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Tumor-penetrating codelivery of siRNA and paclitaxel with ultrasound-responsive nanobubbles hetero-assembled from polymeric micelles and liposomes

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ABSTRACT

Drug resistance is a big problem in systemic chemotherapy of hepatocellular carcinoma (HCC), and nanomedicines loaded with both chemotherapeutic agents (e.g. paclitaxel, PTX) and siRNA's targeting antiapoptosis genes (e.g. BCL-2) possess the advantages to simultaneously overcome the efflux pump-mediated drug resistance and antiapoptosis-related drug resistance. However, tumor-penetrating drug delivery with this type of nanomedicines is extremely difficult due to their relatively big size compared to the single drug-loaded nanomedicines. Aiming at address this problem, US-responsive nanobubbles encapsulating both anti-cancer drug paclitaxel (PTX) and siRNA (PTX–NBs/siRNA) for HCC treatment were developed by hetero-assembly of polymeric micelles and liposomes in the present study. Utilizing an external low-frequency US force imposed to the tumor site, effective tumor-penetrating codelivery of siRNA and PTX was achieved *via* tail vein injection of PTX–NBs/siRNA into nude mice bearing human HepG2 xerografts. Consequently, the PTX treatment-inducible antiapoptosis in HepG2 cells was effectively suppressed by the codelivered siRNA targeting an antiapoptosis gene (BCL-2 siRNA) during chemotherapy. Owing to the synergistic anti-cancer effect of two therapeutic agents, tumor growth was completely inhibited using low-dose PTX in animal study. Our results highlight the great potential of this type of US-responsive hetero-assemblies carrying both anti-cancer drug and siRNA as an effective nanomedicinal system for HCC therapy.

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1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant cancers that seriously threatens human health [1]. Surgical approaches including liver resection and liver transplantation are known as the curative treatments for HCC. Unfortunately, a large number of patients lost their opportunity for surgical operations when the cancerization was confirmed. Therefore, chemotherapy still remains a common approach for most of the patients even without sufficient benefits [2].

The first major obstacle limiting the chemotherapeutic outcomes lies in the insufficient drug concentration in tumor tissue. Consequently, high concentration and repeated applications of anti-cancer agents are commonly needed in systematic chemotherapy, which however results in severe side effects [3]. To date, although various nanoparticles (NPs) have been developed to alter the pharmacokinetics of drugs and to improve the therapeutic effects on the basis of enhanced permeability and retention (EPR) effect [4], poor tumor targeting ability still hinders their clinical applications [5]. In addition, after plenty of preclinical and clinical studies of EPR effect for decades, the significant heterogeneity within or between tumor types has been revealed as a formidable barrier for NPs to act more effectively [6,7]. For instance, large amount of drug-encapsulated NPs would be limited perivascularly by the intrinsic high pressure of tumor tissue, resulting in heterogeneous therapeutic effects. Thus, development of drug delivery

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systems that may improve tumor targeting and meanwhile deeply infiltrate tumor tissue is of great importance at present.

Another challenge is that the drug resistance, which is developed from different pathways, is one of the most common reasons for clinical chemotherapy failure. The predominant mechanism for cancer cells to escape from cell death relates to the over-expression of P-glycoprotein (P-gp), which is known as the drug-efflux pump mediated drug resistance [8,9]. Despite decades of endeavor in reducing the efflux-pump mediated drug resistance during the procedure of chemotherapy, the therapeutic index is still often unsatisfactory [10]. In addition, the non-pump related drug resistance induced by up-regulation of some anti-apoptosis genes, which reduces the cellular sensitivity to chemotherapeutic drugs, also remains a big problem [11]. BCL-2 (B-cell lymphoma-2), known as a common anti-apoptotic protein, locates on the outer mitochondrial membrane and suppresses the cytochrome *c* and apoptosis-inducing factor (AIF) releasing into cytoplasm to reduce cancer cell apoptosis [12,13]. Hence, down-regulation of the BCL-2 gene in cancer cells (especially non-P-gp overexpressing ones) during chemotherapy may lead to further breakthrough in reversing drug resistant effects.

On the other hand, acoustic microbubbles (MBs), which are designed as ultrasound (US) contrast agents originally, are now being extended to drug or gene delivery [14]. MBs present within low-frequency US exposed area will oscillate and collapse rapidly, which is called ultrasound targeted microbubble destruction (UTMD) effect and is a principle for increasing the vascular permeability for drug delivery [15]. Nanoscale microbubbles (NBs) with diameter of 300–700 nm are able to pass through the endothelial gaps of defective blood vessels [16]. Under low-frequency US exposure, UTMD effect of NBs occurs simultaneously in the capillary vessels and interstitial space of tumor cells. The intravascular UTMD effect results in higher amount of drug accumulation in tumor due to the US-enhanced permeability of blood vessels, and meanwhile the generation of shock waves, shear forces and microstream from extravascular UTMD effect promotes intratumoral drug delivery to deeper locations far from the vessel endothelium. Hence, anti-cancer agent loaded NBs may be an ideal vector for tumor targeted low-dose drug delivery strategy.

In the previous study, we have successfully fabricated US-sensitive NBs carrying small interfering RNA (siRNA) and obtained remarkable gene silencing efficacy both *in vitro* and *in vivo* [17]. Based on the encouraging results, we prepared US-sensitive NBs which simultaneously encapsulate chemotherapeutic agent PTX and siRNA targeting BCL-2 gene (BCL-2 siRNA) in the present work. It was anticipated that this codelivery system would possess several characteristics leading to improvement of chemotherapy. First, the nanoscale diameter of NBs and the vascular permeability enhancement induced by UTMD effect would increase the drug concentration in tumor tissue. The EPR-based and US-related dual targeting ability would make low-dose drug application and reduction of systemic side effects possible. Second, the NBs-based UTMD effect would be induced by local US exposure of tumor tissue. The high power releasing from the bubble destructions would deliver the encapsulated PTX into deeper locations of tumor tissue. Third, codelivery of PTX and BCL-2 siRNA would overcome drug resistance relevant to the non-pump mechanism by silencing the anti-apoptosis gene BCL-2. To explore these potential, the synergistic therapeutic effects of PTX and BCL-2 siRNA codelivered by NBs were evaluated both *in vitro* and *in vivo* in the current study.

2. Material and methods

2.1. Materials

The phospholipids in powdery form (Avanti Polar Lipids Inc., USA) were used in this study without further purification: 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC; M_w: 734.05), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE; M_w: 748.06) and 1,2-dipalmitoyl-sn-glycero-3-phosphate (DPPA; M_w: 670.88). Pluronic F-68 and fluorescein diacetate (FDA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Paclitaxel (PTX) in powdery was purchased from Aladdin (Shanghai, China). Octafluoropropane (C_3F_8) gas was purchased from the R&D Center for Specialty Gases at the Research Institute of Physical & Chemical Engineering of Nuclear Industry (Tianjin, China). The fluorescent probe Hoechst 33342 was purchased from Beyotime (Haimen, China).

Two siRNA duplexes designed to target BCL-2 gene (BCL-2-1 and BCL-2-2) were purchased from Genpharm (Suzhou, China). They are 21 nt long double stranded RNA oligos with dTdT overhang at 3′ end and have sequences as shown in Table S1. The siRNA cocktail containing BCL-2-1 and BCL-2-2 (equal molar ratio) was named as BCL-2 siRNA. The Cy3-labeled scrambled siRNA (Cy3-SCR) was obtained from Invitrogen (Carlsbad, CA, USA).

2.2. Preparation of PTX-loaded gas-cored liposomes

PTX-loaded gas-cored liposomes (PTX-loaded nanobubbles, PTX–NBs) were prepared using a thin-film hydration–sonication method. Briefly, all phospholipids (18 mg DPPC, 1 mg DSPE, 1 mg DPPA) and PTX (2 mg) were dissolved in 4 mL of chloroform and transferred into a 9-cm culture dish to form a thin phospholipid film by natural evaporation. The film was hydrated with 4 mL of hydration liquid consisting of 10% glycerol (v/v) and 2 mg/mL Pluronic F-68, and then maintained at 60 °C in a shaking incubator for 1 h to form PTX-loaded liposomes. The liposomal suspension was transferred into a 50 mL centrifuge tube, and the air above the liquid was replaced with C_3F_8 gas using a 50 mL syringe equipped with a long and fine needle. Finally, the solution was sonicated using a VCS 130 PB ultrasonic processor (Sonics, USA) at 130 W for 5 min. Large bubbles were separated as a thin layer from the suspension by low-speed centrifugation (50× *g*, 5 min) and discarded. Subsequently, PTX–NBs were washed with PBS for three times to remove the excess unincorporated phospholipids and PTX. Gas-cored liposomes containing fluorescent probe FDA (FDA–NBs) was prepared in the same way.

The concentration of gas-cored liposome was determined using a hemacytometer as described previously [17]. Briefly, one drop of FDA–NBs was transferred to a hemacytometer and observed using a Carl Zeiss Aviox-1 inverted fluorescence microscope (Carl Zeiss, Gottingen, Germany). The number of drug-loaded gas-cored liposomes was counted using WCIF ImageJ software (v1.37; National Institutes of Health, Bethesda, MA). Finally, the averaged particle concentration (numbers/mL) was calculated using the same cell counting method from three different field images (400×).

To determine the drug loading content (DLC) of PTX, UV–Vis absorption was measured using the Nanodrop 2000 spectrophotometer (Nanodrop Technologies, Wilmington, DE) at 227 nm. Pre-weighted freeze-dried PTX–NB was re-dissolved in 0.5 mL of chloroform. The freeze-dried lipid admixture of the blank NBs without loading PTX was also dissolved in chloroform for baseline determination. The PTX concentration of PTX–NBs was calculated based on the absorption using a standard curve of freshly prepared PTX solutions (0, 31.25, 62.5, 125, 250 and 500 μg/mL in chloroform). All measurements were carried out in triplicate. The DLC was calculated as the weight percentage of PTX in PTX–NBs.

2.3. Preparation of gas-cored liposomes loaded with PTX and siRNA

The cationic diblock copolymer poly(ethylene glycol)-b-poly(L-lysine) (mPEG-b-PLLys) and the siRNA encapsulated mPEG-b-PLLys micelles were prepared and characterized as described in our previous study [17]. To form PTX-load and siRNA-complexed gas-cored liposomes (PTX–NBs/siRNA: PTX–NBs/BCL-2 or PTX–NBs/SCR), 1×10^7 of PTX–NBs and different amounts of siRNA micelles (N/P = 5) were resuspended in 600 μL PBS, mixed by vigorous pipetting and then incubated at room temperature for 30 min. The liposomal complexes containing FDA and Cy3-labled SCR (FDA–NBs/Cy3) were prepared in the same way (Fig. 1). The amount of siRNA loaded inside the PTX–NBs was determined based upon the designed P/P_1 ratios, which were calculated as the number of phosphate groups in siRNA to that in PTX–NBs. Particle sizes and zeta potentials of the liposomal complexes with different P/P_1 were determined by DLS.

The US sensitivity of PTX–NBs/siRNA was evaluated out *in vitro* by monitoring the size and morphology changes upon US exposure. In brief, 1 mL of PTX–NBs/siRNA with a concentration of 3×10^6 bubbles/mL in 1.5 mL Eppendorf tube was placed in a water tank (10 cm × 10 cm × 10 cm). A 2 cm^2 US probe was placed 2 cm away from the Eppendorf tube below the water surface. Then, the sample was exposed to low-frequency US for 1 min (1 MHz, pulse repetition frequency 1 kHz with a 50% duty cycle, acoustic pressure 500 kPa), which was generated by a therapeutic US system (DCT-700, WELLD, Shenzhen, China). The diameters and zeta potentials of PTX–NBs/siRNA before and after US exposure were detected by DLS. In addition, transmission electron microscope (TEM) detections of the samples were also performed using a Hitachi H-7650 system. A drop of each sample was depositing onto a carbon-coated copper grid and dried at room temperature. Then a small drop of phosphotungstic acid solution (1 wt% in water) was added to the grid and blotted with filter paper for 30 s. Finally, the copper grid was observed under TEM after dried in a desiccator overnight.

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