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Novel alginate-stabilized doxorubicin-loaded nanodroplets for ultrasounic theranosis of breast cancer

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ABSTRACT

Perfluorocarbon nanoemulsions are a new class of multifunctional stimuli-responsive nanocarriers which combine the properties of passive-targeted drug carriers, ultrasound imaging contrast agents, and ultrasound-responsive drug delivery systems. Doxorubicin-loaded alginate stabilized perflourohexane (PFH) nanodroplets were synthesized via nanoemulsion preparation method and their ultrasound responsivity, imaging, and therapeutic properties were studied. Doxorubicin was loaded into the nanodroplets (39.2 nm) with encapsulation efficiency of 92.2%. In vitro release profile of doxorubicin from nanodroplets was an apparently biphasic release process and 12.6% of drug released from nanodroplets after 24 h incubation in PBS, pH = 7.4. Sonication with 28 kHz therapeutic ultrasound for 10 min triggered droplet-to-bubble transition in PFH nanodroplets which resulted in the release of 85.95% of doxorubicin from nanodroplets. Microbubbles formed by acoustic vaporization of the nanodroplets underwent inertial cavitation.

In the breast cancer mice models, ultrasound-mediated therapy with doxorubicin-loaded PFH nanodroplets showed excellent anti-cancer effects characterized by tumor regression. Complete tumor regression was observed for the group in which doxorubicin-loaded nanodroplets were combined with ultrasound, whereas the tumor growth inhibition of doxorubicin –loaded nanodroplets was 89.6%. These multifunctional nanodroplets, with excellent therapeutic and ultrasound properties, could be promising drug delivery systems for chemotherapeutic application.

1. Introduction

Developing stimuli-responsive nanocarriers which release their drug cargo only in response to environmental or physical stimuli, such as pH, hyperthermia, light, or ultrasound is an interesting approach to tumor-targeting [1]. Ultrasound as a drug delivery modality has a number of attractive features and could be applied with a variety of drug carriers. Ultrasound is especially attractive because it is non-invasive, accessible, cost effective, and is possible to direct ultrasound toward deeply located body sites in precise energy deposition patterns and sonicate tumors with millimeter precision [2–9].

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 [2,10,11]. Ultrasound- mediated drug delivery like all energy-based tumor treatment modalities requires tumor imaging prior to treatment. Combining tumor imaging and treatment by ultrasound is especially attractive because it is cost-effective and makes the procedure more simple.

Microbubbles have been used as ultrasound contrast agents in ultrasound imaging for many decades. Nowadays, their therapeutic application as drug carriers and enhancers of drug and gene delivery are being widely investigated [2–5,12–14]. However, these systems present some difficulties which make them hard to utilize. They have very short circulation time (minutes) and large size (within micron range) which hinder their effective extravasation into tumor tissue, which is essential for effective drug targeting.

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Furthermore, injection of microbubbles may cause some side effects, such as high osmotic pressure and blood vessel dilation. The way to overcome these limitations may consist in developing acoustic phase shift nanodroplets that would effectively accumulate in tumor tissue by passive or active targeting and then convert into microbubbles in situ by ultrasound [15,16].

These droplets are usually composed of perfluorocarbon (PFC) and coated materials which are stable in the blood stream. By ultrasound irradiation, PFC nanodroplets undergo an instant phase transition into gas bubbles called acoustic droplet vaporization or ADV. ADV promotes vascular permeability and ultrasound ablation for tumor tissue [17,18], and also triggers the release of encapsulated drugs from nanodroplets [19,20]. The droplet to bubble transition process occurs with inertial cavitation (IC) when the applied acoustic pressure is higher than a particular threshold [21]. IC is the major effect of ADV that cause cell damage via inducing heat and mechanical force in therapeutic ultrasound applications [22–28]. These features of PFC nanodroplets make them promising candidates for overcoming the limitations of contrast bubbles and suggest that acoustic phase shift nanodroplets could be ideal theranostic agents for tumor targeted drug delivery.

Doxorubicin (DOX) is one of the most commonly used chemotherapeutic drugs in current oncological chemotherapy. DOX has been approved for treatment of a number of solid tumors, such as leukemia, aggressive lymphoma, breast, lung, ovarian, and broad types of cancers [29]. However, DOX causes severe side effects when administered at high dose systematically [30,31]. The encapsulation of DOX into micro and nanocarriers would protect normal tissues from exposure and toxicity. Therefore, several studies have been done on delivering DOX to targeted tissue by microspheres [32–34], micelles [31,35–37], nanoparticles [38,39] or synthetic conjugates [40,41] while minimizing the systemic exposure of the drug. Among various methods, polymeric nanoparticles with charges can load ionic drugs such as DOX easily by a simple absorption method with the highest entrapment efficiency [33].

In this work, the aim was to develop DOX-loaded multifunctional alginate-coated perfluorohexane (PFH) nanodroplets which provide efficient local treatment of tumor by a hybrid strategy involving both controlled triggered drug delivery and ADV-mediated destruction of cancer cells and, also serve as contrast-enhanced ultrasound imaging agents. The development and characterization of alginate-coated PFH acoustic nanodroplets that fits these functional demands have been described.

2. Experimental

2.1. Materials

Doxorubicin Hydrochloride (2 mg/ml) was obtained from EBEWE Pharma (Unterach, Austria). Sodium Alginate, Perfluorohexane and Tween 20, phosphate-buffered saline (PBS, pH 7.4) and dialysis membranes (molecular weight cutoff, 12000) were supplied by Sigma-Aldrich (St. Louis, MO, USA). The agar used for in-vitro phantom experiments was purchased from Invitrogen (CA, USA).

All other chemicals and solvents were of analytical grade and used as received without further purification or treatment.

2.2. Preparation of doxorubicin-loaded alginate stabilized PFH nanodroplets

Doxorubicin-loaded nanodroplets were obtained via nanoemulsion process. Briefly, perfluorohexane (PFH), doxorubicin and Tween 20 (surfactant) were homogenized in distilled deionized water for 2 min at 24,000 rpm using Ultra-Turrax SG215 homogenizer. Then, polymer solution (alginate 1.5%w/v) was added drop-wise whilst the mixture was 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. Nanodroplets were characterized for: morphology, by transmission electron microscopy (TEM); size distribution, and polydispersity index by dynamic light scattering; and drug entrapment and release by UV–vis spectroscopy.

2.3. Nanodroplets introduction into gel

To introduce nanoemulsion into agarose gel, 1% agarose solution in phosphate buffered saline (PBS) was prepared and 200 μL of a nanodroplet emulsion was purred into it.

2.4. Cell culture

4T1 human breast cancer cells were obtained from Pasteur Institute (Tehran, IR). The cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). Cells were cultured at 37 °C in humidified air containing 5% CO2.

2.5. Animal procedures

Animals 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 (National Institutes of Health, Bethesda, MD, USA).

Six to 8 weeks old female BALB-c mice were obtained from Pasteur Institute (Tehran, IR). For inoculation, 4T1 human breast cancer cells were suspended in serum-free RPMI-1640 medium and injected subcutaneously to the flank of unanaesthetized mice (7×10^5 cells/100 μL/mouse). One week after transplantation, tumor gelosis formed at the injection site. Mice with similar tumor areas (0.5–0.6 mm length and width of the tumor) were selected as the model animals for the pharmacodynamic study.

2.6. Characterization of doxorubicin-loaded nanodroplets

2.6.1. Particle size analysis

The average size distribution and polydispersity index of nanodroplets was determined by DLS using a Zeta-sizer 3000HS (Malvern Instruments, Malvern, UK).). The analysis was performed at a scattering angle of 90° after the nanodroplet solution diluted adequately with double-distilled water. The measurement yielded the mean size and polydispersity index (PI).

2.6.2. TEM

The morphology of drug-loaded nanodroplets was observed using a transmission electron microscopy (H-7650; Hitachi, Tokyo, Japan) operating at an acceleration voltage of 80 kV. To prepare sample for imaging, one drop of the solution was placed onto a 400-mesh carbon-coated copper grid and dried at room temperature.

2.6.3. Stability of the nanodroplets

Nanodroplets were suspended in phosphate-buffered saline (PBS) at pH 7.4 and incubated at 4 °C for predetermined time intervals. The stability of nanodroplets was determined by changes in their size and drug entrapment efficiency.

2.6.4. Determination of entrapment efficiency

To determine the entrapment efficiency, drug-loaded nanodroplets were separated from the solution by centrifugation