Biomaterials 150 (2018) 1–13

Contents lists available at ScienceDirect

Biomaterials

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

Enhanced antitumor efficacy of arginine modified amphiphilic nanoparticles co-delivering doxorubicin and iSur-pDNA via the multiple synergistic effect

Yudong Song, Cui Tang*, Chunhua Yin

State Key Laboratory of Genetic Engineering, Department of Pharmaceutical Sciences, School of Life Sciences, Fudan University, Shanghai 200438, China

ARTICLE INFO

Article history:
Received 30 July 2017
Received in revised form
1 October 2017
Accepted 3 October 2017
Available online 4 October 2017

Keywords:
Amphiphilic nanoparticles
Co-delivery
Arginine modification
Uptake mechanism
Intracellular transport
Multiple synergistic antitumor effect

ABSTRACT

Arginine and α-tocopherol succinate (α-TOS) double grafted N-trimethyl chitosan chloride (TMC) nanoparticles (TAS NPs) were designed and developed for effective co-delivery of doxorubicin (DOX) and Survivin shRNA-expressing pDNA (iSur-pDNA). With DOX loading into the hydrophobic core and iSur-pDNA combining to the hydrophilic shell, TAS/DOX/pDNA NPs demonstrated favorable structural stability and sustained release properties *in vitro*. With the special non-clathrin-dependent endocytosis, TAS/DOX/pDNA NPs presented higher cellular uptake and mainly distributed in ER and Golgi rather than lysosomes following internalization. The *in vitro* nuclear localization, gene silencing efficiency, cell apoptosis, and growth inhibition of tumor cells were significantly promoted by arginine modification. In the tumor-bearing mice model, TAS/DOX/pDNA NPs possessed the maximum antitumor efficiency as compared with single delivery of DOX or iSur-pDNA. Particularly, blank TAS NPs were selectively be toxic to tumor cells as evidenced by their capabilities to inhibit proliferation and induce apoptosis of tumor cells. The promising tumor treatment of TAS/DOX/pDNA NPs *via* a multiple synergistic manner arising from DOX and pDNA as well as the vectors would provide a potential strategy for a dual-delivery system to improve their therapeutic efficacies.

1. Introduction

Cancer, involving complex signaling pathways and multiple mechanisms, makes the treatment extremely challenging [1,2]. Conventional chemotherapy is largely limited by serious systemic toxicity and development of drug resistance [3]. Recently, the combination of chemotherapy and gene therapy has become a powerful strategy in the treatment of cancer due to its potentially synergistic effects involving increased therapeutic index through simultaneously regulating multiple signaling pathways as well as overcoming intrinsic or acquired multi drug resistance (MDR) in cancer cells [4,5]. To effectively co-encapsulate and co-deliver chemotherapeutics and genes, some amphiphilic cationic copolymer-based vectors with a core-shell structure have been developed [6–8]. To our knowledge, an amphiphilic cationic copolymer-based vector with antitumor activities by itself has not been reported for effective co-encapsulation and co-delivery of chemotherapeutics and genes, though it could possess superior antitumor efficacies *via* a multiple synergistic manner.

The key point for successful formation of co-delivery vectors is to synthesis amphiphilic cationic copolymers which could form nanoparticles (NPs) through the inherent self-assembly in aqueous solution. Chitosan (CS) has been extensively applied for drug delivery systems owing to its well-established safety [9]. However, CS as the hydrophilic component of copolymers shows two major limitations: one is poor solubility as it is only slightly soluble in acid solutions and the other is low transfection efficiency which was resulted from the weak binding affinity with genes [10]. Comparatively, trimethyl chitosan (TMC), a derivative of CS by quaternizing of NH_2 groups on the polysaccharide backbone, possesses satisfactory solubility over a wider pH range and increased transfection efficiency through the efficient complexation of genes [11,12]. To further improve transfection efficiency, arginine modification was performed owing to the increased cellular uptake and nucleus localization of genes [13–15]. α-tocopherol succinate (α-TOS), a hydrophobic natural derivative of vitamin E, consisting of various methylated phenols has attracted attentions for its pharmacological properties to induce apoptosis of various cancer cells without

* Corresponding author.
E-mail address: tangcui@fudan.edu.cn (C. Tang).

https://doi.org/10.1016/j.biomaterials.2017.10.010
0142-9612/

toxicity to normal cells [16,17]. More importantly, it has been reported that α-TOS can act synergistically with doxorubicin (DOX) and paclitaxel (PTX) to reverse MDR [18–20] and the higher antitumor efficiency can be obtained after it was coupled to the water soluble polymer [4,21,22]. Based on the abovementioned understandings, α-TOS as the hydrophobic segment of copolymers is expected to obtain an amphiphilic cationic copolymer-based vectors with antitumor activities.

Therefore, in this study we developed an amphiphilic cationic copolymer-arginine and α-TOS double grafted TMC (TAS) for co-delivery of DOX and Survivin shRNA-expressing pDNA (iSur-pDNA) to achieve the multiple synergistic effect of cancer therapy arising from DOX and pDNA as well as the vectors. As the NPs formed, the size, zeta potential, morphology, drug loading capability, encapsulation efficiency, critical micelle concentration (CMC), bovine serum albumin (BSA) adsorption, *in vitro* drug release, and the structural stability were investigated. Meanwhile, the *in vitro* cytotoxicity, cellular uptake, internalization pathways and mechanisms detailed, subcellular distribution, Survivin gene silencing, cell apoptosis, and antitumor proliferation were studied. Finally, the *in vivo* multiple antitumor efficiency and gene silencing were assessed in tumor bearing mice.

2. Materials and methods

2.1. Materials, cell culture, and animals

Chitosan with deacetylation degree of 85% and molecular weight of 200 kDa was bought from Golden-Shell Biochemical Co., Ltd. (Zhejiang, China). L-arginine hydrochloride, α-TOS, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), Nhydroxysuccinimide (NHS), DOX, pyrene, Hoechst 33258, and fluorescein isothiocyanate (FITC) were purchased from Sigma (St. Louis, MO, USA). iSur-pDNA was amplified in *Escherichia coli* and isolated with an EndoFree Plasmid Mega Kit (Tiangen Biotech Co., Ltd., Beijing, China). ER-Tracker™ Green, NBD- and BODIPY Dye-labeled Sphingolipids (Golgi Dyes, Green), Lysotracker® Green, cholera toxin B subunit (CTB)-Alexa Fluor 488, transferrin-Alexa Fluor 488, FITC labeled dextran (FITC-dextran, Mw of 70 kDa), and Lipofectamine® RNAiMAX (LipoRNAiMAX) were obtained from Invitrogen (Carlsbad, CA, USA). Clathrin heavy chain-1 (CHC-1) siRNA, caveolin-1 siRNA, NPC1 siRNA, and Rac1 siRNA were supplied by GenePharma Co., Ltd. (Shanghai, China). The primers for quantitative Real-Time PCR (qRT-PCR) were bought from Sangon Biotech Co., Ltd. (Shanghai, China). The sequences of siRNA and primers were listed in Supplementary Information Table S1. All other reagents were of analytic grade.

Human hepato-carcinoma (QGY-7703) cells were provided by Chinese Academy of Sciences (Shanghai, China). Raw 264.7 cells were bought from the American Type Culture Collection (Rockville, DS, USA). Both cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, NY, USA) containing 10% fetal bovine serum (FBS) at 37 °C and 5% CO_2.

Kunming mice (Female, 6 weeks, 20 ± 2 g) were acquired from the Animal Centre of Fudan University. Animal procedures were performed following the Guiding Principles of the Institutional Animal Care and Use Committee, Fudan University.

2.2. Synthesis and characterization of TAS

TAS was synthesized *via* a three-step reaction. Firstly, chitosan was reacted with CH_3I in NaOH/N-methy1-2-pyrrolidone solution for 120 min at 65 °C to obtain TMC as previously reported [11,12]. Secondly, TMC was reacted with α-TOS by conjugating the carboxyl of α-TOS and the amino groups of TMC according to a previous report with minor modification [23]. Briefly, the mixture of EDC/NHS/α-TOS (mole ratio, 3:3:1) was dissolved in anhydrous DMSO and continued stirring for 24 h at room temperature (RT) under nitrogen to fully activate the carboxyl group of α-TOS. Subsequently, the resultant DMSO solution was added dropwise to the equal-volume TMC solution (20 mg/mL) under fast stirring and reacted for further 48 h. Then 7-fold volume of acetone was added into the mixture and the resultant precipitates were centrifugated and dissolved in water to obtain α-TOS grafted TMC (TS). TS was further purified by ultrafiltration (MWCO, 10 kDa) with methanol and dialysis against water for 3 days (MWCO, 3.5 kDa), followed by lyophilization. Thirdly, TS (100 mg) was added into a solution (TEMED/HCl, pH 4.8) of arginine and EDC/NHS which has pre-activated for 2 h under RT and reacted for 24 h [14]. Then the final product TAS was dialyzed, lyophilized, and stored at 4 °C. The TS and TAS conjugates were identified by ^{1}H NMR (AVANCE DMX 500, Bruker, Germany) and Fourier transform infrared (FT-IR) spectra (Nexus 470, Nicolet, USA). The quaternization degree of TAS was calculated by ^{1}H NMR. The substitution degrees of α-TOS and arginine were determined through 2,4,6-trinitrobenzenesulfonic acid method and Sakaguchi reaction, respectively, and calculated as the percentage of all sugar rings. The detailed determination methods on the quaternization degree as well as substitution degrees of α-TOS and arginine in TAS were seen in Supplementary Information.

2.3. Preparation and characterization of NPs

Blank TS NPs and TAS NPs were formed *via* self-assembly. DOX and pDNA were loaded into TS NPs and TAS NPs by sonication and electrostatic adsorption, respectively [5]. Briefly, to obtain TS/DOX NPs and TAS/DOX NPs, DOX (10 mg/mL) in DMSO was mixed with TS NPs and TAS NPs (1 mg/mL) at the weight ratio of 1:5, which was further sonicated for 10 min under ice bath. Then the mixture was transferred to ultrafiltration tubes (MWCO 3.5 KDa) to remove the unloaded DOX *via* centrifugal-ultrafiltration. AS for pDNA encapsulation, pDNA (1 mg/mL) was added into DOX loaded TS NPs and TAS NPs at a weight ratio of 1:10 and then vortexed for 10 s to obtain TS/DOX/pDNA NPs and TAS/DOX/pDNA NPs. Besides, TS/pDNA NPs and TAS/pDNA NPs were prepared in the same way as pDNA encapsulated by TS/DOX NPs and TAS/DOX NPs.

The sizes and zeta potentials of the NPs were determined by dynamic light scattering (DLS) using a Zetasizer Nano (Malvern, Worcestershire, UK). Field Emission Transmission Electron Microscope (FETEM, JEM-2100F, JEOL, Japan) were applied to visualize the morphology of TAS/DOX/pDNA NPs.

2.4. Drug loading capability (DL) and encapsulation efficiency (EE)

The EE and DL of DOX was measured *via* centrifugal-ultrafiltration method as previously reported [24]. Briefly, TAS/DOX NPs, TS/DOX/pDNA NPs, and TAS/DOX/pDNA NPs (2 mL, 1 mg/mL) were added into the ultrafiltration tubes (MWCO 3.5 KDa), followed by centrifugation for 30 min at 10,000 rpm. Another 2 mL of above NPs solutions (1 mg/mL) were dissociated by DMSO to extract DOX from the NPs. And the amounts of DOX in the tubes and in DMSO were measured. The DL and EE could be calculated by the equations as follows:

$$EE\ (\%) = m_0 - m_1/m_0 \times 100\%$$

$$DL\ (\%) = m_0 - m_1/(m_0{+}2000\ \mu g) \times 100\%$$

where m_0 represents the amount of DOX (μg) in ultrafiltration tubes; m_1 represents the amount of DOX (μg) in DMSO.