Colloids and Surfaces B: Biointerfaces 169 (2018) 273–279

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Glutathione-triggered dual release of doxorubicin and camptothecin for highly efficient synergistic anticancer therapy

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ARTICLE INFO

Keywords:
Drug delivery
Stimuli-response
Glutathione-response
Co-delivery
Nanomedicine

ABSTRACT

An amphiphilic biodegradable prodrug (PLG-*g*-mPEG/CPT) was synthesized by conjugating disulfide-containing camptothecin (CPT) to poly(L-glutamic acid)-*graft*-methoxy poly(ethylene glycol) (PLG-*g*-mPEG) through esterification reaction. The amphiphilic prodrugs could self-assemble into micellar nanoparticles and encapsulate doxorubicin (DOX) in aqueous solution at pH 7.4. The treatment of the nanoparticles with reducing glutathione (GSH) at cytosolic concentration (10 mM) significantly promoted the *in vitro* dual release of DOX and CPT from the micelles. The results of flow cytometry (FCM) and confocal laser scanning microscopy (CLSM) manifested that the intracellular release of DOX and CPT from the micelles was enhanced by increasing the intracellular GSH level. Consistently, the MCF-7 cell killing mediated by the micelles was also intracellular GSH concentration-dependent. The low combination index (CI) value of < 0.3 demonstrated the high synergistic effect of DOX and CPT co-delivered by the nanoparticles in tumor cell killing. Therefore, this GSH-triggered dual release drug delivery system is a promising strategy for combination cancer therapy.

1. Introduction

Chemotherapy is a widely used method for cancer therapy. However, small-molecule drugs usually suffer from short blood circulation time and present severe side effects due to their lack of selectivity towards cancer cells from the healthy cells [1,2]. To cope with these challenges, polymeric prodrugs have been synthesized by conjugating small-molecule drugs to polymers to improve the drug bioavailability and reduce the adverse effects [3–6]. However, low drug loading content (DLC) of polymeric prodrugs may require increased amount of conjugates to be applied, resulting in problems of cost and dose regime [7,8]. Furthermore, high molecular weight (30–50 kDa) is necessary for the polymeric prodrugs to avoid being eliminated by kidney, and this poses limitations of the polymer selection [2,9].

Another alternative to improve chemotherapy is applying nanoparticle-based drug delivery systems (DDS) such as liposomes and polymeric micelles, which can more efficiently encapsulate one or more kinds of chemotherapeutic drugs by physical interaction [10–12]. The increased size of the drug-loaded nanoparticles over small-molecule drugs can reduce renal filtration; meanwhile, modification of the nanoparticle surface with hydrophilic polymer such as polyethylene glycol (PEG) is supposed to decrease opsonization and recognition by the reticuloendothelial system (RES), thus prolonging the systemic circulation time and promoting the accumulation of anticancer drugs in the solid tumor sites by enhanced permeability and retention (EPR) effect, consequently minimizing the adverse effects [10,12,13]. However, inefficient drug release from the drug-loaded nanoparticles after internalization by cancer cells may significantly reduce the anticancer efficacy [14,15]. Therefore, development of stimuli-responsive smart nanoparticles to obtain “on demand” precise drug release has attracted extensive attention these years [16–18]. Various external and internal stimuli, such as pH, redox status, temperature, light, enzyme activity and small molecules have been exploited to design stimuli-responsive DDS [19–21]. Among these options, reductive cytosolic circumstance containing reducing GSH (2–10 mM) are ideal candidates for the development of intracellular stimuli-responsive DDS because of its significant differences from the extracellular GSH concentration (2–20 μM), benefiting from which the GSH-responsive nanoparticles can keep stable in the extracellular environment and release the loaded drugs after cellular internalization [21–24].

Besides, application of single chemotherapeutic drug for cancer therapy may induce the development of drug resistance and presents

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https://doi.org/10.1016/j.colsurfb.2018.05.025
Received 29 January 2018; Received in revised form 10 May 2018; Accepted 11 May 2018
Available online 17 May 2018
0927-7765/

high toxicity [25–27]. Drug combination therapy has been identified as an effective method to overcome the above challenges while to achieve synergistic effect for cancer therapy [28–30].

Taken together, we synthesized an amphiphilic GSH-responsive CPT prodrug (PLG-*g*-mPEG/CPT) using biodegradable PLG-*g*-mPEG copolymer as a backbone. CPT was coupled to PLG-*g*-mPEG by disulfide bonds. These linkages are supposed to be cleaved in reductive environment. Then, CPT-prodrug micelles were prepared by the self-assembly of PLG-*g*-mPEG/CPT during which doxorubicin (DOX) was simultaneously encapsulated into the nanoparticles by electrostatic and hydrophobic interactions. Our results showed that the prepared nanoparticles presented GSH-responsive dual drug release manner and mediated effective tumor cell killing with high synergistic effect of the co-delivered DOX and CPT.

2. Experimental section

2.1. Materials

Camptothecin and doxorubicin were bought from Beijing HVSF United Chemical Materials CO., LTD., China. Methoxy poly(ethylene glycol) (mPEG, M_n = 5000 g/mol) was purchased from Sigma-Aldrich. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-H-tetrazolium bromide (MTT) was bought from Sigma-Aldrich. Dulbecco's Modified Eagle's Medium (DMEM, GIBCO) and fetal bovine serum (FBS, GIBCO) were purchased from Invitrogen (Carlsbad, CA). CPT-S-S-OH [17] and PLG [31,32] was synthesized as previously reported. All the other reagents and solvents were purchased from Beijing Dingguo Changsheng Biotechnology Co. Ltd., China and used as received.

2.2. Measurements

Dynamic light scattering (DLS) measurements were performed on a WyattQELS instrument with a vertically polarized He–Ne laser (DAWN EOS, Wyatt Technology). The scattering angle was fixed at 90°. Transmission electron microscopy (TEM) measurement was performed on a JEOL JEM-1011 transmission electron microscope with an accelerating voltage of 100 kV. A drop of the micelle solution (0.1 g/L) was deposited onto a 230 mesh copper grid coated with carbon and allowed to dry in air at 25 °C before measurements.

2.3. Synthesis of PLG-g-mPEG

In brief, mPEG was coupled to PLG ($M_n = 2.07 \times 10^4$ g/mol, PDI = 1.36) by esterification reaction in a mass ratio of 2:1. The M_n and PDI of the obtained PLG-*g*-mPEG were 3.73×10^4 g/mol and 1.91, respectively. The chemical structure of PLG-*g*-mPEG was characterized by ^{1}H NMR spectroscopy (Bruker AV 300 MHz NMR spectrometer) using CF_3COOD (TFA-*d*) as the solvent.

2.4. Synthesis of PLG-g-mPEG/CPT

PLG-*g*-mPEG (2.0 g) and CPT-S-S-OH (0.40 g) were firstly dissolved in dimethylsulfoxide (DMSO). Then DMAP (0.03 g) was added and the solution was gently stirred for 10 min. Afterward, EDC·HCl (0.30 g) was added and the resulting mixture was stirred at room temperature for 72 h. After that, the mixture was filtered to remove suspended sediments. The polymer solution was dialyzed against DMSO for 24 h and then dialyzed against deionized water for another 48 h. Finally, the copolymer PLG-*g*-mPEG/CPT was obtained as light yellow solid after lyophilization. CPT loading efficiency was studied using a UV–vis spectrophotometer (Shimadzu UV-2401PC) at an absorbance wavelength of 365 nm[7] The chemical structure of PLG-*g*-mPEG/CPT was characterized by ^{1}H NMR spectroscopy using CF_3COOD (TFA-*d*) as the solvent.

2.5. Preparation of DOX-loaded nanoparticles

PLG-*g*-mPEG/CPT (300 mg) was dissolved in 12 mL of DMF. Then, 5 mg of DOX was dissolved in DMF (5 mL) and mixed with the PLG-*g*-mPEG/CPT solution. The mixture was added dropwise to 60 mL of phosphate buffer (PB) solution (pH 7.4) under vigorously stirring for 12 h. Then the resulting solution was dialyzed (MWCO 3500) against deionized water for 8 h. The DOX-loaded nanoparticles were obtained as red powder after lyophilization in the dark.

2.6. Drug loading content

The weighted DOX-loaded nanoparticles were dissolved in DMSO. The UV-absorption at 485 nm (DOX) or 365 nm (CPT) was measured by a PTI fluorescence spectroscope (Photon Technology International, U.S.A.) [7]. The amount of DOX or CPT was determined using a standard calibration curve method. The drug loading contents (DLC) of DOX and CPT were calculated based on the following equations:

DLC (*wt.* %) = (weight of drug in micelles/weight of drug-loaded micelles) × 100%

2.7. In vitro release of DOX and CPT

In vitro DOX and CPT release behaviors from the nanoparticles were investigated in PB at pH 5.3 and 7.4, respectively. The pre-weighed drug-loaded nanoparticles were suspended in 3 mL of PB with or without 10 mM GSH, and the solution was immediately transferred into a dialysis bag (MWCO 3500 Da). The release experiment was initiated by placing the end-sealed dialysis bag in 50 mL of PB with or without 10 mM GSH at 37 °C under continuous shaking at 72 rpm. At predetermined time, 2 mL of released media was withdrawn and refilled with 2 mL of fresh medium. The amount of released DOX or CPT was measured by fluorescence spectrometer using a standard calibration curve method. The release experiments were performed in triplicate.

2.8. Cell culture

Human breast adenocarcinoma cells (MCF-7) were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). MCF-7 were grown in DMEM, supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. All cells were cultured at 37 °C in an incubator with 5% CO_2 and humidified atmosphere.

2.9. Intracellular DOX and CPT release

Intracellular DOX and CPT release from the DOX-loaded nanoparticles was investigated by confocal laser scanning microscopy (CLSM) and flow cytometry (FCM).

For the CLSM, MCF-7 cells were seeded into 6-well plates at a density of 2×10^5 cells per well. After 24 h, the cells for the GSH pretreated group were pretreated with GSH (final concentration 10 mM) for 2 h. Cells were then washed with PBS and incubated with the prepared nanoparticles at a final DOX concentration of 17.2 μM for 3 or 6 h. Cells without pretreatment were used for comparison. Then the cells were washed with PBS for three times, and fixed in 4% paraformaldehyde. After that, the cells were washed thrice with PBS and mounted on microscope slides for imaging by using a CLSM (Zeiss LSM780). Excitation wavelengths were 365 nm and 480 nm for CPT and DOX, respectively. The results were analyzed by ImageJ software. All the cells in each image were selected using the threshold function after the images were converted to grey scale. The total area and integrated intensity of the selected cells were determined using ImageJ. The mean fluorescence intensity of the selected cells was indicated as follows: mean fluorescence intensity of selected cells = integrated intensity of