as standards (Supporting Information Fig. S10). The fluorescence intensity change in the liver against time was plotted (Fig. 5A), and the time to reach highest fluorescence intensity (T_{max}) and the areas under the curve (AUC_{0–13}) were calculated (Supporting Information Table S1). DSPE-PEG/DiR (i.v.) and DSPE-PEG/DiR (i.p.) showed comparable AUC_{0–13}, while the AUC_{0–13} of C16-N/DiR was slightly lower as a considerable amount of DiR maintained in the liver at day 13 (Fig. 5A). Tissue-to-liver fluorescence intensity ratios were calculated (0–6 days), and mice treated with C16-N/DiR showed ~2-fold lower heart-to-liver, lungs-to-liver, kidneys-to-liver and blood-to-liver ratios, and slightly higher spleen-to-liver ratio, when compared with mice treated with DSPE-PEG/DiR (Fig. 5B–F). The accumulation of DiR in the spleens of C16-N/DiR-treated mice might be associated with lymphatic clearance of nanofibers and subsequent phagocytosis by macrophages or dendritic cells which could accumulate in the lymph nodes and spleens. These results indicated that C16-N hydrogel (i.p.) could deliver encapsulated cargos preferentially to the liver and maintain a steady concentration there for maximized efficacy and minimized side effects.

### 3.4. Anti-tumor efficacy of C16-N/T hydrogel

The anti-tumor activity of C16-N/T was evaluated on orthotopic HCC mouse model which was more clinically relevant. The mice model established through liver transplantation of Bel-7402/Luc tumor block could be easily monitored using bioluminescence imaging using the IVIS spectrum imaging system with acceptable variance among animals (Supporting Information Fig. S11). The anti-tumor efficacy of C16-N/T (1 mg/kg in triptolide) in the model mice was first monitored using IVIS spectrum imaging system over 2 weeks, and PBS (i.v.), DSPE-PEG/T (i.p. or i.v.), and C16-N (i.p.) at the same dosage were used as control (Fig. 6A and Supporting Information Fig. S12). It was found that the bioluminescence signals increased in mice receiving PBS (i.v.), C16-N (i.p.), DSPE-PEG/T (i.p.) and DSPE-PEG/T (i.v.), but the signals decreased in mice treated with C16-N (i.p.) (Fig. 6B). In comparison with PBS, the TIR of C16-N (i.p.) and DSPE-PEG/T (i.v.) were 99.7 ± 0.1% and...
These results were confirmed by further anatomic examination and histological analysis at the end of the experiment (Fig. 6B). Among these triptolide-containing treatments, C16-N/T (i.p.) was the most effective compared with other treatments ($P < 0.05$), followed by DSPE-PEG/T (i.v.) and DSPE-PEG/T (i.p.) (Fig. 6C). Given that DSPE-PEG/T (i.p. or i.v.) have higher AUC0−13 than C16-N/T (i.p.) in the liver (Table S1), these results suggested that the span of time was more important than the extent of amount of drug exposure for HCC treatment when effective concentration was reached. Indeed, no significant difference was observed between the mice treated with C16-N/T or DSPE-PEG/T at day 7 (Fig. 6C), before which time an effective drug exposure in the liver maintained (Fig. 5A). In the second week, an effective concentration of drug maintained only in the liver of mice receiving C16-N/T hydrogel (Fig. 5A), which led to superior activity in inhibiting the growth of orthotopic tumors.

To further investigate the long-term efficacy of C16-N/T hydrogel, a survival experiment was performed with the same control groups ($n = 8$ for each group). C16-N/T hydrogel was the most effective among all the treatments, and the median survival times were 43, 24, 22, 22, and 19 days for C16-N/T (i.p.), DSPE-PEG/T (i.v.), DSPE-PEG/T (i.p.), C16-N (i.p.), and PBS (i.v.) treated mice, respectively (Fig. 7A). In the first 2 weeks after drug administration, body weight loss >10% was not observed in mice except those receiving DSPE-PEG/T (i.v., 12.54%) (Fig. 7B). The body weight loss observed after that was usually associated with tumor progression. The results here were in consistent with the bioluminescence imaging result (Fig. 6), showing that C16-N/T (i.p.) was the most effective treatment.
in treating orthotopic HCC-bearing mice. The observed body weight loss of mice receiving DSPE-PEG/T (i.v.) indicated that the treatment had significant side effects on major organs due to high drug exposure (Fig. 5 and Fig. S9), which might explain why tumor growth inhibition did not translate into survival benefit in mice receiving DSPE-PEG/T (i.v.) (Figs. 6 and 7).

3.5. Safety of the C₁₆-N/T hydrogel

To further investigate the in vivo safety of C₁₆-N/T, the histological change in the major organs and blood biochemical parameters were examined on healthy Balb/c nude mice at day 4 after receiving different treatments. No obvious histological changes were observed in the heart, spleen and lungs of mice receiving any of the treatments (Supporting Information Fig. S13). However, liver tissue necrosis and inflammation were observed in one of the three mice receiving DSPE-PEG/T (i.v.) (Fig. 8A). Further blood biochemical parameter analysis showed that mice receiving DSPE-PEG/T (i.v.) or DSPE-EPG/T (i.p.) had elevated activities of ALT and AST when compared with those receiving PBS, C₁₆-N, or C₁₆-N/T (Fig. 8A). The kidneys of the mice were also analyzed, and histological change in the renal corpuscles was observed in all of the three mice receiving DSPE-EPG/T (i.p.) but not in the rest mice, with widened corpuscle space and glomerulus necrosis (Fig. 8B). In agreement with this result, elevated CRE was recorded only in mice receiving DSPE-EPG/T (i.p.) (Fig. 8B). Elevated BUN was observed in all other groups compared with PBS-treated mice, and thus might not be associated with triptolide treatment (Fig. 8B). The results here demonstrated that C₁₆-N/T hydrogel (i.p.) was better tolerated than DSPE-PEG/T, which was resulted from its prolonged drug release and liver specific accumulation (Figs. 4 and 5).

4. Conclusions

In this study, we explored the potential of C₁₆-N hydrogel as an intraperitoneal depot for triptolide for the treatment of orthotopic HCC. We showed that triptolide could be quantitatively loaded into C₁₆-N nanofibers, and drug encapsulation did not affect self-assembly and gelation. Triptolide released from C₁₆-N/T hydrogel sustainedly, and showed higher cytotoxicity against HCC cells than normal hepatocytes. After intraperitoneal injection, the hydrogel retained in the peritoneal cavity for more than 2 weeks, with preferential accumulation in the liver. The C₁₆-N/T hydrogel significantly inhibited the growth of orthotopic HCC growth, and doubled the median survival time of tumor-bearing mice without noticeable side effects to major organs. Our findings clearly demonstrated that C₁₆-N/T hydrogel has a strong potential in HCC chemotherapy and combined therapy.
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Appendix A. Supporting information

Supporting data to this article can be found online at https://doi.org/10.1016/j.apsb.2019.06.001.

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