Ultrasonics Sonochemistry 28 (2016) 54–61

Contents lists available at ScienceDirect

Ultrasonics Sonochemistry

journal homepage: www.elsevier.com/locate/ultson

Targeted and ultrasound-triggered cancer cell injury using perfluorocarbon emulsion-loaded liposomes endowed with cancer cell-targeting and fusogenic capabilities

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ARTICLE INFO

Article history:
Received 14 May 2015
Received in revised form 30 June 2015
Accepted 30 June 2015
Available online 2 July 2015

Keywords:
Ultrasound
Liposome
Perfluorocarbon
HVJ envelope
Avidin
Cancer cells

ABSTRACT

This study investigated the targeting and ultrasound-triggered injury of cancer cells using anticancer drug-free liposomes that contained an emulsion of perfluoropentane (ePFC5) and were co-modified with avidin as a targeting ligand for cancer cells and the hemagglutinating virus of Japan (HVJ) envelope to promote liposome fusion with the cells. These liposomes are designated as ePFC5-loaded avidin/HVJ liposomes. ePFC5-loaded liposomes were sensitized to ultrasound irradiation. Liposomes modified with avidin alone (avidin liposomes) showed binding to MCF-7 human breast cancer cells, and liposomes modified with HVJ envelope alone (HVJ liposomes) were found to fuse with MCF-7 cells. The irradiation of MCF-7 cells with 1 MHz ultrasound (30 s, 1.2 W/cm^2, duty ratio 30%) combined with ePFC5-loaded avidin/HVJ liposomes resulted in a decrease in cell viability at 1 h after irradiation to 43% of that of controls without ultrasound irradiation or liposomes. The cell viability was lower than that of cells treated with ultrasound irradiation with ePFC5-loaded avidin liposomes or ePFC5-loaded HVJ liposomes. This indicates that co-modification of liposome with avidin and HVJ envelope could enhance ultrasound-induced cell injury in the presence of ePFC5-loaded liposomes.

1. Introduction

Liposomes are self-assembling phospholipid structures formed from a single continuous phospholipid bilayer. The liposomes have a long history of use as carriers for delivering therapeutic and diagnostic agents to cancerous lesions [1,2]. Drug delivery to solid tumors using liposomes is carried out using a combination of passive and active targeting. Liposomes modified with polyethylene glycol (PEG) have been used for passive solid tumor targeting because they can escape the reticuloendothelial system surveillance and can circulate in the blood stream for a long time [3], which results in their accumulation in tumor tissues due to an "enhanced permeability and retention" (EPR) effect [4]. The release of encapsulated drugs from liposomes depends on drug diffusion or slow degradation of the lipid bilayers. Active solid tumor targeting uses liposomes engineered to have a selective affinity for cancer cells and/or allow triggered drug release at the tumor site [5]. To increase their binding affinity for tumors, liposomes have been modified with targeting ligands such as antibody and non-antibody molecules (e.g., RGD peptides to target integrin and folate to target its receptors) [5,6]. A number of studies have reported on liposomes that can release encapsulated drugs in response to environmental stimuli such as acidic pH, high temperature, and ultrasound [5].

Several types of liposomes have been designed for ultrasound-mediated drug release [7]. Many of these use temperature-sensitive liposomes [8–10] that release the encapsulated drug upon ultrasound-induced mild hyperthermia of 40–42 °C, a few degrees above physiological temperature [11–14]. Previous studies developed a new type of liposome for ultrasound-mediated drug release based on an emulsion containing liposomes (eLiposomes) that releases the encapsulated drug by an ultrasound-induced acoustic effect [15,16]. These eLiposomes contain nano-emulsion droplets of the low-boiling-point liquid perfluorocarbon (PFC), which are converted to much larger gas bubbles via ultrasound-induced acoustic droplet

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http://dx.doi.org/10.1016/j.ultsonch.2015.06.032
1350-4177/

vaporization (ADV). The gas bubbles generated by ADV stretch and disrupt the liposome membrane, releasing the encapsulated drug. Previous studies [17–19] reported the targeting of eLiposomes to cancer cells via specific surface ligands. The eLiposomes were incorporated into endosome in cancer cells by endocytosis. Ultrasound irradiation then caused expansion of the emulsion droplets in both the eLiposomes endosomes, resulting in release of the encapsulated anticancer drug doxorubicin into the cytosol.

Cancer cells have higher expression of ATP binding cassette (ABC) transporters, membrane proteins that efflux chemotherapeutic agents from cells, resulting in decreased intracellular accumulation of agents and efficiency of chemotherapy. ABC transporter overexpression results in multidrug resistance (MDR), as these proteins have broad substrate specificity and can efflux a variety of drugs belonging to different classes. Therefore, many research groups are now investigating the use of liposome carriers for the co-delivery of anticancer drugs with either inhibitors or gene silencers of the ABC transporter proteins [20]. In addition, it is important to establish new cancer therapies based on cell-killing mechanisms that differ from those of conventional anticancer drugs and are unrelated to drug efflux transporters. To the best of our knowledge, there are no reports describing targeted, ultrasound-induced cancer cell killing using liposomes without anticancer drugs.

The present study aims to determine the feasibility of targeted, ultrasound-mediated cancer cell injury using anticancer-drug-free eLiposomes containing nanoemulsions of perfluoropentane (PFC5). Here the liposomes are co-modified with 2 components to enhance direct internalization of the encapsulated PFC5 nanoemulsion into the cytosol of cancer cells: avidin, which has an affinity for cancer cells [21–24], and the envelope of the hemagglutinating virus of Japan (HVJ), which promotes the fusion of liposomes to cells [25–27]. These liposomes are designated as ePFC5-loaded avidin/HVJ liposomes. We evaluated ultrasound-triggered disruption of ePFC5-loaded liposomes, avidin-mediated binding of liposomes to cancer cells, and HVJ envelope-mediated fusion of liposomes to cells. We then investigated ultrasound-mediated cancer cell injury by ePFC5-loaded avidin/HVJ liposomes. We hypothesized that cancer cells would be injured by the following sequence of events: (i) avidin-mediated liposome binding to target cancer cells, (ii) subsequent HVJ envelop-mediated liposome fusion to the cells, resulting in internalization of PFC5 emulsion droplets directly into the cytosol, and (iii) ultrasound-mediated vaporization of PFC5 emulsion droplets, physically damaging the cancer cells internally.

2. Materials and methods

2.1. Materials

A freeze-dried empty liposome comprising 1,2-dimyristoyl-*sn*-glycero-3-phosphatidic acid (DMPA), 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), and cholesterol at a molar ratio of 1:4:5 (COATSOME® PL-1614KS), synthesized by NOF Corporation (Tokyo, Japan), was used to prepare ePFC5-loaded avidin/HVJ liposomes. PFC5 (1,1,1,2,3,4,4,5,5,5-decafluoropentane) was purchased from Sigma Aldrich (St. Louis, MO, USA). 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidic acid (DPPA), purchased from Sigma Aldrich, was used as a phospholipid surfactant to stabilize PFC5 emulsion droplets. Bis[*N*,*N*-bis(carboxymethyl)-aminomethyl]fluorescein (Calcein; Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 3,8-Diamino-5-[3-(diethylmethylammonio)propyl]-6-phenylphenanthridinium diiodide (PI; Dojindo Laboratories, Mashiki, Japan) were used as membrane-impermeable fluorescent dyes. Functionalized and PEGylated phospholipid, *N*-[*N*′-(succinimidyloxy glutaryl)aminopropyl polyoxyethylene oxycarbonyl]-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE-PEG-NHS; molecular weight of PEG = 2000), was purchased from NOF Corporation (SUNBRIGHT® DSPE-020GS) and was used to introduce the functional groups into liposomes. Avidin (Nakarai Tesque, Kyoto, Japan) was used as a targeting ligand to cancer cells. HVJ envelope (GenomOne-CF) was purchased from Ishihara Sangyo Kaisha, Ltd. (Osaka, Japan) and was used to endow liposomes with the capability to fuse to cells. The chemicals used in this study were of guaranteed reagent grade and did not require further purification.

2.2. Cell culture and media

The human breast cancer cell line MCF-7 was purchased from the RIKEN cell bank, Tsukuba, Japan. MCF-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Nakarai Tesque) supplemented with 10% (v/v) fetal bovine serum (FBS; Life Technologies Corporation, Waltham, MA, USA), 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 0.25 μg/mL of amphotericin B (Nakarai Tesque). Cells were maintained at 37 °C in a 5% CO_2 atmosphere.

2.3. Preparation of ePFC5-loaded avidin/HVJ liposomes

A nano emulsion of PFC5 was prepared according to methods described previously [21–24]. Specifically, 1 mL of DPPA solution (10 mg/mL in chloroform/acetic acid [95:5, v/v]) was vacuum dried in a round-bottomed flask and then hydrated by rotation in Dulbecco's phosphate buffered saline (2 mL) without calcium chloride or magnesium chloride [D-PBS(−)]. After hydration, PFC5 (600 μL) was added to the sample. An emulsion was made by sonicating the sample on ice using a 24-kHz ultrasonic processor (UP200S with sonotrode S1, Hielscher Ultrasonics GmbH, Teltow, Germany)at an emission power of 35 W for 90 s (30 s each with 1 min interval between sonications). The emulsion was then sequentially extruded 10 times through a 50-nm polycarbonate filter using an Avanti® Mini-Extruder (Avanti Polar Lipids, Inc., Alabaster, AL, USA).

To prepare the ePFC5-loaded avidin/HVJ liposomes, freeze-dried empty liposome (20 mg), DSPE-PEG-NHS (4 mg), and avidin (2 mg) were added to the prepared PFC5 nano-emulsion. To assay for binding or fusion of avidin/HVJ liposome to cancer cells, 1 mL of calcein solution (10 mM) or PI solution (200 μg/mL in saline) was loaded into liposomes instead of the PFC5 nano-emulsion. After mixing, the suspension was incubated for 8 h at 4 °C in the dark using a tube rotator. The 1.5-mL tube was then centrifuged (15000 rpm) for 60 min at 4 °C and the supernatant was removed. The recovered liposomes were washed 3 times with D-PBS(−), and finally suspended in D-PBS(−) (1 mL). The obtained suspension of avidin-modified liposomes was sequentially extruded 10 times through an 800-nm polycarbonate filter using an Avanti® Mini-Extruder. This suspension was then mixed with HVJ envelope (0.6 mg) and incubated for 2 h at 37 °C in the dark using a tube rotator. After washing 3 times (15000 rpm for 60 min at 4 °C) to remove the free HVJ envelope, the precipitate was suspended in 1 mL of D-PBS(−) to obtain avidin/HVJ liposomes. As a control, liposomes modified with avidin alone (avidin liposomes) were prepared as mentioned above but without the addition of HVJ envelope. Liposomes modified with HVJ alone (HVJ liposomes) were prepared without avidin. Naked liposomes, without modification, were prepared without avidin or HVJ. The liposome concentration of the prepared suspension was determined by measuring the optical density at a wavelength of 600 nm (OD_{600}).