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Enhanced anti-tumor efficacy by co-delivery of doxorubicin and paclitaxel with amphiphilic methoxy PEG-PLGA copolymer nanoparticles

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ABSTRACT

The use of single chemotherapeutic drug has shown some limitations in anti-tumor treatment, such as development of drug resistance, high toxicity and limited regime of clinical uses. The combination of two or more therapeutic drugs is feasible means to overcome the limitations. Co-delivery strategy has been proposed to minimize the amount of each drug and to achieve the synergistic effect for cancer therapies. Attempts have been made to deliver chemotherapeutic drugs simultaneously using drug carriers, such as micelles, liposomes, and inorganic nanoparticles (NPs). Here we reported core-shell NPs that were doubly emulsified from an amphiphilic copolymer methoxy poly(ethylene glycol)-poly(lactide-co-glycolide) (mPEG-PLGA). These NPs offered advantages over other nanocarriers, as they were easy to fabricate by improved double emulsion method, biocompatible, and showed high loading efficacy. More importantly, these NPs could co-deliver hydrophilic doxorubicin (DOX) and hydrophobic paclitaxel (TAX). The drug-loaded NPs possessed a better polydispersity, indicating that they are more readily subject to controlled size distribution. Studies on drug release and cellular uptake of the co-delivery system demonstrated that both drugs were effectively taken up by the cells and released simultaneously. Furthermore, the co-delivery nanocarrier suppressed tumor cells growth more efficiently than the delivery of either DOX or TAX at the same concentrations, indicating a synergistic effect. Moreover, the NPs loading drugs with a DOX/TAX concentration ratio of 2:1 showed the highest anti-tumor activity to three different types of tumor cells. This nanocarrier might have important potential in clinical implications for co-delivery of multiple anti-tumor drugs with different properties.

1. Introduction

Existing chemotherapeutic drugs are far from perfect with undesirable severe side effects, low bioavailability or development of drug resistance. Overcoming these limitations requires effective delivery of chemotherapeutic drugs to tumor tissues with a minimal amount to other sites. To improve therapeutic efficacy and reduce toxicity and frequency of drug administration, various drug delivery systems have developed. Over the past few decades, there has been an increasing interest in the potential use of copolymer nanoparticles (NPs) as delivery vehicles for chemotherapeutic drugs and the studies have demonstrated that these nanocarriers can significantly enhance the anti-tumor efficiency of various chemotherapeutic drugs [1–3].

Generally, synergistic combination of two or more drugs is a promising strategy to overcome undesirable toxicity and other side effects that limit the utility of many potential drugs by countering biological compensation, allowing reduced dosage of each agent or accessing context-specific multiple targets [4–7]. Co-delivery systems, loading different drugs with different physiochemical properties simultaneously to the same tumor cells *in vitro* and *in vivo*, have been proposed to minimize the amount of drug and to achieve the synergistic therapeutic effect in treating cancers after a single injection [8,9]. Recently, many multifunctional delivery systems have been designed for co-delivery of different guests, including micelles [10–12], liposomes [13], and inorganic nanoparticles [14]. Although many efforts have been

Abbreviations: NPs, nanoparticles; mPEG-PLGA, methoxy poly(ethylene glycol)-poly(lactide-co-glycolide); DOX, doxorubicin; TAX, paclitaxel; W/O/W, water-in-oil-in-water; FITC, fluorescein isothiocyanate; PVA, polyvinyl alcohol; HPLC, High pressure liquid chromatography; DLS, dynamic light scattering; FTIR, fourier-transform infrared spectroscopy; NMR, nuclear magnetic resonance; TEM, transmission electron microscopy; EE, encapsulation efficiency.

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made on a single carrier for two or more therapeutic agents, such as chemicals, siRNA, plasmid DNA or peptide [15], co-delivery of chemotherapeutic drugs with distinct water solubility properties is not easy to realize. Because of the compartmentalized internal structure, double emulsion method can make amphiphilic copolymers form nanoparticles with the ability to encapsulate both polar and non-polar cargos and can improve the controlled release of therapeutic molecules [16,17]. Water-in-oil-in-water (W/O/W) emulsion, an example in double emulsion, utilizes a two-step process to form an 'inverse' water-in-oil emulsion first, followed by emulsification of this mixture in water using a combination of surfactants [18–20].

Doxorubicin (DOX) and paclitaxel (TAX) are among the most common chemotherapeutic drugs in clinical use currently, due to their excellent anti-tumor efficiency against various solid tumors [21]. They, however, are drugs with distinct solubility characteristics and different anticancer mechanisms [22–24]. DOX is a hydrophilic compound which binds to DNA by intercalation and induces a series of biochemical events inducing apoptosis in a number of different tumor cells [25,26]. TAX, a naturally occurring antimitotic agent, is a highly hydrophobic drug, which can inhibit microtubules disassembly and promote the formation of unusually stable microtubules, thereby disrupting normal dynamic reorganization of the microtubule network required for mitosis and cell proliferation, and in turn causing cell apoptosis [27–31]. Some clinical studies have shown that incorporation of DOX and TAX increases tumor regression rates relative to the individual drugs [21] and has been used as first-line treatment for metastatic breast cancer [24].

As the key point for successful combination therapy is to design simple co-delivery systems. Double emulsion method is an active process to incorporate hydrophilic drugs into amphiphilic copolymers nanoparticles. This method generating nanoparticles with "core-shell droplet" morphology [32,33] is important for industrial applications, such as in the food, cosmetic and pharmaceutical industries. However, encapsulation of a drug in a double emulsion can be achieved only when the drug is soluble in the inner aqueous phase (W) but insoluble in the intermediate oil phase (O) of the emulsion [34]. In this study, we have developed a convenient method for improved double emulsion suitable for a large-scale production. Inspired from W/O/W form prepared by double emulsion method, we utilized the improved double emulsion method to load hydrophobic drugs at the O layer and hydrophilic drugs at the hydrophilic core. We chose mPEG-PLGA as the amphiphilic copolymer model to realize co-delivery and controlled release of DOX and TAX, mainly because PEG and PLGA are met with FDA's approval for clinical use as drug adjuvants. Furthermore, this double emulsion method also suits other amphiphilic copolymers to form nanocarriers for co-delivering other hydrophilic and hydrophobic drugs.

2. Methods

2.1. Materials

DOX was purchased from Beijing Huafeng United Technology Co. Ltd., and TAX was obtained from Beijing Norzer Pharmaceutical Co. Ltd.. Poly(lactic-co-glycolic acid) (PLGA, molar ratio of D, L-lactic to glycolic acid, 75:25), monomethoxy poly(ethylene glycol) (mPEG) was purchased from Jinan Daigang Biotechnology Co. Ltd.. Fluorescein isothiocyanate (FITC) was obtained from Sigma–Aldrich (St. Louis, MO, USA). Cell Counting Kit-8 was purchased from Dojindo Molecular Technologies (Tokyo, Japan). Dulbecco's modified Eagle's medium (DMEM), RMPI 1640 medium, and fetal bovine serum were purchased from Gibco BRL (Grand Island, NY, USA). Propidium iodide (PI), Hoechst 33258, penicillin, and streptomycin were provided by Sigma–Aldrich (St. Louis, MO, USA). All chemicals used were of analytical reagent quality.

2.2. Synthesis and characterizations of copolymer

Diblock copolymer mPEG-PLGA was synthesized as described in the reference with minor modifications [35,36]. In brief, PLGA (8 g) and mPEG (2 g) were copolymerized in freshly distilled toluene (150 mL) using stannous 2-ethylhexanoate (20 mg) as a catalyst at 114 °C for 8 h with stirring at 250 rpm. After vacuum evaporation of the solvent, the residue was dissolved in 200 mL of DCM, filtered, and then added into vigorously stirred water (1000 mL) at 60 °C. After DCM evaporation, the mPEG-PLGA solid was isolated from the aqueous phase by decantation. Subsequently, fluorescein isothiocyanate (FITC, 20 mg) was conjugated to mPEG-PLGA (1.5 g) in 30 mL of DMSO at 90 °C for 2 h in darkness. The resultant solution was dialyzed against distilled water for 3 days to remove DMSO and unreacted FITC, and then lyophilized [37]. Characterization of products was confirmed by ^{1}H NMR spectrometer (Bruker AVANCE 400 NMR spectrometer, Billerica, MA, USA) with DMSO as the solvent and Fourier-transform infrared spectrometer (FTIR, Magna FTIR-750, Nicolet, USA). FTIR spectra were obtained from a neat film cast from the chloroform copolymer solution between KBr tablets.

2.3. Preparations of mPEG-PLGA NPs, NPs-DOX, NPs-TAX and NPs-DOX-TAX

mPEG-PLGA NPs and NPs-DOX were prepared by the double emulsion (W/O/W) method with a little modification. Briefly, 20 mg of mPEG-PLGA dissolved in 1 mL of methylene chloride and 0.2 mL of water or DOX solution were transferred to a centrifuge tube, and the mixture was emulsified by sonication for 3 min. Then the emulsion and 2 mL of 2% polyvinyl alcohol (PVA) were emulsified by sonication for 5 min. The emulsion was then slowly dropped into 10 mL of 0.6% PVA and stirred for 10 min at room temperature. After vacuum evaporation of the solvent, the NPs were collected by centrifugation at 13,000 rpm for 10 min at room temperature and washed twice using distilled water.

NPs-DOX-TAX was prepared by the improved double emulsion method. After the first emulsification, the mixture and 2 mL of 2% PVA were stirred for 3 min at room temperature. Meanwhile, 0.2 mL of TAX-dissolved methylene chloride was added slowly, and then emulsified again. The subsequent steps were identical with the preparation of mPEG-PLGA NPs and NPs-DOX.

Distinctively, NPs-TAX was produced using an emulsion/solvent evaporation technique. 20 mg of copolymer and 1 mg of TAX were dissolved in 1 mL of methylene chloride. The solution was stirred for 10 min at room temperature and emulsified by sonication in 10 mL of aqueous solution with 1% PVA. After vacuum evaporation of the solvent, the NPs were collected by centrifugation at 13,000 rpm for 10 min at room temperature and washed twice using distilled water.

2.4. Measurements of particle size distribution and zeta potential

The NPs size (diameter, nm), polydispersity index and surface charge (zeta potential, mV) were determined using a ZetaSizer Nano series Nano-ZS (Malvern Instruments Ltd., Malvern, UK) equipped with a He–Ne Laser beam at a wavelength of 633 nm and a fixed scattering angle of 90°. Determinations were performed at 25 °C for samples appropriately diluted in distilled water.

2.5. Morphological characterization

The morphology of NPs-DOX-TAX was confirmed using a transmission electron microscopy (TEM, JEM-200CX, Jeol Ltd., Japan) after negative staining with sodium phosphotungstate solution (2%, w/w).

2.6. Stability and in vitro drug release

Nanoparticles (50 mg) were suspended in phosphate-buffered saline at pH 7.4 or pH 4.4, under stirring at 110 rpm/37 °C. The nanoparticles sizes were determined at 0, 1 and 2 days to examine the stability of the nanoparticles at different pH values by dynamic light scattering (DLS).

For *in vitro* drugs release study, drug-loaded nanoparticles (20–30 mg) were reconstituted in PBS (5 mL, pH 4.4 or 7.4) and transferred to dialysis bags (MWCO: 3500 Da) placed in 30 mL of PBS with stirring at 110 rpm/37 °C. At appropriate intervals, the environmental buffer solution was replaced with fresh PBS, and the concentration of the released DOX in the removed PBS was determined using a calibration curve at the wavelength where DOX showed its maximum absorbance (483.5 nm), quantitatively monitored by Lambda 950 Visible-UV spectrophotometer (PerkineElmer Fremont, CA, USA). Then, the accumulative ratios of the released DOX were calculated as a function of time.

High performance liquid chromatography (HPLC) was used to determine the released amount of TAX [38]. The PBS used for DOX detection in the quartz cell also used for TAX detection. The accumulative percentage of the released TAX was calculated as a function of incubation time. An Agilent 1200 series quaternary pump and a Rheodyne sample injector with a 20 μL loop, an Agilent 1200 series UV–Vis DAD detector, and a chemstation data processing system were used. The mobile phase was water/acetonitrile (1:1 v/v). The HPLC analysis was carried out with an Agilent Zorbax Eclipse XDB C18 reversed-phase column (150 × 4.6 mm, 5 μm, 1 mL/min, Agilent Technologies).

Encapsulation efficiency of the NPs was calculated by the following equation.

$$\text{Encapsulation Efficiency(EE)} = \frac{A}{B} \times 100\%$$