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Targeted and ultrasound-triggered drug delivery using liposomes co-modified with cancer cell-targeting aptamers and a thermosensitive polymer

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ABSTRACT

In this study, we demonstrated the feasibility of targeted and ultrasound-triggered drug delivery using liposomes co-modified with single stranded DNA aptamers that recognized platelet-derived growth factor receptors (PDGFRs) as targeting ligands for breast cancer cells and poly(NIPMAM-*co*-NIPAM) as the thermosensitive polymer (TSP) to sensitize these liposomes to high temperature. TSP-modified liposomes (TSP liposomes) released encapsulated calcein under 1 MHz ultrasound irradiation for 30 s at 0.5 W/cm^2 as well as the case under incubation for 5 min at 42 °C. Ultrasound-triggered calcein release from TSP liposomes was due to an increased local temperature, resulting from cavitation bubble collapse induced by ultrasound, and not due to an increase in the bulk medium temperature. Liposomes modified with PDGFR aptamers (APT liposomes) bound to MDA-MB-231 human breast cancer cells through PDGFR aptamers; however, they did not bind to primary human mammary epithelial cells (HMECs). The binding of APT liposomes was greatest for MDA-MB-231 cells, followed by MCF-7, WiDr, and HepG2 cancer cells. In a cell injury assay using doxorubicin (DOX)-loaded APT/TSP liposomes and ultrasound irradiation, cell viability of MDA-MB-231 at 24 h after ultrasound irradiation (1 MHz for 30 s at 0.5 W/cm^2) with DOX-loaded APT/TSP liposomes was 60%, which was lower than that with ultrasound irradiation and DOX-loaded TSP liposomes or with DOX-loaded APT/TSP liposomes alone.

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]. Especially, liposomes, which were first proposed in the 1970s and are self-assembled structures of phospholipids that form a single continuous phospholipid bilayer, have a long history as carriers for delivering therapeutic and diagnostic agents to cancerous lesions [2]. Strategies for drug delivery to solid tumors using liposomes are divided into passive targeting and active targeting. For passive solid tumor targeting, liposomes modified with polyethylene glycol (PEG) have been used because they can evade reticuloendothelial system surveillance 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 on drug diffusion or slow degradation of these lipid bilayers.

For active solid tumor targeting, liposomes have been engineered to have mechanisms of triggered drug release in the region of a tumor and/or have an affinity to bind to cancer cells. With regard to triggered drug release, a number of studies have been reported on liposomes that could release 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 lipid [6]. TSLs release encapsulated drugs near the melting phase transition temperature (T_m) of the lipid bilayer, at which the structure of lipid membrane changes concomitant with its transition from a gel to a liquid crystalline phase. Drug release from TSLs had been 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 its non-invasiveness as compared with other methods to induce hyperthermia such as microwave, IR laser, and radio frequency heating that require an interstitial needle or insertion of an antenna.

With regard to liposomes that have binding affinity for tumors, several investigators have studied liposomes that were modified

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with targeting ligands such as antibody and non-antibody molecules (e.g., RGD peptides to target integrins, folate to target its receptors, and others; [5,9]). TSLs have also been modified with cancer cell targeting ligands (antibodies or peptides), which exhibited binding affinity for cancer cells and triggered drug release induced by hyperthermia [10,11].

Another strategy for designing TSLs was reported by Kono et al. [12]. Liposomes were modified with a thermosensitive polymer (TSP) such as poly(*N*-isopropylacrylamide), which had a lower critical solution temperature (LCST) and aggregated at temperatures above the LCST due to hydrophobic interactions [13]. These TSP-modified liposomes (TSP liposomes) released their encapsulated drugs above the LCST, at which the liposomes were destabilized concomitant with a transition of the TSP from a hydrophilic to a hydrophobic state, although the hydrophilic TSP extended into solution and stabilized these liposomes below the LCST [14]. To date, many studies have demonstrated that drug release from TSP liposomes had been induced by mild hyperthermia at 39–40 °C using an incubator for *in vitro* studies [12,15] and by radio frequency for *in vivo* studies [16,17]. However, so far as we know, there have been no reports on ultrasound-triggered drug release from TSP liposomes, except for our previous report [18]. Moreover, there have been no reports regarding TSP liposomes that were co-modified with affinity ligands for targeting cancer cells.

Therefore, in this study, we demonstrated the feasibility of targeted, ultrasound-mediated drug delivery using TSP liposomes that were co-modified with aptamers as targeting ligands for cancer cells (APT/TSP liposomes). We evaluated both ultrasound-triggered drug release from TSP liposomes and the binding affinity of APT liposomes for cancer cells. We also demonstrated that ultrasound-mediated cancer cell injury was induced in the presence of APT/TSP liposomes.

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 APT/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 [19], and used as a TSP to provide liposomes with temperature sensitivity. PEGylated phospholipids and distearoylphosphatidylethanolamine–polyethylene glycol–*N*-hydroxysuccinimide (DSPE–PEG–NHS), synthesized by NOF Corporation, were used to introduce the functional groups into liposomes. Avidin was purchased from Nakarai Tesque, Kyoto, Japan. Single stranded DNA (ssDNA) aptamers that recognized the platelet-derived growth factor receptor (PDGFR; 5′- CAGGCTACGGCACGTAGAGCATCACCAT GATCCTG -3′) [20] were used as targeting ligands for cancer cells. 5′-biotinylated aptamers for PDGFRs were synthesized and purified by reverse phase high-performance liquid chromatography (HPLC) (Operon Biotechnologies, Tokyo, Japan). 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 cultures and media

Human breast cancer cells MDA-MB-231, used as target cells, were purchased from the RIKEN cell bank, Tsukuba, Japan. Primary human mammary epithelial cells (HMECs), used as a control for MDA-MB-231 cells, were purchased from Life Technologies Corporation, Carlsbad, CA, USA. For some experiments, other human breast cancer cell line (MCF-7), human liver cancer cells (HepG2), and human colon cancer cells (WiDr), purchased from the RIKEN cell bank, were also used. MDA-MB-231, MCF-7, HepG2, and WiDr 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). HMEC cells were cultured in Medium 171 with Mammary Epithelial Growth Supplement (MEGS; Life Technologies Corporation). For HepG2 cells only, collagen type I coated culture dishes (AGC Techno Glass Co., Ltd., Tokyo, Japan) were used to ensure cell attachment to the dish. 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 APT/TSP liposomes

To prepare APT/TSP liposomes, 10 mg of the freeze-dried lipid mixture and 0–20 mg of $2C_{12}$-poly(NIPMAM-*co*-NIPAM), 1.6 mg of DSPE–PEG–NHS, and 0.5 mg of avidin were added to a 1.5-mL tube containing 1 mL of a calcein solution (10 mM) or a 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 finally suspended in 3.5 mL of D-PBS(−) to obtain liposomes co-modified with avidin and TSP. This liposome suspension was mixed with 1 mL of a PDGFR aptamer solution (100 nM) in D-PBS(−) and incubated for 5 h at 4 °C in the dark to immobilize the biotinylated aptamers on the liposomes by avidin–biotin coupling. After washing 3 times (15,000 rpm for 40 min at 4 °C) to remove the free aptamers, the precipitate was suspended in 3.5 mL of D-PBS(−) to obtain APT/TSP liposomes. As a control, liposomes modified with APT alone (APT liposomes) were prepared as mentioned above but without using $2C_{12}$-poly(NIPMAM-*co*-NIPAM). Liposomes modified with TSP alone (TSP liposomes) were prepared without using avidin and PDGFR aptamers. Naked liposomes, without modification, were prepared without using $2C_{12}$-poly(NIPMAM-*co*-NIPAM), avidin, and PDGFR aptamers. 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 a calcein-loaded TSP liposome suspension in D-PBS(−) at OD_{540} = 0.5 was added to a 35-mm culture dish, and incubated at 37 °C for 15 min. 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: reading output intensity = 0 and 0.5 W/cm^2 which correspond to 0 and 0.252 W/cm^2 based on calorimetry [21]; irradiation time = 0–120 s at room temperature. As a control experiment, a 1.5-mL tube containing 1 mL of a