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Bypassing multidrug resistant ovarian cancer using ultrasound responsive doxorubicin/curcumin co-deliver alginate nanodroplets

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ABSTRACT

Ultrasound-responsive perfluorocarbon nanoemulsions are a class of new multifunctional smart nanocarriers which combine diagnostic properties with therapeutic properties and release their drug payload in a controlled manner in response to ultrasound. Therefore, combination therapy using chemotherapeutic and chemosensitizing agents co-entrapped in these nanocarriers seems beneficial for cancer treatment. In the present study, multifunctional smart alginate/perfluorohexane nanodroplets were developed for co-delivery of doxorubicin and curcumin (a strong chemosensitizer). The nanodroplets with the average particle size of 55.1 nm were synthesized via nanoemulsion process. The entrapment efficiency of doxorubicin was 92.3%. To improve curcumin entrapment into the alginate shell, Span 60 was added to the formulation as a co-surfactant and finally curcumin entrapment of about 40% was achieved. Ultrasound-mediated drug release kinetic was evaluated at two different frequencies of 28 kHz (low frequency) and 1 MHz (high frequency). Low frequency ultrasound resulted in higher triggered drug release from nanodroplets. The nanodroplets showed strong ultrasound contrast via droplet to bubble transition as confirmed via B-mode ultrasound imaging.

Enhanced cytotoxicity in adriamycin-resistant A2780 ovarian cancer cells was observed for Dox-Cur-NDs compared to Dox-NDs because of the synergistic effects of doxorubicin and curcumin. However, ultrasound irradiation significantly increased the cytotoxicity of Dox-Cur-NDs. Finally, *in vivo* ovarian cancer treatment using Dox/Cur-NDs combined with ultrasound irradiation resulted in efficient tumor regression. According to the present study, nanotherapy of multidrug resistant human ovarian cancer using ultrasound responsive doxorubicin/curcumin co-loaded alginate-shelled nanodroplets combined with ultrasound irradiation could be a promising modality for the future of cancer treatment.

1. Introduction

In the past decades, the advances in nanotechnology have overcome the serious problems of conventional chemotherapy such as severe systemic toxicity of chemotherapeutic drugs, low bioavailability and low tumor growth inhibition efficiency and development of drug resistance in tumor cells [1,2].

The development of ultrasound responsive phase-shift perfluorocarbon (PFC) nanodroplets that release their drug cargo locally in the target tissue under the action of ultrasound has allowed the combination of different functionalities in on carrier system. In addition to passive targeting of tumor tissue and ultrasound-controlled drug release which allow double tumor targeting, these nanodroplets serve as ultrasound contrast agents which provide image-guided tumor targeted cancer therapy [3–6]. Combination of ultrasound contrast property with ultrasound-controlled drug release in these nanodroplets makes them promising nanocarriers which can provide real-time information in a safe and cost effective way.

These nanodroplets with a proper design and size (less than 200 nm) are expected to have relatively long circulation time, avoid extravasation to normal tissues [7] and effectively accumulate in tumor tissue via EPR effect (the enhanced permeability and retention) [8]. Nanoparticles in order to effectively extravasate in tumor tissue need to have sufficient circulation time via avoiding recognition by the reticuloendothelial system (RES) [9]. Therefore, alginate-Tween 20-Span60 composite was chosen to cover and stabilize the PFC nanodroplets. Alginate is a biocompatible hydrophilic

Abbreviations: Dox, doxorubicin; Cur, curcumin; Dox-NDs, doxorubicin-loaded nanodroplets; Cur-NDs, curcumin-loaded nanodroplets; Dox-Cur-NDs, doxorubicin/curcumin-loaded nanodroplets; Dox- Cur-NDs-US, doxorubicin/curcumin-loaded nanodroplets combined with ultrasound.

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biopolymer which suppresses blood protein adsorption and Tween 20-Span 60 eliminate particle recognition by the cells of the reticuloendothelial system.

PFC nanodroplets are stable at physiological environment (37 °C) while under the action of ultrasound vaporize and convert into microbubbles [10,11]. This effect is called acoustic droplet vaporization (ADV) [12]. Coalescence of primary small microbubbles results in formation of larger microbubbles with long-lasting stronger ultrasonic contrast [13]. The expansion of nanodroplets via ADV results in local triggered release of encapsulated drug from nanodroplets at the site of ultrasound irradiation and enhances the intracellular uptake of drug molecules by the target cells, which results in effective tumor treatment.

Doxorubicin is one of the most active drugs in current chemotherapy. Doxorubicin is an anthracycline antibiotic which has been effective in the treatment of a wide range of solid tumors [14]. However, doxorubicin treatment efficiency in clinical trials has been very poor which might be because of its severe toxicity, multidrug resistance and narrow therapeutic window [15]. One effective way to improve doxorubicin treatment efficiency is nanoparticle based combination therapy. It has been shown that the combination of chemotherapeutic agents with chemosensitizers in one nanocarrier improves the chance of overcoming multidrug resistance (MDR) in cancer cells, decreases side effects and significantly enhances the therapeutic effect [16–18]. Therefore, co-delivery of doxorubicin, a chemotherapeutic agent, and a chemosensitizer using novel stimuli-responsive multifunctional alginate/PFH nanodroplets is expected to result in effective treatment of MDR cancers. It has been reported that combination therapy using chemotherapeutic agents and chemosensitizers relsuts in more efficient anti-tumor effects and overcomes MDR more effectively compared to combined multiagents [18,19]. Therefore, curcumin, which is an ideal chemosensitizer, was chosen for combination therapy with doxorubicin in PFH nanodroplets.

It has been reported that curcumin, the polyphenol constituent of turmeric, reverses MDR in cancerous cells by suppressing the overexpression of P-glycoproteins and reduces the side effects of chemotherapy [20,21]. However, its clinical applications have been hindered due to its poor water solubility, low bioavailability and poor absorption [22,23]. Therefore, development of a stable nanoformulation of curcumin with enhanced solubility via alginate/PFH nanodroplets will improves its clinical application.

Novel doxorubicin-loaded multifunctional ultrasound-responsive alginate/PFH nanodroplets were successfully developed in our previous study [24]. In the present study, alginate/PFH nanodroplets were developed for co-delivery of doxorubicin and curcumin, and their cancer treatment efficacy was evaluated *in vitro* and *in vivo*.

2. Experimental

2.1. Materials

Doxorubicin hydrochloride (2 mg/mL) was purchased from EBEWE Pharma (Unterach, Austria). Curcumin sodium alginate, perfluorohexane, and Tween 20, phosphate-buffered saline (PBS, pH 7.4) and dialysis membranes (molecular weight cutoff, 12000) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and solvents were of analytical-grade.

Non-resistant (A2780) and adriamycin resistant A2780 ovarian cancer cells (A2780 ADR) were supplied from Pasteur Institute (Tehran, IR). High and low frequency ultrasounds were generated via a 1-MHz therapeutic unit (SM3670, Shrewsbury Medical Ltd., Shropshire, UK) and a custom-made 28 kHz system. Ultrasound imaging was performed using a 12-MHz linear transducer (Acuson Sequoia 512, Siemens, Mountain View, CA) to monitor ADV of nanodroplets.

2.2. Preparation of Alginate/PFH nanodroplets

Cur/Dox-loaded alginate-shelled PFH nanodroplets were synthesized via nano-emulsion process [24]. Briefly, doxorubicin and curcumin dissolved and dispersed in Tween 20 solution were added to perfluorohexane (PFH) and distilled deionized (DD) water under stirring and homogenized for 2 min at 24,000 rpm using Ultra-Turrax SG215 homogenizer. Then, polymer solution (alginate 1.5%w/v) was added drop-wise to the emulsion while homogenized at 13,000 rpm for 3 min. Finally, $CaCl_2$ solution (0.2%w/v) was added dropwise to the emulsion under homogenization at 3000 rpm for 3 min. The detail of formulation of nanodroplets are presented in Table 1.

2.3. Cells

Non-resistant (A2780) and adriamycin resistant (A2780 ADR) human ovarian cancer cells (Pasture Institute, Tehran, IR) were seeded (1×10^4 cells/well) in 96-well plates and incubated in RPMI medium at 37 °C in a humidified atmosphere containing 5% CO_2.

2.4. Animals

Animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by Pasteur Institute of Iran, Tehran, Iran. To establish a human ADR-resistant ovarian cancer xenograft tumor, 6–8 weeks old female BALB/c mice (Pasteur Institute, Tehran, IR) were inoculated with A2780 ADR human ovarian cancer cells as followed: cell suspension (7×10^5 cells/100 μl/mouse) was injected subcutaneously to the flank of un-anaesthetized mice. Tumor appeared at the injection site one week after transplantation.

2.5. Characterization of alginate/PFH nanodroplet

The average particle size (hydrodynamic diameter, nm), polydispersity index (PDI) and zeta potential of nanodroplets were determined by dynamic light scattering (Zeta-sizer 3000HS, Malvern Instruments, Malvern, UK). 100 μl of nanodroplet solution was diluted with 2 ml of ultrapure water and the measurements were done at a scattering angle of 90°. All samples were prepared in triplicate.

The shape and morphology of drug-loaded nanodroplets were observed under a transmission electron microscope (TEM, H-7650; Hitachi, Tokyo, Japan). Before imaging, the nanodroplets were placed on a carbon-coated copper grid and dried at room temperature.

2.5.1. Entrapment efficiency

The amount of entrapped doxorubicin in DOX/Cur-NPs was determined by centrifuging the nanodroplet solution and measuring the absorbance of doxorubicin in supernatant with a UV–vis spectrophotometer at 480 nm (UV-1601; Shimadzu, Japan). The entrapment efficiency of curcumin was measured as followed: 1 mg of freeze-dried nanoparticles was dispersed in 2 ml of PBS and ethyl acetate (1:1) solution and shaked for 2 min. Then, ethyl acetate phase was separated and the amount of free curcumin in ethyl acetate was determined via UV–vis spectrophotometery at 424 nm.

The entrapment efficiency (EE) was calculated by the following equation:

$$EE(\%) = \frac{(M_0) - (M_f)}{M_0} \times 100, \quad (1)$$