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Ultrasonic nanotherapy of breast cancer using novel ultrasound-responsive alginate-shelled perfluorohexane nanodroplets: *In vitro* and *in vivo* evaluation

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ABSTRACT

Development of a new class of multifunctional ultrasound-responsive smart nanocarriers that combine therapeutic properties with diagnostic imaging has gained great attention in recent years. Here, we describe the results of ultrasonic nanotherapy of breast cancer using novel alginate-stabilized perfluorohexane nanodroplets. Doxorubicin (Dox)-loaded multifunctional nanodroplets (Dox-NDs) were synthesized via nanoemulsion process and evaluated in vitro and in vivo with focus on cytotoxicity, hemolytic activity, biodistribution, biosafety, and antitumor activity. Echogenic property of nanodroplets was confirmed by B-mode ultrasound imaging. Tumor therapy using Dox-NDs combined with sonication (Dox-ND-US) resulted in strong in vivo antitumor activity characterized by tumor regression which could be because of on demand efficient ultrasound-aided drug release from nanodroplets in tumor tissue under the action of ultrasound. Dox concentration in tumor area for Dox-ND-US treated group reached 10.9 μg/g after sonication for 4 min (28 kHz, 0.034 W/cm^2), which was 5.2-fold higher compared to non-sonicated Dox-NDs group. The cardiotoxicity of Dox-NDs was much lower than that of free Dox and no hemolytic activity was observed for Dox-NDs.
Strong therapeutic effect of these multifunctional nanodroplets combined with their ultrasound-contrast property indicated that this drug delivery system has a great potential in smart cancer-therapy.

1. Introduction

In the past decades, a lot of progress has been made in order to decrease the negative side-effects of chemotherapeutic drugs, and improve the utilization of drugs through the following two methods: (1) Active targeted nano-drug delivery (ATDD), which uses specific interactions between ligand-receptor and antibody-antigen to target specific cells [1–3] and (2) Stimuli-responsive nano-drug delivery (SRDD), which makes use of different properties to release the drug load from carriers only in response to environmental or physical stimuli, such as pH, temperature, light, or ultrasound [4–7].

These two methods have some advantages and disadvantages. For example, active targeting enhances the accumulation of chemotherapeutic drugs in tumor tissues and increases cellular uptake by receptor-mediated effect [3]; however, this method has a major limitation. Active targeted nano-drug delivery systems have no control on their drug release process in order to release drug locally in a specific target tissue. They release their drug payload in a burst or sustained passive manner in vivo [8]. Stimuli-responsive nanocarriers are able to overcome this limitation by controlling the location and amount of drug release [4,9]. However, the enhanced permeability and retention effect of tumor microvasculature allows extravasation of active targeted nanocarriers through large interendothelial gaps; yet, their intracellular drug concentration remains low because of insufficient cellular uptake. Therefore, it is necessary to develop a class of stimuli responsive nanocarriers which are able to increase the intracellular drug uptake by enhancing cell membrane permeability.

Besides, local application of external stimuli in vivo requires tumor imaging prior to, and during treatment. Biodistribution of drug carriers can also be monitored by imaging. Such information allows optimal timing of external stimulus application. Among feasible external stimuli, ultrasound is especially attractive because it is non-invasive, accessible, cost effective, and it is possible to combine imaging and therapeutic

Abbreviations: Dox, doxorubicin; Dox-NDs, doxorubicin-loaded Nanodroplets; Dox-ND-US, doxorubicin-loaded Nanodroplets combined with sonication.

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capabilities in one ultrasound-responsive agent [10,11]. Ultrasonic irradiation of tumor triggers drug release from the accumulated particles and transiently alters the permeability of cell membrane, which results in effective intracellular drug uptake by tumor cells [10,12,13]. Ultrasound irradiation can also increase the temperature in target tissue [14], and cause local hyperthermia which can trigger the release encapsulated drug from thermo-responsive drug carriers such as thermosensitive liposomes and micelles [15–17].

Ultrasound as an imaging modality provides real time information which can be improved using ultrasound contrast agents, i.e. microbubbles.

Microbubbles have been used as ultrasound contrast agents for many decades and their therapeutic application in drug and gene delivery are now being widely investigated [10,18,19]. However, some characteristics of microbubbles including their very short circulation time (minutes) and relatively large size (within micron range) hinder their effective extravasation into tumor tissue, which is essential for effective drug targeting. One way to overcome this problem is to use long-lasting acoustic phase shift perfluorocarbon nanodroplets that would effectively accumulate in tumor tissue and convert into microbubbles in situ by ultrasound exposure. Phase shift nanodroplets are composed of a liquid perfluorocarbon core and a stabilizing shell of lipid, polymer and/or protein [20–22].

Perfluorocarbon nanodroplets easily get converted into microbubbles under the action of ultrasound at sufficiently high rarefactional pressures [17,18]. The ultrasound-induced droplet-to-bubble transition is called acoustic droplet vaporization (ADV) [23,24]. Droplet-to-bubble transition via AVD and bubble oscillation-or so-called cavitation- triggers release of encapsulated drug and enhances intracellular uptake [20–22]. Also, the bubbles respond non-linearly to ultrasound, which makes them ideal ultrasound contrast agents for both B-mode and contrast- specific imaging techniques such as pulse-inversion [25].

Properly designed nanodroplets avoid extravasation to normal tissues and recognition by cells of the reticulo-endothelial system (RES); these properties prolong circulation time of nanoparticles after systemic injection. This in turn allows passive targeting of cancerous tissues [26]. Alginate was chosen for the stabilizing shell due to its favorable properties, including biocompatibility, low toxicity, relatively low cost and ease of gelation, and hydrophilic nature. The small size of nanodroplets (<100 nm) and their presence of non-ionic surfactant of Tween 20 in the composition of alginate shell which improves stealth properties from RES increase their circulation time which results in higher accumulation of drug in the tumor site through EPR effect (enhanced permeability and retention) [27].

In this study, in vitro and in vivo therapeutic and ultrasound contrast properties of doxorubicin-loaded alginate stabilized perfluorohexane (PFH) nanodroplets were evaluated using human breast carcinoma tumor xenografts inoculated in BALB/c mice. The results were expressed in terms of cytotoxicity, hemolysis activity, in vivo distribution, safety, cardiotoxicity and antitumor effects.

2. Materials and methods

2.1. Materials

Perfluorohexane (boiling point, 58–60 °C), sodium alginate and Tween 20 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Doxorubicin Hydrochloride (2 mg/ml) was obtained from EBEWE Pharma (Unterach, Austria). All other chemicals and solvents were obtained commercially as analytical-grade reagents and used as received without further purification or treatment. 28 kHz ultrasound was generated via a custom-designed sonicator in our co-worker's lab (Ultrasound Lab, Tarbiat Modares University, Tehran, Iran). Schematic of ultrasound setup is presented in our previous work [28].

2.2. Preparation of doxorubicin-loaded PFH/alginate nanodroplets

Doxorubicin-loaded nanodroplets were synthesized via nanoemulsion process as described in detailed in our previous work [28]. Briefly, perfluorohexane (PFH), doxorubicin and Tween 20 (surfactant) were homogenized in distilled de-ionized water for 2 min at 24,000 rpm using Ultra-Turrax SG215 homogenizer. To stabilize the PFH droplets, alginate solution was added drop-wise to the mixture under homogenization at 13,000 rpm for 3 min. Finally, $cacl_2$ solution was added drop-wise to the emulsion under homogenization to crosslink alginate chains. Nanodroplets were characterized for: morphology, by transmission electron microscopy (TEM); size distribution, polydispersity index and zeta-potential by dynamic light scattering; drug entrapment and release by UV–vis spectroscopy and echogenic properties by B-mode ultrasound imaging. Entrapment efficiency and passive and ultrasound-aided drug release were determined by the procedures described in our previous work [28].

2.3. Animals

Animal study was performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health. All experiments were conducted in accordance the Guiding Principles for the Use of Laboratory Animals (Pasteur Institute of Iran, Tehran, Iran, permit number: 91/27, 2.13). Six to 8 weeks old female BALB/c mice were obtained from Pasteur Institute (Tehran, IR). For tumor inoculation, 4T1 human breast cancer cells suspension (7×10^5 cells/100 μL/mouse) was injected subcutaneously to the flank of unanesthetized mice. One week after transplantation, tumor formed at the injection site.

2.4. Monitoring acoustic droplet vaporization

Acoustic droplet vaporization and formation of microbubbles from nanodroplets at 37 °C was monitored by ultrasound imaging using a 12-MHz linear transducer (Acuson Sequoia 512, Siemens, Mountain View, CA). In order to simulate the viscous extracellular matrix, nanodroplets were introduced into warm 1%w/v agarose solution in phosphate buffered saline (PBS) and cooled down to room temperature for gel formation.

2.5. Cytotoxicity

The cytotoxic effects of Dox-loaded nanodroplets were evaluated by MTT assay. 4T1 Cells were seeded into 96-well plates at a density of 10^4 cells/well for 24 h, then incubated in RPMI medium with various doses of free doxorubicin (Dox) and Dox-loaded nanodroplets (Dox-NDs) for 24 h and 48 h. Blank control was the cells incubated only with RPMI medium. To examine the effect of ultrasound on cell viability, another 96-well plate containing the same dosage of nanodroplets was prepared and the cell underwent ultrasound exposure for 4 min (28 kHz ultrasound, 0.034 W/cm^2). The concentration of Dox in each nanodroplets dose was the same as the free Dox. Then, MTT solution was added to the cells for 4 additional hours and washed with PBS. The formazan crystals were dissolved using 100 μL of dimethyl sulfoxide (DMSO). The plates were then read by a microplate reader at 570 nm and at a reference wavelength of 650 nm. The cellular uptake was observed using a fluorescence microscope (BX51; Olympus, Japan). Cytotoxicity of blank nanodroplets was also evaluated at droplet concentration of up to 1 mg/ml.

2.6. Hemolysis studies

Blood samples from healthy volunteers were collected in heparin-coated tubes and fresh red blood cell (RBCs) suspensions were obtained by PBS washing and centrifugation (1500 rpm for 5 min). The RBC