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Ultrasound-mediated drug delivery using liposomes modified with a thermosensitive polymer

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ABSTRACT

Ultrasound-mediated drug delivery was established using liposomes that were modified with the thermosensitive polymer (TSP) poly(NIPMAM-*co*-NIPAM), which sensitized the liposomes to high temperatures. TSP-modified liposomes (TSP liposomes) released encapsulated calcein under 1 MHz ultrasound irradiation at 0.5 W/cm^2 for 120 s as well as the case under incubation at 42 °C for 15 min. In addition, uptake of the drug released from TSP liposomes by cancer cells was enhanced by ultrasound irradiation. In a cell injury assay using doxorubicin (DOX)-loaded TSP liposomes and ultrasound irradiation, cell viability of HepG2 cells at 6 h after ultrasound irradiation (1 MHz, 0.5 W/cm^2 for 30 s) with DOX-loaded TSP liposomes (TSP/lipid ratio = 1) was 60%, which was significantly lower than that of the control conditions such as DOX-loaded TSP liposomes alone and DOX-loaded intact liposomes under ultrasound irradiation.

1. Introduction

Drug delivery systems (DDSs) using lipid- or polymer-based nanoparticles have been intensively investigated to improve the pharmacological properties of administered drugs [1]. In particular, since proposed in the 1970s, liposomes, self-assembled structures of phospholipids that form single continuous phospholipid bilayers, have had a long history as carriers for delivering therapeutic and diagnostic agents to cancerous lesions [2]. The strategies used with liposomes for drug delivery to solid tumors are categorized as passive targeting and active targeting. For passive targeting of solid tumors, liposomes modified with polyethylene glycol (PEG) have been used because they can evade surveillance by the reticuloendothelial system and circulate in the blood stream for long time [3], which results in their accumulation at tumor tissues due to an "enhanced permeability and retention" (EPR) effect [4]. However, the release of encapsulated drugs from liposomes depends either on the diffusion of the drug or the slow degradation of liposome lipid bilayer.

For active targeting of solid tumors, liposomes have been engineered to mechanism to release drug in the region of a tumor. A number of studies have been reported on liposomes that released encapsulated drugs in response to environmental stimuli such as acidic pH, light, and high temperature [5]. The most promising liposomes are temperature-sensitive liposomes (TSLs) that use dipalmitoylphosphatidylcholine (DPPC) as the primary liposome lipid [6]. TSLs release encapsulated drugs near the melting phase transition temperature (T_m) of the lipid bilayer, at which the structure of the lipid membrane changes in conjunction with its transition from a gel to a liquid crystalline phase. Currently, drug release from TSLs can be induced by mild hyperthermia at 39–40 °C, a few degrees above physiological temperature [7]. As a source of hyperthermia to trigger drug release from TSLs, Dromi et al. [8] employed high intensity, focused ultrasound that was more advantageous in terms of non-invasiveness as compared with other hyperthermia methods such as microwave, IR laser, or radio frequency heating that require an interstitial needle or insertion of an antenna.

Another strategy for designing TSLs was proposed by Kono et al. [9]. They modified liposomes using a thermosensitive polymer (TSP), such as poly(*N*-isopropylacrylamide), which exhibited a lower critical solution temperature (LCST) and aggregated at temperatures above the LCST due to hydrophobic interactions [10]. These TSP-modified liposomes (TSP liposomes) released an encapsulated drug above the LCST, at which the liposomes were destabilized along with a transition of the TSP from a hydrophilic to a hydrophobic state, although the hydrophilic TSP extended into solution and stabilized the liposomes below the LCST [11]. To date, drug release from TSP liposomes has been induced by mild hyperthermia at 39–40 °C using an incubator for *in vitro* studies [9,12] and radio frequency for *in vivo* studies [13,14]. However, to the best of our knowledge, there have been no reports on ultrasound-mediated drug release from TSP liposomes.

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Therefore, we investigated ultrasound-mediated responses of TSP liposomes. We examined both ultrasound-mediated drug release from TSP liposomes and ultrasound-mediated cellular uptake of the drug released from TSP liposomes. Moreover, ultrasound-mediated cancer cell injury in the presence of TSP liposomes was also evaluated.

2. Materials and methods

2.1. Materials

A freeze-dried lipid mixture comprising dimyristoylphosphatidic acid, dipalmitoylphosphatidylcholine, and cholesterol at a molar ratio of 1:4:5 (COATSOME® PL-1614KS; NOF Corporation, Tokyo, Japan) was used as the starting material to prepare TSP liposomes. A copolymer of *N*-isopropylmethacrylamide (NIPMAM) and *N*-isopropylacrylamide (NIPAM) with two dodecyl groups at the chain end of the copolymer [$2C_{12}$-poly(NIPMAM-*co*-NIPAM)] (number-average molecular weight = 4850, weight-average molecular weight = 7550, and composition of comonomer NIPMAM:NIPAM = 46:54) was synthesized according to the method reported previously [15], and used as a TSP to provide liposomes with temperature sensitivity. Doxorubicin (DOX; Adriacin®; Kyowa Hakko Kirin Co., Ltd., Tokyo, Japan) was used as an anticancer drug, and 3,3′-Bis[*N*,*N*-bis(carboxymethyl)aminomethyl]fluorescein (Calcein; Wako Pure Chemical Industries, Ltd., Osaka, Japan) was used as a model drug. The chemicals used in this study were of guaranteed reagent grade and did not require further purification.

2.2. Cell culture and medium

Human liver cancer cells HepG2, were purchased from the RIKEN cell bank, Tsukuba, Japan. HepG2 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). Collagen type I coated culture dishes (AGC Techno Glass Co. Ltd., Tokyo, Japan) were used to ensure cell attachment. All culture media were supplemented with 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 TSP liposomes

TSP liposomes were prepared by the reported manner [15] with minor modification. Concretely, 10 mg of the freeze-dried lipid mixture and 0–20 mg of $2C_{12}$-poly(NIPMAM-*co*-NIPAM) was added to a 1.5-mL tube containing 1 mL of calcein solution (10 mM) or DOX solution (2 mM) in saline. The weight ratios of TSP per lipid mixture (TSP/lipid ratio) were set at 0, 0.5, 1, and 2. After vortexing and incubation for 8 h at 4 °C in the dark, the 1.5-mL tube was centrifuged (15,000 rpm) for 40 min at 4 °C and the supernatant was removed. The recovered liposomes were washed three times with Dulbecco's phosphate buffered saline without calcium chloride and magnesium chloride [D-PBS(−)] and then suspended in 3.5 mL of D-PBS(−) to obtain TSP liposomes. The concentrations of calcein-loaded liposomes and DOX-loaded liposomes in the prepared suspensions were evaluated by measuring the optical density at wavelengths of 540 nm (OD_{540}) and 480 nm (OD_{480}), respectively.

2.4. Assay for ultrasound-mediated drug release from TSP liposomes

Two milliliters of calcein-loaded or DOX-loaded TSP liposome suspension in D-PBS(−) at OD_{540} = 0.5 or OD_{480} = 0.2 was added to a 35-mm culture dish. The culture dish was placed on the transducer of an ultrasonic apparatus (Sonic Master ES-2, OG Giken Co., Ltd., Okayama, Japan) after the transducer surface was covered with 3 mL of water. Ultrasound (frequency = 1 MHz; duty ratio = 100%) was then irradiated from the bottom of the dish under the following conditions: output power = 0–0.5 W/cm^2; irradiation time = 0–120 s at room temperature. As a control experiment, a 1.5-mL tube containing 1 mL of a calcein-loaded TSP liposome suspension (OD_{540} = 0.5) was heated using a block incubator at 35–42 °C for 15 min.

After ultrasound irradiation or heating, 980 μL aliquot of the calcein-loaded liposome suspension was mixed with 20 μL of $CoSO_4$ solution (10 mM) to quench any free calcein released from the liposomes. To evaluate ultrasound-mediated calcein release from liposomes, the fluorescent signals from 200 μL of the liposome suspension were measured with a microplate spectrofluorometer (Gemini XPS, Molecular devices, Sunnyvale, CA, USA) at excitation and emission wavelengths of 485 nm and 530 nm, respectively. Fluorescence derived from calcein, entrapped in the liposomes, (F_C) was determined from the difference between the fluorescent signal of calcein-loaded liposome suspension and background signal from calcein-unloaded liposome suspension. Ultrasound-mediated calcein release from liposomes was calculated by the following equation:

$$\text{Calcein release } [\%] = \left(\frac{F_{C,0} - F_{C,t}}{F_{C,0}}\right) \times 100 \quad (1)$$

where $F_{C,0}$ and$F_{C,t}$ were the F_C values before and after ultrasound irradiation or heating, respectively, for a specified time, *t*.

2.5. Assay for ultrasound-mediated drug uptake and cell injury in the presence of TSP liposomes

Prior to performing this assay, 4×10^5 HepG2 cells suspended in 2 mL of culture medium in a 35-mm culture dish and incubated for 48 h to ensure confluent growth. Subsequently, DOX-loaded TSP liposome suspension was added to the cell culture for a liposome concentration equivalent to OD_{480} = 0.2, which was approximately equivalent to 20 μM DOX in the medium. The culture dish was placed on the transducer of an ultrasonic apparatus (Sonic Master ES-2) after the transducer surface was covered with 3 mL of water. Ultrasound was then irradiated from the bottom of the dish under the following conditions: frequency = 1 MHz; duty ratio = 100%; output power = 0.5 W/cm^2; and irradiation time = 30 s at room temperature. After ultrasound irradiation, the culture dishes were incubated for 60 min and then washed three times with 2 mL of D-PBS(−) to remove floating cells and liposomes. The cells were then analyzed for DOX uptake and cellular injury.

Microscopic and flow cytometry analyses were used to evaluate DOX uptake by cells. For microscopy, cell monolayers were observed using a fluorescence microscope (BZ-8000, KEYENCE, Osaka, Japan). For flow cytometry, a cell monolayer was detached using a solution with 2.5 g/L of trypsin and 1 mM EDTA (Nakarai Tesque). The cells were then washed twice with D-PBS(−) and suspended in 300 μL of D-PBS(−). The fluorescent signals from the cells were detected using a flow cytometer (EPICS XL-MCL ADC, Beckman Coulter, Inc., Brea, CA, USA) by counting 10,000 events. DOX-uptake index was calculated as the mean fluorescence intensity determined from a flow cytometry histogram.

To evaluate cancer cell injury, 2 mL of culture medium was added to the culture dish and incubated for 24 h. Cells were harvested by enzymatic treatment using a solution with 2.5 g/L of trypsin and 1 mM EDTA (Nakarai Tesque). Subsequently, viable cells were counted with a hemocytometer using trypan blue exclusion test. Cell viability was expressed as the percentage of viable