Surface Modification of Liposomes Using IR700 Enables Efficient Controlled Contents Release Triggered by Near-IR Light

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INTRODUCTION

Liposomes are one of the most used nanoparticulate drug carriers in both preclinical and clinical studies. Liposomes have several advantages over other drug carriers; for example, liposomes are capable of loading hydrophilic and hydrophobic low-molecular-weight drugs as well as proteins and genes. These incorporated drugs are protected from attack by serum proteins and enzymatic degradation in vivo. Moreover, it is possible to easily control the physicochemical properties of liposomes, such as particle size and surface charge, and modify their surface using functional molecules. These characteristics of liposomes enable efficient drug delivery to the targeted tissues and lesions. The most progressing research field for drug delivery using liposomes is cancer therapy. Because liposomes can prolong the duration of drug circulation and consequently increase their accumulation in tumors, several liposomal anti-cancer agents have been developed, and some of them are clinically used. However, their therapeutic efficacy remains limited because the release of anti-cancer drugs from liposomes is uncontrollable and therefore it is difficult to maintain sufficient concentrations of free anti-cancer drugs in tumors to obtain cytotoxic effects. To achieve efficient and safe cancer treatment using liposomal anti-cancer drugs, it is necessary to control the release of drugs from liposomes at an appropriate rate in the tumor and regulate drug release in healthy tissues.

Stimuli-responsive liposomes have been widely recognized as promising systems for controlled drug release. There are two main stimuli used to trigger drug release from liposomes, namely environmental and external stimuli. Environmental stimuli exploit the different factors between the tumor microenvironment and normal tissue, such as pH and enzymes, and a number of pH-responsive and enzymatically degradable liposomes have been constructed. However, the tumor microenvironment differs between cancer types and between patients with the same cancer type, and it also dynamically changes during tumor progression and treatment. Therefore, it would be difficult to properly regulate drug release from environmental stimuli-responsive liposomes. By contrast, external stimuli, such as heat, light, ultrasound and magnet, can achieve spatiotemporally controlled drug release from liposomes. Among them, near-IR (NIR) light has several advantages, such as less attenuation in biological tissues and resultant deeper tissue penetration, low invasiveness, and high spatiotemporal precision. Recently, NIR light-responsive liposomes have been developed for the efficient delivery of anticancer drugs into tumors.

We and other groups previously demonstrated that a silicon phthalocyanine derivative, IRDye700DX (IR700), conjugated to antibodies has the capacity to induce plasma membrane damage and selective cytotoxicity against cancer cells when the conjugate binds to the targeted cell membrane, followed by irradiation with NIR light. This newly developed cancer therapy, called NIR photoimmunotherapy, has also been reported to induce immunogenic cell death via cell membrane rupture and the release of intracellular molecules, including damage-associated molecular patterns. These previous
findings prompted us to utilize IR700 for controlled drug release from liposomes triggered by NIR light.

In this study, we constructed IR700-modified liposomes (IR700 liposomes) as NIR light-responsive drug carriers and evaluated their drug release properties triggered by NIR light irradiation. The aim of this study is proof of concept that IR700 modification enables liposomes to release their contents in response to NIR light irradiation.

MATERIALS AND METHODS

Materials 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE) were purchased from NOF Corporation (Tokyo, Japan). IRDye700DX NHS ester (IR700) was purchased from LI-COR Bioscience (Lincoln, NE, U.S.A.). Cholesterol was purchased from KANTO CHEMICAL Co., Inc. (Tokyo, Japan). Calcein was purchased from DOJINDO Laboratories (Kumamoto, Japan). Hoechst 33342 was purchased from Thermo Fisher Scientific K.K. (Tokyo, Japan). Doxorubicin hydrochloride (Dox) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). All other chemicals were of reagent grade.

Synthesis of IR700-Conjugated Phospholipids DPPE was dissolved in chloroform/methanol (9:1). IR700 was dissolved in dimethyl sulfoxide. They were mixed at an equal molar ratio with 1.2% triethylamine and incubated at 40°C for 20h. The resultant mixture was lyophilized and redissolved in methanol. Then, IR700-conjugated DPPE (IR700-DPPE) was purified via preparative layer chromatography.

Preparation of IR700 Liposomes Liposomes were prepared according to a previous report. Briefly, DPPC and cholesterol were mixed in chloroform at a molar ratio of 6:4. The mixture was dried in a rotary evaporator, followed by vacuum desiccation. The resultant lipid film was suspended in saline with or without 50 mM calcein or 35 µM Hoechst 33342 and hydrated at 55°C for 30 min under mechanical agitation. To prepare Dox-encapsulated liposomes, the lipid film was suspended in 120 mM ammonium sulfate (pH 4.0). The obtained dispersion was extruded 10 times through a polycarbonate membrane filter with a 0.2-µm pore size (GE Healthcare, Milwaukee, WI, U.S.A.). IR700 liposomes were prepared by incubating the liposomes with IR700-DPPE at a molar ratio of 100:1 at 55°C for 60 min under mechanical agitation. The particle size and ζ-potential of liposomes were measured using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, U.K.).

Dox was encapsulated in the liposomes via the remote loading method. Briefly, the external phase of liposomes was replaced with phosphate-buffered saline (PBS, pH 8.0) via gel filtration using a Sephadex G25 column (PD-10, GE Healthcare). The liposomes were incubated with Dox at a weight ratio of 10:2 at 55°C for 60 min. The free Dox was removed via gel filtration.

Cell Lines U-87 MG human glioblastoma and CT-26 murine colon carcinoma cells were purchased from the American Type Culture Collection (Manassas, VA, U.S.A.). These cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin G (100 U/mL), and streptomycin (100 µg/mL) at 37°C in 5% CO₂/95% air.

In Vitro Calcein Release Experiment Calcein-encapsulated unmodified liposomes or calcein-encapsulated IR700 liposomes were diluted in PBS at a liposome concentration of 100 µg/mL with or without 5 mM d-cysteine hydrochloride monohydrate and 10 mM sodium azide (NaN₃). The liposomes were irradiated with NIR light for 0.3–10.7 min at an intensity of 0.1 W/cm² (2–64 J/cm²) using an MLL-III-690 laser at 690 nm (Changchun New Industries Optoelectronics Technology Co., Ltd., Changchun, China). To prepare the deoxygenated solution, Ar bubbling was performed. Five minutes after irradiation, the released calcein was detected by measuring the fluorescent intensity of the liposome solution at a wavelength of 490 (excitation)/ 515 (emission) nm using an Infinite M200 microplate reader (Tecan Japan Co., Ltd., Kanagawa, Japan). In addition, the absorption spectrum was measured with a UV spectrophotometer UV-1800 (Shimadzu Co., Kyoto, Japan) before and after NIR light irradiation. The fluorescence emission spectrum excited at 660 nm was also measured with an FP-8600 (JASCO Co., Tokyo, Japan).

Cellular Uptake of Hoechst 33342 Encapsulated in IR700 Liposomes U-87 MG cells were seeded in 35-mm culture dishes at a density of 1 × 10⁶ cells/cm² and cultured for 1 d. Cells were exposed to 200 µg of Hoechst 33342-encapsulated IR700 liposomes and irradiated with NIR light (503/J/cm²). Ten minutes after irradiation, the cells were washed with PBS and observed using a fluorescence microscope (CKX41; Olympus, Tokyo, Japan). Then, the cells were collected in PBS and homogenized by repeated freeze-thawing. The fluorescent intensity of the resultant samples was measured at a wavelength of 350 (excitation)/461 (emission) nm using an Infinite M200 microplate reader. The amount of Hoechst 33342 was normalized with respect to the protein content of the cells using a BCA protein assay.

Cell Viability Assay CT-26 cells were plated in 96-well culture plates at a density of 5 × 10⁴ cells/cm² and cultured for 1 d. Then, Dox-encapsulated unmodified liposomes or Dox-encapsulated IR700 liposomes (0.5 µM Dox) were added to each well, and cells were irradiated with NIR light (503/J/cm²). After an additional incubation for 2 d, cell viability was measured using Cell Counting Kit-8 (DOJINDO Laboratories) and an Infinite M200 microplate reader. The results were expressed as viability (%).

Statistical Analysis The results are presented as the mean ± standard deviation (S.D.) of four experiments. ANOVA was used to test the statistical significance of differences between groups. Two-group comparisons were performed using Student’s t-test. Multiple comparisons between control groups and other groups were performed using Tukey’s test.

RESULTS

Calcein Release from IR700 Liposomes Following NIR Light Irradiation In this study, we synthesized IR700-conjugated DPPE (Fig. 1) and prepared NIR light-responsive IR700 liposomes composed of DPPC/cholesterol/IR700-DPPE (60:40:1, molar ratio). The particle size of IR700 liposomes was 282.7 ± 3.3 nm, which was similar to that of unmodified liposomes (302.2 ± 10.7 nm). The ζ-potential of IR700 liposomes was -21.5 ± 0.2 mV, which was lower than that of unmodified liposomes (-9.2 ± 0.6 mV).

As shown in Fig. 2, we evaluated the NIR light-triggered
release of calcein from IR700 liposomes. Calcein release from IR700 liposomes in PBS was significantly enhanced by NIR light irradiation, and the effect depended on the duration of irradiation (Fig. 2A). Approximately 60% of calcein was released by NIR light irradiation for approximately 10 min. This enhanced calcein release was significantly suppressed by the addition of NaNO₃, a singlet oxygen quencher. In addition, calcein release from IR700 liposomes by NIR light irradiation was significantly promoted under conditions of deoxygenation and the presence of an electron donor (d-cysteine) (Fig. 2B). Approximately 80% release of calcein was achieved within 2 min under this condition, and insoluble precipitation was observed in IR700 liposome solution after NIR light irradiation (Fig. 2C). Moreover, Fig. 2D showed that the absorbance at 688 nm of IR700 liposome solution was much decreased by NIR light irradiation under conditions of deoxygenation and the presence of an electron donor. We also observed that the fluorescence intensity at around 700 nm of IR700 liposome solution was approximately 0.6-fold decreased by the irradiation (Fig. 2E).

**Cellular Uptake of Hoechst 33342 Released from IR700 Liposomes** To evaluate drug release from IR700 liposomes and its cellular uptake, we prepared Hoechst 33342-encapsulated IR700 liposomes. Once Hoechst 33342 is released from liposomes, it easily permeates cells, and subsequently, we can detect the emission of blue fluorescence from Hoechst 33342 due to its specific binding to double-stranded DNA. As shown in Fig. 3, U-87 MG cells treated with Hoechst 33342-encapsulated IR700 liposomes exhibited much stronger blue fluorescence following NIR light irradiation compared with the results for no light irradiation.

**In Vitro Cytotoxic Effect of Dox-Encapsulated IR700 Liposomes in CT-26 Cells** We evaluated the cytotoxic effect of Dox-encapsulated IR700 liposomes following NIR light irradiation. As shown in Fig. 4, Dox-encapsulated unmodified liposomes exhibited no significant cytotoxic effect against CT-26 cells with or without NIR light irradiation. On the contrary, significant cytotoxicity was observed with the combination of Dox-encapsulated IR700 liposomes and NIR light irradiation.

**DISCUSSION**

In this study, we described a novel formulation of NIR light-responsive liposomes, the surface of which was modified using IR700, to achieve controlled drug release for effective cancer therapy. We observed that IR700 liposomes could release encapsulated calcein in a NIR light irradiation time-dependent manner (Fig. 2A). This type of membrane permeabilization of NIR light-responsive liposomes has been reported to occur via lipid oxidation. NIR light-responsive liposomes incorporate photosensitizers, such as porphyrin and phthalocyanine, in their lipid bilayers or on their surfaces, and these photosensitizers can generate singlet oxygen by absorbing NIR light. The generated singlet oxygen induces phospholipid oxidation, which increases the polarity of the lipid phase and subsequently increases liposomal membrane permeability. Similarly as other photosensitizers, IR700 has also been reported to produce singlet oxygen via the absorption of NIR light. In the present study, the enhanced calcein release from IR700 liposomes in PBS was substantially suppressed by NaNO₃ (Fig. 2A), suggesting that singlet oxygen is involved in the permeabilization of the IR700 liposomal membrane. Conversely, Pashkovskaya et al. demonstrated that liposomes composed of unsaturated phospholipids, such as Egg phosphatidylcholine (EggPC) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), can leak their contents in the presence of aluminum phthalocyanine with red light exposure, whereas content leakage from saturated DPPC liposomes hardly occurs. They described that the oxidizability of fully saturated phospholipids was much lower than that of unsaturated phospholipids, resulting in the absence of photodynamically induced content leakage from DPPC liposomes. This observation is likely inconsistent with our present result. One possible reason why IR700 liposomes can be permeabilized by NIR light irradiation is the involvement of cholesterol oxidation. Cholesterol is known to be oxidized by singlet oxygen, and oxidized cholesterol causes the disruption of lipid membranes. IR700 liposomes contain 40 mol% cholesterol, whereas the DPPC liposomes used in Pashkovskaya’s study are composed of only phospholipids. In fact, Luo et al. reported that porphyrin-modified saturated 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) liposomes containing 40 mol% cholesterol can release up to 75% of their content following NIR light irradiation. This observation is in accordance with the present result. Contrarily, light-triggered drug release was not observed when porphyrin-modified DSPC liposomes contained no cholesterol. These findings suggest that the permeabilization of IR700 liposomes triggered by NIR light irradiation could occur by the oxidation of...
cholesterol rather than DPPC.

Interestingly, NIR light-triggered calcein release from IR700 liposomes was also observed under deoxygenated conditions (Fig. 2B). Moreover, this calcein release was observed within 2 min after NIR light irradiation (2–8 J/cm²). Because IR700 cannot produce singlet oxygen under deoxygenated conditions, the oxidation of cholesterol and/or phospholipids would hardly occur. Therefore, the membrane permeabilization of IR700 liposomes under deoxygenated conditions should be induced by a different mechanism from membrane oxidation. Recently, we revealed that cell membrane damage induced by an antibody-IR700 conjugate following NIR light irradiation is attributable to physical change within the conjugate.31) IR700 rapidly releases its axial ligands following NIR light irradiation in the presence of electron donors, and this reaction is accelerated under deoxygenation. The release of axial ligands from IR700 induces the transition of its physical property from highly hydrophilic to hydrophobic, which induces both water- and oil-insoluble aggregation with π-stacking of phthalocyanines. This physical change of IR700 on the cell surface causes stress in the cellular membrane, impairing membrane function. In addition, this reaction can be induced by NIR light irradiation at a relatively low energy level (4–16 J/cm²). Considering these findings, it is conceivable that the NIR light-triggered membrane permeabilization of IR700 liposomes under conditions of deoxygenation would occur via the physical transition of IR700 following axial ligand release. The observation of an insoluble precipitation
after the exposure of IR700 liposomes to NIR light under deoxygenated conditions strongly supports our view (Fig. 2C). As well as the appearance of the insoluble aggregation, our previous report also demonstrated that the absorbance at around 690 nm and fluorescence intensity related to IR700 were lost when the axial ligands dissociated from IR700 by NIR light irradiation. We observed the reduction of the absorbance at 688 nm and fluorescence intensity at around 700 nm of IR700 liposome solution by NIR light irradiation under deoxygenated condition and in the presence of an electron donor (Figs. 2D, E). These results are in accordance with our previous observation.

We also observed that IR700 liposomes can release their contents in response to NIR light irradiation in cell culture medium (Fig. 3), and Dox-encapsulated IR700 liposomes exhibited the highest cytotoxicity against cancer cells following NIR light irradiation (Fig. 4). These results suggest that the controlled release of anti-cancer drugs from IR700 liposomes triggered by NIR light irradiation is effective for cancer therapy.

Several reports described phthalocyanine-based NIR light-responsive liposomes. In most cases, the light-triggered drug release from these liposomes was based on the photodynamic or photothermal effect of phthalocyanine. For example, Machiček et al. demonstrated that the light-triggered release of Dox from zinc phthalocyanine-incorporated liposomes occurred via the oxidation of unsaturated lipids in liposomal membranes. In addition, Yuan et al. illustrated that NIR light irradiation of IR780-modified liposomes generated hyperthermia and facilitated the release of Dox from the liposomes. However, the photodynamic effect is restricted in the hypoxic tumor microenvironment, and therefore, the oxidative destruction of liposomes may not efficiently occur in tumors. By contrast, IR700 liposomes can release their contents in response to NIR light irradiation under conditions of deoxygenation. This is a potential advantage as a drug carrier for cancer therapy. Furthermore, the previously constructed phthalocyanine-based NIR light-responsive liposomes were composed of unsaturated lipids, which are unstable against oxidation and hyperthermia. Therefore, the stability of these liposomes under physiological conditions has generally been limited. Meanwhile, IR700 liposomes consist of fully saturated lipids, indicating their potentially superior stability compared with conventional NIR light-responsive liposomes.

In order to utilize IR700 liposomes in vivo, polyethylene glycol (PEG) modification on the surface of IR700 liposomes is required for its stability and long-circulation in blood and subsequent efficient accumulation in tumor tissues. Previous reports demonstrated that NIR light-responsive liposomes could release their contents in response to NIR light irradiation whether PEG modification was performed or not. In addition, Luo et al. showed that the Dox release rate from porphyrin-incorporated liposomes after NIR light irradiation was not changed when 3 or 5 mol% of PEG was modified on the surface of liposomes, whereas 8 mol% PEG modification decreased the Dox release.

Several reports demonstrated that NIR light-responsive liposomes using IR700. IR700 liposomes can release their contents following NIR light irradiation under both normoxic and anoxic conditions. Under normoxic conditions, the membrane permeabilization of IR700 liposomes occurs via membrane oxidation, whereas it occurs via a physical change in the solubility of IR700 under anoxic conditions. Further studies using PEG-modified IR700 liposomes are needed to use them for cancer treatment in vivo. Nevertheless, IR700 liposomes would be useful for the efficient controlled release of anticancer drugs in tumors, even under hypoxic conditions.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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