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PLGA nanoparticles introduction into mitoxantrone-loaded ultrasound-responsive liposomes: *In vitro* and *in vivo* investigations

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ABSTRACT

A novel ultrasound-responsive liposomal system for tumor targeting was prepared in order to increase the antitumor efficacy and decrease serious side effects. In this paper, PLGA nanoparticles were used ultrasound-responsive agents instead of conventional microbubbles. The PLGA-nanoparticles were prepared by an emulsion solvent evaporation method. The liposomes were prepared by a lipid film hydration method. Particle size, zeta potential, encapsulation efficiency and drug loading capacity of the liposomes were studied by light scattering analysis and dialysis. Transmission electron microscopy (TEM) and atomic force microscope (AFM) were used to investigate the morphology of liposomes. The release *in vitro* was carried out in the pH 7.4 phosphate buffer solutions, as a result, liposome L3 encapsulating PLGA-nanoparticles displayed good stability under simulative physiological conditions and quickly responsive release under the ultrasound. The release *in vivo* was carried out on the rats, as a result, liposome L3 showed higher bioavailability than traditional intravenous injectable administration, and liposome L3 showed higher elimination ratio after stimulation by ultrasound than L3 without stimulation. Thus, the novel ultrasound-responsive liposome encapsulating PLGA-nanoparticles has a potential to be developed as a new drug delivery system for anti-tumor drug.

1. Introduction

Cancer is one of the most challenging problems in the modern medicine. Despite rapid advances in diagnostic procedures and treatments, the overall cancer survival rate has not improved substantially during the past thirty years (Ghanghoria et al., 2016). Mitoxantrone (MXT) is widely used in cancer therapy including breast cancer, acute leukemia, lymphoma, prostate cancer and melanoma (Amato et al., 2013; Huber et al., 1995; Neuhaus et al., 2006; Rossato et al., 2013). In current clinical trials, MXT is made into freeze-dried powder and administered by intravenous injection. Lacking of cell cycle phase specificity, MXT causes serious side effects, such as neuropathy, nausea, general discomfort, myelosuppression, nephrotoxicity, and so on. Thus, it's necessary to design a new drug delivery system for MXT with low systemic toxicity.

Liposomes are self-enclosed spherical vesicles consisting of a lipid bilayer and aqueous inner cavity with a range of size from 50 nm to 1000 nm (Mehta, 2016). Liposomes show many advantages including improved solubility, bioavailability, stability, targeting delivery and sustained release. Moreover, liposomes enhance the intracellular uptake of drugs (Muthu et al., 2012), and protect encapsulated drugs from the destruction by the external environment (Alexander et al., 2016). In order to improve the bioavailability of MXT, Pedrosa et al. (2015) prepared a liposome containing a short chain sphingolipid, C8-glucosylceramide or C8-galactosylceramide, this liposome promoted MXT transporting through the plasma membrane, and improved bioavailability. Xing et al. (2016) reported a MXT-entrapped liposome that could strengthen chemotherapy efficacy and safety.

However, how to control drug release at a specific tissue and at a predestinate time is still a challenge in the development of liposomes. The researchers developed stimuli-responsive smart liposomes for controlling release including magnetism, pH, temperature and ultrasound responsive liposomes. Among them, the ultrasound as a mature technology has been extensively applied in clinical trials (Boissenot et al., 2016). Low frequency ultrasound (<100 kHz) has been proven to accelerate drug permeation across the animal's skin (Liao et al., 2016; Prausnitz and Langer, 2008; Schoellhammer et al., 2015) and the gastrointestinal tract (Kost and Langer, 1990; Schoellhammer and Traverso,

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2016). High frequency ultrasound (>1 MHz) is usually used in ultrasonography, tumor ablation and lithotripsy in clinics (Kennedy, 2005).

The mechanisms of ultrasound enhancing drug delivery include physics of ultrasound, hyperthermia and cavitation (Pitt et al., 2004). Liposomes encapsulating a gas core show an obvious response to ultrasound due to the action of ultrasonic cavitation. This gas core is usually sulfur hexafluoride gas (Chetty et al., 2008) or perfluorocarbon gas (Díaz-López et al., 2010). Chumakova et al. (2008) reported ultrasound enhanced gene delivery *in vitro* and *in vivo* without cell damage due to the interaction of ultrasound and air-filled PLGA NPs. However the liposomes encapsulating a gas core performed poor stability and short blood half-life time (<10 min) during exposure to ultrasound (Mousnier et al., 2014). An experimental phenomenon that the movement tract of suspended solid particles under the ultrasound with 2.5 MHz frequency was recorded by a camera (Garcia-Sabaté et al., 2014). This result inspires us design a novel type of liposome that the core encapsulated in the liposome is not yet a gas, but a more stable solid nanoparticle. When this liposome is exposed to ultrasound, the inner solid nanoparticle will cause movement and break down the lipid membrane. For this response to ultrasound is due to ultrasound physics rather than ultrasound cavitation, this novel liposome will be more stable.

To human drug products, the stimulus conditions and pharmaceutical components need to meet strict requirements of including biocompatibility, nontoxicity and ease of application (Li et al., 2016). In this paper, high frequency ultrasound (>1 MHz) was used to trigger MXT delivery at an anticipated time, and poly(lactic-co-glycolic acid) nanoparticles (PLGA-NPs) were used to prepare inner core. PLGA is a pharmaceutical excipient for intravenous administration approved by FDA due to excellent biodegradability and biocompatibility (Das and Khuda-Bukhsh, 2016). Many kinds of microspheres products on the market use PLGA as the excipient, such as Lupron Depot®, Arestin® and Somatuline®LA. Moreover, Anderson and Shive (1997) reported that the half-life (50% loss of weight) of the PLGA (50:50) microspheres was 15 days. Thus, here PLGA (50:50) nanoparticles was used as a stable solid inner core to respond to ultrasound.

In this paper, ultrasound responsive MXT liposomes encapsulating PLGA-NPs were prepared. The novel MXT liposomes were constructed by core-shell structure, the inner core was PLGA NPs and MXT molecules, the outer layer was lipid membranes. When the liposomes were stimulated by the ultrasound, PLGA NPs would produce vibrations. The vibrating PLGA NPs would break the lipid membranes, and MXT molecules easily release through the lipid membranes. Furthermore, the release *in vitro* and *in vivo* of the MXT-entrapped liposomes was evaluated. This MXT liposome integrating stability of the liposome and rapid-response of ultrasound would be potential for anticancer treatment.

2. Materials and methods

2.1. Materials

2.1.1. Reagents

Mitoxantrone hydrochloride was purchased from Beijing HWRK Chem Co., Ltd. (Beijing, China). Cholesterol (Chol) and cationic exchange resin were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Poloxamer188 was purchased from BASF (Ludwigshafen, Germany). 1-Palmitoyl-2-stearoyl-*sn*-glycero-3-phosphatidylcholine (HSPC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (DSPE-PEG2000) were purchased from Xi'an Ruixi (Lipoid, Germany). Poly (D, L-lactic-*co*-glycolic acid) (PLGA, 50:50, M_W = 15 kDa) was purchased from Jinan Daigang Biomaterial Co., Ltd. (Jinan, China). Ultrapure water was prepared using a Milli-Q water purification system (Millipore Corp., Bedford, MA, USA), acetonitrile and methanol were of chromatographic grade, other reagents were of analytical grade.

2.1.2. Animals

Adult Sprague-Dawley rats weighing 200–300 g (half male and female) were purchased from Shanghai Laboratory Animal Co., Ltd. (Shanghai, China).

2.2. Preparation of MXT liposomes

2.2.1. Preparation of PLGA-NPs

PLGA-NPs were prepared by an emulsion solvent extraction/evaporation method modified by Rodríguez-Nogales et al. (2016). Here oil phase was a mixture of acetone and dichloromethane (1:1, v/v), aqueous phase was poloxamer188 in water (3%, w/w). At first, 10 mg of PLGA was dissolved in 2 mL of oil phase, and then this PLGA solution was added slowly into 4 mL of aqueous phase at a rate of 1 mL/min by a needle with an inner diameter of 0.17 mm. PLGA emulsion (O/W) was formed with stirring at 1000 rpm for 10 min. Next, the PLGA emulsion was continually stirred for 4 h at room temperature to evaporate oil phase, and the suspension of PLGA was formed. Large aggregates in the suspension were precipitated by centrifugation (Avanti J-30I Centrifuge, Beckman Coulter, Fullerton, CA, USA) at 4 °C, 5000 rpm for 5 min. The PLGA NPs in the supernatant was collected by ultracentrifugation at 20,000 rpm for 10 min. At last, 2 mg of PLGA-NPs sample was gained by vacuum drying. The PLGA-NPs sample was diluted to be 0.2 mg/mL with deionized water for later experiment.

2.2.2. Preparation of PLGA-NPs-encapsulated liposomes

Liposomes were prepared by a lipid film hydration method (He et al., 2014). At first, a lipid mixture composed of HSPC, DSPC, Chol and DSPE-PEG2000 (Table 1) was dissolved in 10 mL of above-mentioned oil phase. This mixed solution was dried at 30 °C under

Table 1
Formulations of the liposomes.

Formulation	HSPC (mmol)	Chol (mmol)	DSPE-PEG2000 (mmol)	DSPC (mmol)	PLGA-NPs (mL)
L1	0.2	0.1	0.006	0	9
L2	0.18	0.12	0.006	0	9
L3	0.15	0.15	0.006	0	9
L4	0.15	0.15	0.006	0	NA[a]
L5	0.1	0.15	0.006	0.05	9
L6	0	0.15	0.006	0.15	9

[a] "NA" meant not application.