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Novel ultrasound-responsive chitosan/perfluorohexane nanodroplets for image-guided smart delivery of an anticancer agent: Curcumin

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ABSTRACT

Ultrasound-responsive nanodroplets are a class of new emerging smart drug delivery systems which provide image-guided nano-therapy of various diseases, especially cancers. Here, we developed multifunctional smart curcumin-loaded chitosan/perfluorohexane nanodroplets for contrast-ultrasound imaging and on-demand drug delivery. The nanodroplets were synthesized via nanoemulsion process. The optimal formulation with the size of 101.2 nm and 77.8% curcumin entrapment was chosen for release study and cytotoxicity evaluation. Sonication at the frequency of 1 MHz, 2 W/cm^2 for 4 min triggered the release of 63.5% of curcumin from optimal formulation (Cur-NDs-2). Ultrasound aided release study indicated that the concentration of perfluorohexane and the degree of acoustic droplet vaporization play important role in ultrasound-active drug release. B-mode ultrasound imaging confirmed strong ultrasound contrast of chitosan nanodroplets even at low concentrations via droplet to bubble transition. Finally, cytotoxicity of the ultrasound-responsive nanodroplets in the presence of ultrasound was evaluated in-vitro on 4T1 human breast cancer cells. Cell growth inhibitory effects of curcumin-loaded nanodroplets significantly increased by ultrasound exposure. According to the obtained results, these ultrasound responsive curcumin-loaded chitosan/perfluorohexane nanodroplets have a great potential for imaged-guided cancer therapy.

1. Introduction

Ultrasound is a widely available, noninvasive, and cost-effective diagnostic modality [1–4]. A novel ultrasound-mediated chemotherapeutic modality is based on systemic injection of phase-shift drug-loaded nanodroplets that vaporize and convert into microbubbles under the action of ultrasound. Acoustic phase shift nanodroplets effectively accumulate in tumor tissue by passive or active targeting and then convert into microbubbles in situ by ultrasound [5,6]. Under the action of ultrasound, perfluorocarbon (PFC) nanodroplets vaporize into gas bubbles via acoustic droplet vaporization or ADV which results in triggered release of encapsulated drugs from nanodroplets [7,8]. Expansion of nanodroplets from ADV induces mechanical tissue erosion and cell damage [9–10] and promotes vascular permeability and ultrasound ablation for tumor tissue [11,12]. These features of PFC nanodroplets make them promising candidates for overcoming the limitations of contrast bubbles such as 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 [5,6].

The polyphenol curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione),is one of the most active component of the perennial herb *Curcuma longa* (commonly known as turmeric) [13]. Curcumin has a wide range of therapeutic properties such as anticancer [14,15], antioxidant [15,16], anti-inflammatory [17], anti-arthritic [18], anti-ischaemic [19], and antiamyloid [20]. Curcumin suppresses many types of cancers [21], cures multidrug-resistant cancers [22], and shows a synergistic antitumor effect with other anticancer agents. However, curcumin is a hydrophobic molecule and has a poor aqueous solubility [23] which eliminates its bioavailability and therapeutic efficiency [24]. Therefore, developing a delivery system to improve the aqueous solubility and stability and subsequently the bioavailability of curcumin is important.

Ultrasound-responsive nanodroplets comprise a perfluorocarbon (PFC) core and a stabilizing shell of lipid, polymer and/or protein. In first generation of perfluorocarbon nanodroplets, perfluoropentane (PFP) was used as a droplet core [7,25–31]. PFP has a low boiling temperature (29 °C); therefore, PFP droplets have low stability. They easily turn to foam in blood circulation before reaching the target tissue and it is hard to control their droplet-to-bubble transition [7,29]. In order to obtain nanodroplets with higher stability, perfluorohexane (PFH) was chosen for the droplet core in this work because of its higher boiling

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point (58–60 °C) compared to other common perfluorocarbons including perfluoropentane.

Various PFC nanodroplet formulations have been developed for ultrasound controlled drug delivery and gene delivery. Most of them are comprised a block copolymer shell such as poly(ethylene oxide)-*co*-poly(L-lactide) (PEG-PLLA) or poly(ethylene oxide)-copolycaprolactone (PEG-PCL) [26,29–31], a lipid shell (DPPC, DSPE-PEG/cholesterol) [32], a protein shell (lung surfactant, albumin) [33–35] or a surfactant shell (perfluoroocatanoic acid) [36]. In order to design a cost effective nanodroplet formulation with high curcumin entrapment efficiency and no in-vivo toxicity, we considered using the well-known biocompatible polysaccharide, chitosan as the stabilizing shell. Chitosan, [(1–4)-2-amino-2-deoxy-_-D-glucan], is a cationic linear polysaccharide with interesting physicochemical properties such as its abundance, flexibility, nontoxicity, hydrophilicity, biocompatibility [37], biodegradability [38], and high resistance to heat [39] which makes it suitable for biomedical application including drug delivery [40]. Also, chitosan binds curcumin with high affinity at considerably high pH (pH = 7.0–10.5) through its functional amine groups [41]. Moreover, chitosan effectively interacts with different surfactants in aqueous solutions [42]. This could be used to tailor the size and other characteristics of nanoparticles.

The aim of the current study was to formulate and characterize a new curcumin-loaded multifunctional nanodroplet formulation composed of a chitosan/surfactant shell and a perfluorohexane (PFH) core to improve the bioavailability of curcumin and enhance its anti-tumor efficiency by image guided ultrasound-mediated triggered drug release.

2. Experimental

2.1. Materials

LMW chitosan, curcumin, Perfluorohexane and Tween 20, lecithin, phosphate-buffered saline (PBS, pH 7.4) and dialysis membranes (molecular weight cutoff, 12,000) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and solvents were obtained commercially as analytical-grade reagents. 4T1 human breast cancer cells were obtained from Pasteur Institute (Tehran, IR). Ultrasound was generated via a 1-MHz therapeutic unit (SM3670, Shrewsbury Medical Ltd., Shropshire, UK). 12-MHz linear transducer (Acuson Sequoia 512, Siemens, Mountain View, CA) was used to monitor ADV of nanodroplets.

2.2. Preparation of curcumin-loaded chitosan/PFH nanodroplets

Curcumin-loaded chitosan-stabilized PFH nanodroplets were prepared via nano-emulsion process [43]. Briefly, curcumin and lecithin (surfactant) solution in 98% ethanol 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 (chitosan 1.5%w/v in 1%v/v acetic acid, pH, 5) was added drop-wise to the emulsion while homogenized at 13,000 rpm for 3 min. For co-surfactant inclusion, Tween 20 was first added to the mixture of PFH and DD water, then the rest of the precursors were added as before. In order to separate the free curcumin from curcumin-loaded nanodroplets, the nanodroplets were centrifuged, then they were dispersed in PBS and ethyl acetate (1:1). The mixture was shaken to dissolve the free curcumin content in ethyl acetate and finally, the ethyl acetate phase containing free curcumin was separated.

2.3. Characterization of chitosan/PFH nanodroplet

2.3.1. Physicochemical properties

The average size, polydispersity index and zeta potential of nanodroplets were measured dynamic light scattering at a scattering angle of 90° using Zeta-sizer 3000HS (Malvern Instruments, Malvern, UK).

Morphology and shape of curcumin-loaded nanodroplets were observed using transmission electron microscopy, TEM, (H-7650; Hitachi, Tokyo, Japan). Sample was prepared for imaging, by pouring one drop of the solution on a 400-mesh carbon-coated copper grid and drying at room temperature.

The structural features of pure curcumin, chitosan and curcumin-loaded chitosan nanodroplets were determined by Fourier transform infrared (FTIR, Bomem MB 100 spectrometer, Bruker IFS 28) in the 4500–400 cm^{-1} spectral range using KBr pellets.

2.3.2. Determination of entrapment efficiency

To determine the entrapment efficiency of curcumin, nanodroplets were freeze dried (1 mg) and dispersed in 1 ml PBS, and then 1 ml of ethyl acetate was added. The mixture was shaken and ethyl acetate phase being separated. Free curcumin content in ethyl acetate phase was determined using a UV–Vis spectrophotometer at 424 nm (U.V-1601; Shimadzu, Japan). Samples were prepared and measured in triplicate. The percentage of drug entrapment efficiency (EE) was calculated using the following equation:

$$\textit{Entrapment Efficiency}\ (\%) = \frac{(\textit{Total amount of drug added}) - (\textit{Free amount of drug})}{\textit{Total amount of drug added}} \times 100$$

2.3.3. Stability of 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.

2.3.4. Stability of curcumin loaded into nanodroplets

To estimate the stability of curcumin in its free form and loaded into nanodroplets, they were mixed with PBS (pH 7.2), at the same curcumin concentration and poured into microcentrifuge tubes. The samples were incubated at 37 °C under shaking at 100 rpm. The concentration of remained curcumin at various time intervals was measured using a UV–vis spectrophotometer (λ = 424 nm). Each sample was tested in triplicate. The percentage of remaining curcumin was calculated as followed:

$$\textit{Remaining curcumin}\ (\%) = \frac{\textit{amount of curcumin at a fixed time}}{\textit{Total amount of curcumin added}} \times 100$$

2.3.5. In-vitro drug release study

2.3.5.1. Passive drug release. Release study was performed using dialysis sack method by dialysis tubing (MW cutoff 12,000 g/mol, Sigma, Canada). Two milliliters of nanodroplet solution was placed in dialysis membrane and immersed in 20 ml phosphate buffer pH 7.4 and citrate buffer pH 5.4 containing 0.1% Tween 20. The release study was carried out in a shaker incubator (MS MP8 Wise Stir Wertheim, Germany) with shaking rate of 100 rpm at 37 °C for 24 h. Two milliliters samples were withdrawn in predetermined time intervals and replaced with an equivalent volume of fresh buffer. The amount of released curcumin was measured at 424 nm using UV–vis spectrophotometer (U.V-1601; Shimadzu, Japan). To ensure that sustained release profile is not due to membrane, curcumin dispersion in the same concentration with curcumin nanodroplets was studied under the same condition for release.

2.3.5.2. Ultrasound-induced drug release. To determine the influence of 1-MHz ultrasound on curcumin release from nanodroplets, the nanodroplet solution was purred into Plexiglas scaffold with a latex cover and placed into a water bath at 37 °C, and then was sonicated for 0.5, 1, 2, 4 and 7 min with 2 W/cm^2. After processing, the nanodroplet