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Cholic acid-functionalized nanoparticles of star-shaped PLGA-vitamin E TPGS copolymer for docetaxel delivery to cervical cancer

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ABSTRACT

We developed a system of nanoparticles (NPs) of cholic acid functionalized, star-shaped block copolymer consisting of PLGA and vitamin E TPGS for sustained and controlled delivery of docetaxel for treatment of cervical cancer, which demonstrated superior *in vitro* and *in vivo* performance in comparison with the drug-loaded PLGA NPs and the linear PLGA-*b*-TPGS copolymer NPs. The star-shaped block copolymer CA-PLGA-*b*-TPGS of three branch arms was synthesized through the core-first approach and characterized by ^{1}H NMR, GPC and TGA. The drug- or coumarin 6-loaded NPs were prepared by a modified nanoprecipitation technique and then characterized in terms of size and size distribution, surface morphology and surface charge, drug encapsulation efficiency, *in vitro* release profile and physical state of the encapsulated drug. The CA-PLGA-*b*-TPGS NPs were found to have the highest cellular uptake efficiency, the highest antitumor efficacy compared with PLGA-*b*-TPGS NPs and PLGA NPs. The results suggest that such a star-shaped copolymer CA-PLGA-*b*-TPGS could be used as a new molecular biomaterial for drug delivery of high efficiency.

1. Introduction

Cervical cancer is one of the most serious health problems among women worldwide, especially in developing countries. In the recent decades, the morbidity and mortality of cervical cancer have greatly increased [1]. However, the current approaches for cervical cancer treatment are still limited to surgical resection, radiotherapy, chemotherapy in clinics, which are highly aggressive and/or non-specific, and often accompanied by serious side effects because the anticancer agents also show conspicuous toxicity to normal cells and tissues [2,3]. Nanomedicine, especially drug formulation by polymeric nanoparticles (NPs), could provide sustained and controlled delivery of anticancer agents, and has shown significant promise to solve such problems in cancer therapy [4,5]. Recently, the biodegradable polymeric NPs have been received considerable attention for their active and passive drug targeting therapeutics after parenteral administration [6,7]. The NPs used as drug carriers also have other advantages such as high encapsulation efficiency, high cellular uptake, more reasonable pharmacokinetics and more desirable biodistribution as well as preferentially accumulate at the tumor site due to the enhanced permeability and retention (EPR) effect [8]. Moreover, NPs could reduce the drug resistance of the cancer cells [9].

The amphiphilic block biodegradable copolymers have the potential to form polymeric NPs by self-ensemble effects due to the hydrophobic–lipophilic interactions. Poly(lactide-*co*-glycolide) (PLGA) is one of the most widely investigated biodegradable polymers for biomedical applications [10]. However, it is well-known that PLGA is extremely hydrophobic and its degradation is too slow, more than one year, to meet the needs to be applied for drug delivery [11]. Furthermore, the PLGA NPs could be rapidly filtration in the liver and captured by the reticuloendothelial system (RES) when they are injected into the blood stream [12,13]. These drawbacks can be overcome by introduced D-α-tocopheryl polyethylene glycol 1000 succinate (vitamin E TPGS or simply

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TPGS) into PLGA [14,15]. TPGS, a water-soluble derivative of natural vitamin E, is formed by esterification of vitamin E succinate with PEG 1000. TPGS is an excellent emulsifier. It was found to enhance the solubility of drugs including taxanes, cyclosporines, antibiotics and steroids. It has been reported that TPGS could serve as an excipient for surmounting multidrug resistance and as an inhibitor of P-glycoprotein for increasing the cytotoxicity and oral bioavailability of anticancer drugs [16]. PLGA and TPGS have been used in multiple pharmaceutical products and are approved by the FDA in drug delivery systems [17,18].

Although the PLGA NPs and the TPGS-*b*-PLGA NPs have been extensively investigated as drug delivery vehicle in the literature, most of the research was focused on utilizing linear copolymers. Recently, branched polymers, such as hyper-branched polymers, star-shaped polymers and dendrimers have attracted much attention because they exhibit useful rheological and mechanical properties [19,20]. Star-shaped block polymers are a simple example of branched polymer with all branches extending from a single point (core) [21]. Compared with the linear polymers of the same molar mass, the drug carriers based on star-shaped polymers show a lower solution viscosity, smaller hydrodynamic radius, higher drug loading content (LC) and higher drug encapsulation efficiency (EE) [22,23]. It is thus worthy to investigate a type of star-shaped block copolymer based on TPGS and PLGA with unique architectures, which may provide bright insights for preparing excellent drug carriers for nanomedicine applications [24,25]. We assume that nanoparticles of star-shaped PLGA-TPGS copolymers may combine the advantages of both the PLGA and TPGS NPs, which may have much better performance in drug delivery than the linear PLGA-TPGS copolymer.

Cholic acid (CA), the main bile acid in body, is composed of a steroid unit with three hydroxyl groups and one carboxyl group. CA was selected as the poly-hydroxy initiator because of its biological origin, which may generate better biocompatibility for polymers incorporated with CA moiety [26]. Furthermore, papers reported that CA functionalized star-shaped copolymers exhibited faster hydrolytic degradation rates compared with PLGA and poly(ε-caprolactone). Interestingly, the existence of CA moiety in materials could also significantly enhance both cell adherence and proliferation [27].

In the present study, in order to develop a superior drug carrier of docetaxel used as a model anticancer drugs with satisfactory drug loading content and drug encapsulation efficiency for cervical cancer treatment, the star-shaped block copolymer CA-PLGA-*b*-TPGS with three branch arms was prepared through the core-first approach. The drug-loaded star-shaped CA-PLGA-*b*-TPGS NPs were characterized and the antitumor effect of NPs was evaluated both *in vitro* and *in vivo*. The results were made in close comparison with the current clinical formulation of DTX (Taxotere® as well as the DTX-loaded linear copolymers PLGA and PLGA-*b*-TPGS NPs [28].

2. Materials and methods

2.1. Materials

D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS), cholic acid (CA), stannous octoate ($Sn(Oct)_2$), 1,3-diisopropylcarbodiimide (DCC), 4-(dimethylamino) pyridine (DMAP), D,L-lactide (LA) and glycolide (GA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). PLGA (LA: GA = 75:25, M_n 20,000) was purchased from Jinan Daigang Biomaterial Co. Ltd (Jinan, China). Docetaxel (DTX) was provided by Beijing InnoChem Science & Technology Co., Ltd (Beijing, China). Acetonitrile and methanol were purchased from EM Science (HPLC grade, Mallinckrodt Baker, USA). All other chemicals of the highest quality were commercially available and used as received. Human cervix carcinoma cell line HeLa was purchased from American Type Culture Collection (ATCC, Rockville, MD).

2.2. Synthesis of linear copolymer PLGA-*b*-TPGS and star-shaped copolymer CA-PLGA-*b*-TPGS

Synthesis of the linear copolymer *PLGA-b-TPGS* and the star-shaped block copolymer CA-PLGA-*b*-TPGS was carried out as described in the literature [29,30]. The reaction scheme for synthesis of CA-PLGA-*b*-TPGS was presented in Fig. 1.

2.2.1. Synthesis of carboxyl-terminated CTPGS

TPGS (M_n = 1500, 7.50 g, 5.0 mmol), succinic anhydride (0.60 g, 6.0 mmol), DMAP (0.61 g, 5.0 mmol) and TEA (0.51 g, 5.0 mmol) were dissolved in 60 mL of anhydrous dioxane and stirred for 24 h at room temperature. The solvent was completely evaporated in a rotary evaporator. The residue was dissolved in DCM and filtered to remove unreacted succinic anhydride. After filtration, the solution was precipitated in anhydrous ether. The precipitated product carboxyl-terminated TPGS (CTPGS) was dried under vacuum at room temperature for 24 h (82.3% yield).

2.2.2. Synthesis of star-shaped CA-PLGA

D,L-lactide (12.96 g, 90 mmol), glycolide (3.48 g, 30 mmol), initiator CA (0.41 g, 1 mmol), and catalyst $Sn(Oct)_2$ (0.049 g, 0.1 mol% of monomers) were added in a glass tube, which was connected to a vacuum system. An exhausting-refilling with argon process was then repeated three times. The tube was sealed and heated to 150 °C in oil bath for 12 h. After the reaction, the tube was cooled to room temperature. The resulting product was dissolved in CH_2Cl_2 and then precipitated in excess cold methanol to purify the products. The product named CA-PLGA was collected by filtration and dried under vacuum at 40 °C for 24 h (92.6% yield). In addition, the linear copolymer named PLGA-*b*-TPGS was synthesized in the same way except the initiator CA was replaced by TPGS.

2.2.3. Coupling reaction of star-shaped CA-PLGA and CTPGS

CA-PLGA (M_n = 17,360, 3.47 g, 0.2 mmol), CTPGS (1.36 g, 0.85 mmol), DCC (0.18 g, 0.85 mmol) and DMAP (0.021 g, 0.17 mmol) were dissolved in 40 mL of anhydrous dichloromethane and reacted at room temperature for 24 h under dry argon. The reaction byproduct dicyclohexylcarbodiurea (DCU) was removed by filtration and then precipitated in anhydrous ether twice. The obtained star-shaped block polymer CA-PLGA-*b*-TPGS was purified by solvent extraction using ether and benzene as a co-solvent. The final product white powder was dried in vacuo at 40 °C for 24 h (75.2% yield).

2.3. Characterization of copolymers

^{1}H NMR (Bruker AMX 500) was used to confirm the structure of synthesized CA-PLGA and CA-PLGA-*b*-TPGS with $CDCl_3$ used as a solvent. Molecular weight and molecular weight distribution were determined by gel permeation chromatography (Waters GPC analysis system with RI-G1362A refractive index detector, Waters Corp., Milford, MA, USA). Thermogravimetric analysis (TGA, TGA Q500 thermogravimetric analyzer, USA) was carried out to investigate the thermal properties of the copolymers. The copolymers were heated from 40 to 600 °C at a rate of 20 °C/min and N_2 flow rate of 20 mL/min.

2.4. Formulation of DTX-loaded nanoparticles

DTX-loaded CA-PLGA-*b*-TPGS nanoparticles (NPs) were prepared by a modified nanoprecipitation method using an acetone-water system [31]. In brief, a pre-weighed amount of DTX powder and 100 mg of copolymer CA-PLGA-*b*-TPGS were dissolved in 8 ml of acetone by vortexing and sonication. This mixture was dropwise added into 100 mL aqueous solution (including 0.03% TPGS) under stirring. The resulting suspension was then stirred uncovered overnight to remove acetone completely. The NPs suspension was centrifuged at 20,000 rpm for 30 min and then washed three times to remove the emulsifier TPGS and unencapsulated drug. Finally, the dispersed solution was lyophilized 2 days for further use. DTX-loaded PLGA NPs and PLGA-*b*-TPGS NPs NPs and fluorescent couramin 6-loaded CA-PLGA-*b*-TPGS NPs were prepared in a similar manner. The lyophilized NPs were redispersed in PBS before use.

2.5. Characterization of DTX-loaded NPs

2.5.1. Size, zeta potential and morphology of the NPs

The particle size and zeta potential were measured by Malvern Mastersizer 2000 (Zetasizer Nano ZS90, Malvern Instruments Ltd., UK). Before measurement, the freshly prepared nanoparticles were appropriately diluted. All measurements were measured at room temperature after equilibration for 10 min. The data were achieved with the average of three measurements.

The surface morphology of NPs was examined by a field emission scanning electron microscopy (FESEM, JEOL JSM-6301F, Tokyo, Japan). To prepare samples for FESEM, the nanoparticles were fixed on the stub by a double-sided sticky tape and then coated with platinum layer by JFC-1300 automatic fine platinum coater (JEOL, Tokyo, Japan) for 60 s.

The NPs were further observed by transmission electron microscopy (TEM, Tecnai G2 20, FEI Company, Hillsboro, Oregon, USA). Sample was dropped onto a copper grid coated with a carbon membrane. The grid was allowed to dry before characterization.

2.5.2. Drug loading and drug encapsulation efficiency

To determine the drug loading content (LC) and drug encapsulation efficiency (EE) of the DTX-loaded NPs, a predetermined amount of NPs were dissolved in 1 mL