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Dual Functional Gd(III)-DOTA Liposomes for Cancer Therapy and Diagnosis as a Theragnostic Carrier

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Development of dual functional liposome has been studied for cancer theragnostics. Therefore, we focused on ultrasound-sensitive liposomes with doxorubicin (DOX) and gadolinium (Gd) as a theragnostic carrier having a potential for cancer therapy and diagnosis. In this study, Gd(III)-DOTA-modified sonosensitive liposomes (GL) was developed using chemically synthesized Gd(III)-DOTA-DPPE lipid. Sonosensitivity of GL to 1 MHz ultrasound induced 25% of DOX release. The relaxivities (r_1) of GL were 7.33-10.34 $mM^{-1}s^{-1}$, which was higher than that of MR-bester®. Intracellular delivery of DOX from GL by ultrasound irradiation was evaluated according to ultrasound intensity, resulting in increase of uptake of DOX released from ultrasound-triggered GLs compared to GL3 or Doxil® without ultrasound. Taken together, this study shows that the paramagnetic and sonosensitive liposomes, GL, is a novel and highly effective delivery system for drug with the potential for broad applications in human disease.

Key Words : Liposome, Ultrasound sensitivity, Contrast agent, Doxorubicin

Introduction

Liposome-based approaches hold great potential for anticancer drug delivery system.[1,2] Among these approaches, many studies focused on structural modification of liposomes to increase circulation time in bloodstream and to enhance drug release from liposomes in target cells. However, one of the key challenges to the use of liposomes for cancer chemotherapy is the need for efficient intracellular delivery of target drug in tumor cell to enhance therapeutic efficacy. Therefore, to overcome this hurdle, targeted drug delivery systems such as thermo-, pH-, ultrasound- and optical-sensitive liposomes have been studied.[3,4] In particular, ultrasound-sensitive (sonosensitive) liposomes for controlled drug release at the target site have been studied to increase antitumor efficacy of drugs and decrease the associated side effects.[5,6]

Magnetic resonance (MR) is widely used in diagnostic medicine in pathological areas. Usually, accumulation of contrast agents is essential to achieve MR imaging (MRI) and high-resolution images.[7,8] Most of contrast agents for MRI are based on either iron oxide particle or gadolinium (III) (Gd(III))-chelated complexes. Gd(III)-based contrast agents have a low r_2/r_1 ratio and are frequently used to generate positive contrast (increased signal intensity) in T_1-weighted images. Recently, various nanoscale carriers such as liposomes, micelles, and polymeric nanoparticles have been modified or incorporated with the MRI contrast agent Gd(III).[8,9] Liposomal nanocarriers are able to carry multiple reporter moieties such as peptides and antibodies for the efficient and selective delivery of contrast agents into the pathological sites.[10]

Here, we developed a novel dual functional liposomal carrier, Gd(III)-DOTA modified liposomes (GL) loaded with doxorubicin (DOX) for theragnostic therapy by MR-image guidance. The GL was prepared using synthesized Gd(III)-DOTA-DPPE lipid. Sonosensitivity and MR properties of the GLs with varying lipid ratios were examined. Furthermore, intracellular uptake of the drug from GL was evaluated according to the intensity of ultrasound. These data provide a significant conceptual advance in our understanding of the theragnostic dual cancer therapy and diagnosis, and support the potential for drug release from liposomes.

Experimental

Materials. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (DSPE-mPEG2000), and cholesterol (CHOL) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Doxil® was purchased from ALZA Corporation (Mountain View, CA, USA). Doxorubicin hydrochloride, *N,N'*-diisopropylethylamine (DiPEA), *N,N,N',N'*-tetramethyl-O-(*N*-succinimidyl) uranium hexafluorophosphate (HSTU), trifluoroacetic acid (TFA) and gadolinium(III) acetate hydrate (Gd(III) $(OAc)_3$) were purchased from Sigma-Aldrich Chemical Co (St. Louis, MO, USA). Fetal bovine serum (FBS), penicillin-streptomycin, and Dulbecco's modified Eagle medium (DMEM) were purchased from Gibco BRL/Life Technologies (New York, NY, USA). Tri-*tert*-butyl 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetate (protected DOTA) was purchased from Tokyo Chemical Industry Corporation (TCI; Tokyo, Japan). All other materials were of analytical grade and used without further purification.

Synthesis of Gd(III)-DOTA-DPPE. Gd(III)-DOTA-DPPE

was chemically synthesized as shown in Figure 1.[11-13] Protected DOTA (0.9 mmol) was added to 5 mL of dry dimethylformamide containing 0.9 mmol of HSTU and 3.2 mmol of DiPEA under a nitrogen atmosphere and the mixture was stirred for 1 h at room temperature. The obtained solution was added to 10 mL of chloroform containing 0.8 mmol of DPPE, stirred for 3 h at 65 °C and and incubated overnight at room temperature. After evaporation of the solution with toluene (2 × 20 mL) and chloroform (2 × 20 mL) using a rotary evaporator (Buchi Rotavapor R-200, Flawil, Switzerland), the product was precipitated in water/methanol (1:1 v/v) and the precipitate was isolated by centrifugation. The resulting pellet was dissolved in chloroform and the solution was concentrated under vacuum. The resulting solid was redissolved in 20 mL of diethyl ether and washed with water (2 × 10 mL). The organic phase was collected and concentrated in vacuum to obtain powder. The prepared powder was dissolved in 10 mL of TFA/chloroform (3:7 v/v), and stirred for 17 h at room temperature. The solution was evaporated with toluene (2 × 20 mL) and chloroform (2 × 20 mL) using a rotary evaporator, precipitated in acetonitrile and dried under vacuum. A solution of the yellowish solid in 9 mL of chloroform was mixed with a solution of 0.42 mmol Gd(III) $(OAc)_3$ in 5 mL of methanol/water (10:1 v/v), adjusted to pH 6.5 with pyridine and stirred overnight at room temperature. The solution was concentrated under reduced pressure and co-evaporated with methanol/toluene (1:1 v/v, 2 × 10 mL) and chloroform (2 × 10 mL), resulting in Gd(III)-DOTA-DPPE as a yellowish solid. The yield of Gd(III) chelated Gd(III)-DOTA-DPPE was 67%. Gd(III)-DOTA-DPPE; ^{1}H-NMR ($CDCl_3$) δ 7.73 (s, 1H, *NH*), 4.24 (m, 1H, *CH*), 2.34 (m, 4H, $CH_3(CH_2)_{13}CH_2CO$), 1.32-1.27 (m, 60H), 0.94-0.91 (t, 6H, $(CH_2)_{13}CH_3$) and MALDI-TOF (negative mode): *m/z* [M-H$^-$], calculated = 1231.7 Da.; observed = 1231.155 Da.

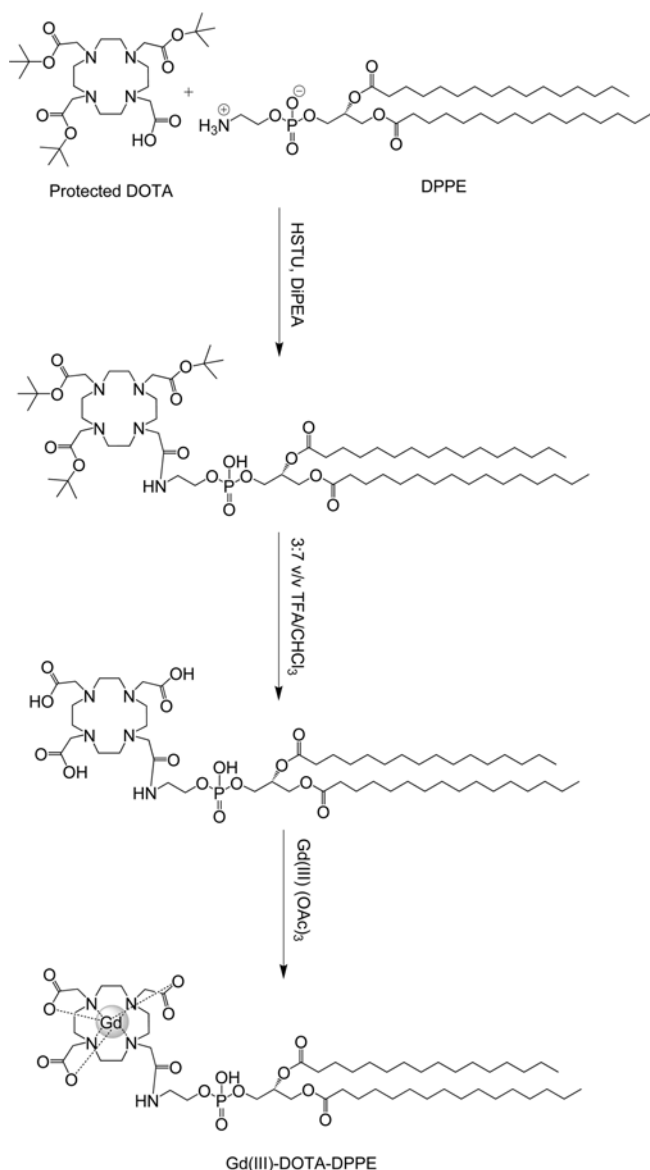

Figure 1. Synthesis of Gd(III)-DOTA-DPPE for preparation of GLs.

Preparation of Gd(III)-DOTA-DPPE Incorporated Liposomes. Gd(III)-DOTA-DPPE incorporated liposomes (GL) and control liposomes (CL) without Gd(III)-DOTA-DPPE were prepared by thin-film hydration and sequential extrusion method. The loading of DOX into the aqueous core of the liposomes was carried out using the remote loading method using an ammonium sulfate transmembrane gradient.[14,15] The lipid compositions and molar ratios for the preparation of the liposomes were as follows: (1) GL1; Gd(III)-DOTA-DPPE:CHOL:DSPE-mPEG = 62:30:8, (2) GL2; Gd(III)-DOTA-DPPE:CHOL:DSPE-mPEG:DPPE = 40:30:8:22, (3) GL3; Gd(III)-DOTA-DPPE:CHOL:DSPE-mPEG:DPPE = 20:30:8:41 and (4) CL; CHOL:DSPE-mPEG: DPPE = 30:8:62. The lipids for each liposome formulation were dissolved in 3 mL of chloroform to give 16.67 mM of total lipid concentration (GL1: 18.40 mg/mL, GL2: 16.41 mg/mL, GL3: 14.60 mg/mL and CL: 12.84 mg/mL) and dried to a thin film using a rotary evaporator. The film was hydrated with 3 mL of 300 mM ammonium sulfate solution and the liposome suspension was extruded sequentially 5 times through polycarbonate membrane filters (Whatman, Piscatawqy, NJ, USA) having a pore size of 200 and 100 nm using a high-pressure extruder (Northern Lipids Inc., Burnaby, Canada). Unloaded ammonium sulfate was removed by dialysis in distilled water for 24 h at 4 °C using a cellulose dialysis tube (MWCO, 12000-14000; Viskase Co., Darien, IL, USA). DOX solution (2 mg/mL) was added to the liposomal solution (1:1 v/v) and incubated for 2 h at 75 °C. The mixture was dialyzed to remove the unloaded DOX for 48 h at 4 °C. The DOX-loaded liposomes were stored at 4 °C until use.

The concentration of DOX in the liposomes was measured using a UV-vis spectrophotometer at 497 nm (UV-mini; Shimadzu, Tokyo, Japan) and the loading efficiency was calculated as below.

$$\text{Loading efficiency (\%)} = F_t/F_i \times 100 \quad (1)$$

where F_t is the concentration of DOX in the liposomes after dissolution of DOX-loaded liposomes in an organic solvent

mixture consisting of chloroform:methanol (2:1, v/v) and F_i is the initial concentration of DOX. The particle size and zeta potential of the liposomes were measured using an electrophoretic light scattering spectrophotometer (ELS-Z; Otuska Electronics Co., Tokyo, Japan). The amount of chelated Gd(III) was measured using an inductively coupled plasma-atomic emission spectrometer (Ultima-C; Jobin Yvon, Longjumeau, France).

Ultrasound-triggered Drug Release from Liposomes. Ultrasound-triggered release of DOX from GL and CL was conducted using a 1 MHz high intensity focused ultrasound system (EofE Ultrasonics Co., Seoul, Korea). The liposomal solutions were diluted in a ratio of 1:4 (v/v) with PBS (pH 7.4) and exposed to a continuous mode (100% duty cycle) of ultrasound for 1 min at intensity levels of 283, 678 and 1094 W/cm^2, respectively. During the ultrasound irradiation, the temperature of each sample was controlled to be below 50 °C. The release of DOX from liposomes was measured by fluorescence spectrophotometry. The excitation and emission wavelengths were 487 and 595 nm, respectively. The percentage of DOX release from the liposomes was calculated as follows:

$$\text{Drug release (\%)} = (F_t - F_0)/(F_{max} - F_0) \times 100 \quad (2)$$

where F_t is the fluorescence intensity of the liposome sample after a given duration (t) of ultrasound irradiation, F_0 is the initial background fluorescence of the liposome sample prior to ultrasound irradiation and F_{max} is the fluorescence intensity of DOX in the liposomes after dissolution of DOX-loaded liposomes in an organic solvent mixture consisting of chloroform:methanol (2:1 v/v).[16] The release test was performed on 3 independent samples of each liposome.

Relaxivity Measurement. The liposomal samples were prepared in the range of 0.05-0.40 mM of Gd(III) concentration. The longitudinal relaxation time (T_1) of each sample was measured by saturation recovery method using a 4.7-T MR system (Bruker-biospin, Ettlingen, Germany). Relaxivity (r_1, in units of mM^{-1}s^{-1}) was obtained from the slope of the linear fit of the inverse of T_1 as a function of Gd(III) concentration. T_1-weighted MR images were obtained using a heavily T_1-weighted fast spoiled gradient echo sequence. Scans were performed with the following imaging parameters: repetition time (TR) = 8.0, 6.0, 4.0, 2.5, 0.5, 0.2 and 0.07 s; echo time (TE) = 7.8 ms; flip angle (FA) = 180°; field of view (FOV) = 40 × 50 mm^2; image matrix = 128 × 128 mm^2; and number of signal average = 5.

Intracellular Uptake of DOX from Ultrasound-Triggered Liposomes. For the experiments on intracellular uptake of DOX from liposomes, B16F10 murine melanoma cells were cultured in DMEM supplemented with 10% (v/v) FBS and 10 μL/mL penicillin-streptomycin. The cultures were sustained at 37 °C in a humidified incubator containing 5% CO_2. The cells were maintained within their exponential growth phase. The intracellular uptake of DOX from liposomes was determined by flow cytometry analysis.[16,17] B16F10 cells were transferred to 24-well tissue culture plates at a density of 1 × 10^5 cells/well and incubated for 12 h at 37 °C. The liposomal DOX solutions were diluted in a ratio of 1:4 (v/v) with PBS (pH 7.4) just prior to the experiments. The diluted liposomal solutions were irradiated by a 1 MHz high intensity focused ultrasound transducer in a continuous mode (100% duty cycle) at an intensity level of 283, 658 or 1,094 W/cm^2 for 2 min at 37 °C. The culture medium was replaced with the ultrasonically irradiated liposomal DOX solution diluted in culture media at a concentration of 15 μg of DOX/mL and then incubated for 45 min. The culture medium was then removed and each well was washed with PBS (pH 7.4). The fluorescence intensities of sample were determined by flow cytometry with a FACScan (Becton Dickinson, San Jose, CA, USA). Cell-associated DOX was excited with an argon laser (488 nm), and fluorescence was detected at 560 nm. Data of 10,000 gated events were collected and analyzed with the CELL Quest software.

Results and Discussion

Physical Properties of Gd(III)-DOTA-DPPE Liposomes. The physical properties of GLs and CL were evaluated by measuring their mean particle size, zeta potential, DOX loading efficiency and amount of Gd(III) (Table 1). While particle size of CL was around 121 nm, GLs were slightly increased from 136 nm to 153 nm according to Gd(III)-DOTA-DPPE mole ratio in the lipid composition and zeta potential was around −27 mV due to the PEG and the Gd(III)-DOTA complex having lone pairs of electrons of oxygen atom. We performed pegylation of liposomes with PEG to inhibit opsonization and RES uptake. by forming hydrodynamic layer and hence prolong circulation time of the liposomes in bloodstream.[18] Additionally, incorporation of CHOL to liposome could increase stability of them in bloodstream.[19] The amount of Gd(III) in GLs was proportional to the Gd(III)-DOTA-DPPE content in the lipid com-

Table 1. Physical properties of liposomes

Liposomes	Gd(III)-DOTA-DPPE:CHOL: DSPE-mPEG2000:DPPE (mol %)	Mean particle diameter (nm)	Zeta potential (mV)	DOX loading efficiency (%)	Amount of Gd(III) (mM)
GL1	62:30:8:0	153.9 ± 12.3	-26.0 ± 2.1	68.8 ± 23.9	5.02 ± 0.20
GL2	40:30:8:22	159.7 ± 14.5	-28.1 ± 8.8	67.0 ± 1.9	2.70 ± 0.14
GL3	20:30:8:42	136.1 ± 16.1	-28.9 ± 4.1	58.6 ± 4.3	1.64 ± 0.02
CL	0:30:8:62	121.2 ± 1.5	-26.7 ± 2.2	93.5 ± 9.2	ND

ND; Not detected. Amount of Gd(III) could not be measured because there is no Gd(III).

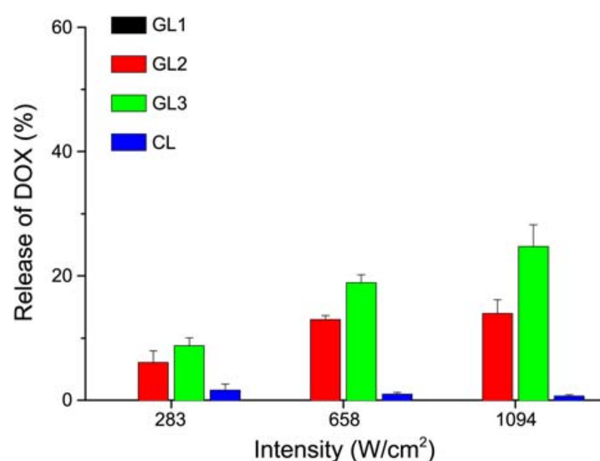

Figure 2. Release of DOX from liposomes after irradiation of high intensity focused ultrasound for 1 min with various intensities at 1 MHz. Mean and S.D. are shown (n = 3).

position.

The Gd(III)-DOTA-DPPE was used as a lipid composition for magnetic resonance effect. The DOX loading efficiency of GLs was lower by approximately 25-35% than that of CL because the Gd(III)-DOTA complex may induce steric effect and mechanical stress in the membrane of lipid bilayers.[5,20]

Ultrasound-Triggered Drug Release. The release of DOX from GLs and CL by ultrasound irradiation was investigated under various intensities (283, 658 and 1094 W/cm^2) of a 1 MHz high intensity focused ultrasound. The ultrasound-triggered release of DOX from GL was proportional with increase of DPPE mole ratio in the lipid composition. GL3 showed a high DOX release of 25% by ultrasound irradiation (Figure 2).

Recently, Evjen *et al.* reported that sonosensitivity of the liposomes is related to the ability of DSPE to form inverted hexagonal structures under high temperature or pressure.[21] Ultrasound irradiation-induced cavitations can generate high pressure or temperature in the liposome membrane.[4,5] Phase transitions of liposomes have been known to be induced by pressure and/or temperature changes. Similarly, DPPE in the liposomal bilayer might be undergoes a thermotropic phase transition from the lamellar liquid-crystalline to the inverted hexagonal phase by cavitation because the long fatty acids occupy larger volume than the polar head groups.[21,22] The phase transition might induce local defects or polymorphic phase transitions within micro-rafts or the whole liposome bilayer during ultrasound irradiation, further leading to drug release by membrane rupture, as shown in Figure 2.

Magnetic Resonance Property of Liposomes. MRI is one of the most powerful techniques currently used in medical diagnostics such as tumor detection and vascular imaging. Gd-based complexes, such as Gd(III)-DOTA and Gd(III)-DTPA using paramagnetic material, are known as the most effective T_1-agents.[10] The MR images of contrast agents are based on the same principles of nuclear magnetic resonance (NMR). The MR image of contrast agents is related to relaxation behavior of hydrogen nuclei of water. The principle mechanism for Gd(III)-complexes is due to the interaction of an inner sphere water molecule with the paramagnetic Gd(III) ion having 9th coordination site, leading to the subsequent magnetic relaxation of the water molecule.[23] The signal intensity of the image is related to the longitudinal relaxation time (T_1) and a shortened T_1 provides improved images.[24]

Figure 3(a) shows T_1-weighted MR images of GLs and a commercial contrast agent, MR-bester® at different concentrations of Gd(III) (0.4, 0.2 and 0.05 mM). The MR images of various GLs showed similar brightness and looked brighter compared to MR-bester® at the same concentration of Gd(III). The brightness of MR imaging of GL was proportional to the increase in Gd(III) concentration. The MR images of CL were very dark because they did not contain Gd(III). The relaxivity (r_1) values of GLs were 7.33-10.34 mM^{-1}s^{-1}, which was approximately 5.9-8.4 times higher than that of MR-bester® (Figure 3(b)). This result indicates that GLs could induce strong relaxivity compared to MR-bester®. Among these, we selected GL3 for subsequent study due to their high sonosensitivity and relaxivity. Generally, Gd(III) complexes on the liposomal surface are known to improve ionic relaxivity compared to Gd(III)-entrapped liposomes.[10] The nuclear magnetic resonance dis-

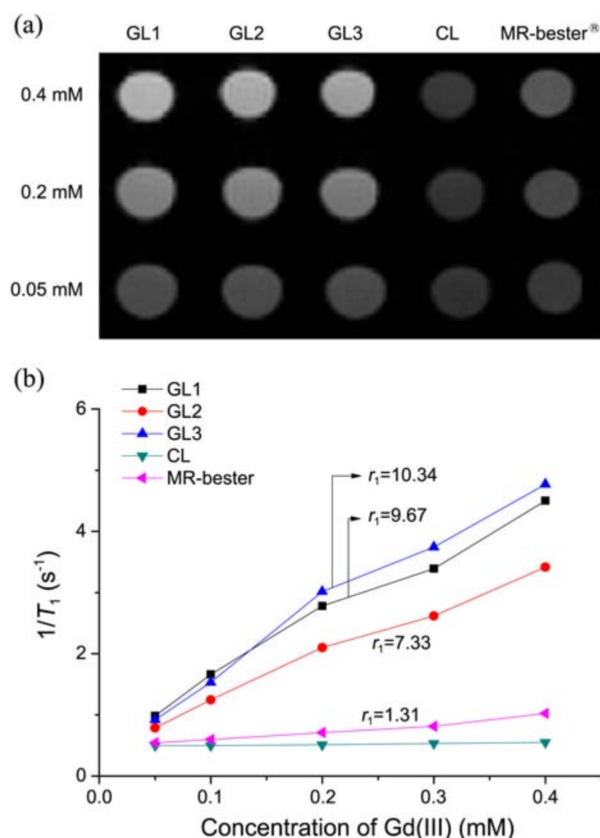

Figure 3. Magnetic resonance properties of GLs. (a) T_1-weighted MR images of GLs and MR-bester® at different concentrations of Gd(III). The MR images were taken together for comparison. (b) Relaxivities (r_1, in units of mM^{-1}s^{-1}) of GLs and MR-bester®. Relaxivity was obtained from the slope of the linear fit of the inverse of the measured T_1 (longitudinal relaxation time) as a function of Gd(III) concentration. 1/T_1 Data of CL without Gd(III)-DOTA-DSPE were presented as negative controls.

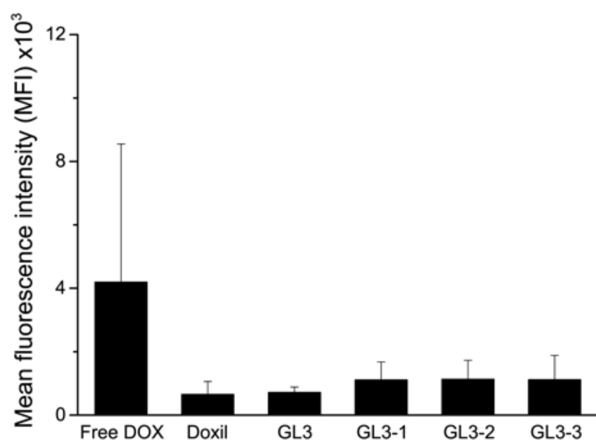

Figure 4. Intracellular uptake of DOX released from ultrasound-triggered GL3. Mean fluorescence intensity (MFI) was determined by flow cytometry analysis. GL3-1, GL3-2, and GL3-3 were irradiated by high intensity focused ultrasound at 1 MHz for 2 min with different intensity levels of 283, 658 and 1,094 W/cm^2, respectively. GL3, free DOX and Doxil® were not irradiated with ultrasound. Mean and S.D. are shown (n = 3).

persion (NMRD) profiles of liposomal contrast agents show a typical peak at higher frequencies.[8,9]

Intracellular Uptake of DOX Released from Ultrasound-Triggered Liposomes. We thought that GLs can be delivered to not only tumor vessel but also tumor cell in tissue microenvironment. Therefore, we designed to confirm in vitro intracellular uptake of DOX, which already released out from liposomes by ultrasound in tumor microenvironment. To confirm the cellular uptake of DOX released from ultrasound-triggered liposomes, the amount of DOX in B16F10 cells was measured by flow cytometry. Intracellular uptake of DOX released from ultrasound-irradiated GL3-1, GL3-2, and GL3-3 resulted in a 1.57-fold increased compared to GL3 without ultrasound irradiation (Figure 4). This result indicates that the DOX release from GLs by ultrasound irradiation could increase cellular uptake of drug at target cell in tumor microenvironment. However, Free DOX shows high uptake efficiency through diffusion mechanism, leading to high cellular uptake compared to liposomal DOX.[16,17] Although free DOX shows high uptake efficiency, it is limited for the use of clinic due to serve side effect. Additionally, GL3 and Doxil without ultrasound irradiation show low intracellular uptake by negative surface charge caused by electrostatic repulsion. However, ultrasound-irradiated GL3 showed high intracellular uptake of DOX because it induced the burst release of DOX by cavitation in 2 min and this data was highly matched with Figure 2. However, the DOX uptake from GLs according to ultrasound intensity showed a similar increase in intracellular uptake.

Conclusion

Dual functional Gd(III)-DOTA-modified sonosensitive liposomes (GL) as a theragnostics carrier were developed and evaluated for their sonosensitivity, MR properties, and intracellular uptake. GL showed potential for contrast agent compared to a commercial contrast agent, MR-bester® and increased intracellular uptake due to the ultrasound-triggered release compared to GL without ultrasound. This study suggests that the novel liposomal carrier may provide a convenience for dual function of cancer chemotherapy and diagnosis by MR-image guidance and moreover a possibility of ultrasound-mediated targeted drug delivery. Although the ultrasound-triggered delivery system can be useful for ultrasound-mediated therapies, additional intensity approaches may be useful. Nevertheless, ultrasound targeted delivery strategy presented here has broad potential as a systemic delivery platform in human disease and could be adapted for other targeting approaches.

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