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Evaluation of the potential of doxorubicin loaded microbubbles as a theranostic modality using a murine tumor model

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ABSTRACT

In this study, a novel phospholipid-based microbubble formulation containing doxorubicin and perfluoropropane gas (DLMB) was developed. The DLMBs were prepared by mechanical agitation of a phospholipid dispersion in the presence of perfluoropropane (PFP) gas. An anionic phospholipid, distearoyl phosphatidylglycerol (DSPG) was selected to load doxorubicin in the microbubbles by means of electrostatic interaction. The particle size, zeta potential, echogenicity and stability of the DLMBs were measured. Drug loading was ⩾92%. The potential of the DLMBs for use as a theranostic modality was evaluated in tumor bearing mice. Gas chromatography analysis of PFP showed significant enhancement of PFP retention when doxorubicin was used at concentrations of 10–82% equivalent to DSPG. The inhibitory effects on the proliferation of B16BL6 melanoma murine cells *in vitro* were enhanced using a combination of ultrasound (US) irradiation and DLMBs. Moreover, *in vivo* DLMBs in combination with (US) irradiation significantly inhibited the growth of B16BL6 melanoma tumor in mice. Additionally, US echo imaging showed high contrast enhancement of the DLMBs in the tumor vasculature. These results suggest that DLMBs could serve as US triggered carriers of doxorubicin as well as tumor imaging agents in cancer therapy.

1. Introduction

In cancer therapy, targeted drug delivery to the tumor is a great challenge [1,2]. Many recent studies have shown that microbubbles (MBs) serve as a potential modality for both tumor therapy and imaging [3]. When systemically administrated, the interaction between the perfluoropropane (PFP) gas in MBs and an ultrasound (US) wave result in contrast signal enhancement in the blood vessels. However, irradiating MBs with higher pressure amplitude US causes cavitations [4,5]. These effects, known as sonoporation, enhance the delivery of drugs and nucleic acids into cells for improving therapeutic outcome [6]. Although US is a safe, easily performed, and relatively cheap technology, US imaging lacks high resolution especially in the case of deep tissues. Therefore, the formulation allows us not only to determine MB distribution but also to achieve treatment response at the tumor site in a non-invasive manner [7,8].

Doxorubicin is one of the most frequently used anti-cancer agents for a variety of solid tumors. Combination of doxorubicin loaded MBs (DLMBs) with US irradiation has been found to enhance doxorubicin uptake through US induced cavitation effects [9,10]. Regarding the stability of doxorubicin encapsulation into liposomes, the use of anionic phospholipids may be suitable for loading via electrostatic interaction [11,12]. For theranostic applications concerning the tumor, not only doxorubicin but also PFP gas should be stably encapsulated into the formulation. However, MBs lack stability under *in vivo* conditions as a result of gas diffusion and leakage [8,13].

For the stable encapsulation of PFP gas into bubble formulations, the selection of lipid is an important factor [14,15]. So far, our group has developed positively charged bubble liposomes for gene or siRNA delivery in combination with US irradiation [16,17]. Additionally, we have developed negatively charged

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bubble lipopolyplex using 1,2-distearoyl-sn-glycero-3-phosphatidylglycerol (DSPG) as an anionic lipid for safe and efficient transfection by diminished interaction with erythrocytes [18,19]. Recently, Sax et al. have reported that DSPG incorporated bubble (acoustic) liposomes had an enhanced half-life both *in vitro* and *in vivo* [20]. Taking all of these factors into consideration, we expect that the use of anionic MBs partly containing DSPG can offer a platform for the development of more stable DLMBs with theranostic applications in cancer therapy.

In the present study, we optimized the conditions required for the preparation of stable DLMBs using negatively charged MBs in relation to the encapsulation of doxorubicin and PFP gas by means of a simple agitation method. The potential of DLMBs for use in theranostics was evaluated under both *in vitro* and *in vivo* conditions for cancer therapy and US imaging.

2. Materials and methods

2.1. Phospholipids

DSPG, 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC) and PEG_{2000}-DSPE were purchased from Avanti Polar Lipid Inc. (Alabaster, AL, USA) and NOF Co. (Tokyo, Japan).

2.2. Cells

The B16BL6 murine melanoma cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's modified eagle medium Nissui Pharmaceutical Co., Ltd., (Tokyo, Japan) supplemented with 10% fetal bovine serum and 100 U/ml penicillin/streptomycin at 37 °C in 5% CO_2.

2.3. Animals and tumor models

Female 6-week-old C57BL6 mice were purchased from the Shizouka Agricultural Cooperation Association for Laboratory Animals (Shizouka, Japan) and female 6-week-old HR1 hairless mice were obtained from Sankyo Laboratory Service Corporation, Inc. (Tokyo, Japan). For preparing tumor bearing mice, 1×10^6 cells in phosphate buffered saline were injected subcutaneously into the left flanks of mice with a 26-gauge needle. Experiments were initiated when tumors reached 5–10 mm in diameter after 9–14 days. All experiments were carried out in accordance with the principles of laboratory animal care in accordance with the Principles of the National Institutes of Health and Guidelines for Animal Experiments of Kyoto University; all experiments were approved by Teikyo University School of Medicine Animal Ethics Committee, reference number of approval was: 14–027.

2.4. Doxorubicin liposome and DLMBs preparation

DPPC, DSPG and PEG_{2000}-DSPE in a 7:2.5:0.5 molar ratio were dissolved in chloroform, followed by evaporation of chloroform in a rotary evaporator at 25 °C for 30 min; this was followed by further drying under vacuum at room temperature overnight. Ten mg of lipid film was hydrated with 3 ml of 5% glucose solution at 65 °C for 60 min under mild agitation. The final lipid concentration after hydration was adjusted to 3 mg/ml. The glucose solution also contained 2 mg doxorubicin. For preparing MBs 0.5 ml of liposomes was added to a 5-ml sterilized vial. The air in the vial was replaced with PFP gas (Takachiho Chemical Industries Co., Tokyo, Japan) and after capping 6 ml of PFP was injected. To obtain the MBs a shaking machine (Ultra Mate 2, Victoria, Australia) was used. Temperature was measured after agitation using a needle type thermometer (Custom Co., Tokyo, Japan). Perfluoropropane content analysis was performed as reported previously [21]. The particle size and zeta potential of the liposomes and MBs were determined using a Zetasizer Nano ZS instrument (Malvern Instruments Ltd., Worcestershire, UK).

2.5. Doxorubicin binding efficiency

To determine the binding efficiency of doxorubicin in DLMBs, a sample consisting of a 0.5-ml DLMBs dispersion (1.65 mg lipid and 0.33 mg doxorubicin) was centrifuged at 16,000 g for 2 min. Then the sample was divided to three fractions: a foaming cake at the top which contained doxorubicin loaded MBs; a pellet at the bottom, which contained liposomes; and in-between a solution containing free doxorubicin. The fractions were collected, and then the concentration in each fraction was determined by measuring doxorubicin fluorescence (480 nm was used as excitation wavelength and doxorubicin signal was detected at 590 nm) (FluoroMax4, Horiba, Ltd., Kyoto, Japan).

2.6. In vitro echogenicity

The DLMBs were injected into a beaker filled with 500 ml of degassed distilled water at 37 °C under magnetic stirring. Ultrasound contrast enhancement was observed using an ultrasonography system (Vevo 2100, Visual Sonics, Inc. Toronto, Canada). For additional investigations of DLMBs destruction by higher energy US burst, an external US probe at an intensity of (2 W/cm^2) was used. The process of US burst was repeated until most of the DLMBs had disappeared.

Table 1
Mean particle size and zeta potential of liposomes and microbubbles. $n = 3$; mean ± SEM.

	Mean particle size (nm)	Zeta potential (mV)
Unloaded liposome	250 ± 1	−0.076 ± 0.02
DOX loaded liposome	270 ± 6	0.038 ± 0.02
Unloaded microbubbles (MBs)	1051 ± 4	−0.066 ± 0.04
DOX loaded microbubbles (DLMBs)	1022 ± 5	0.031 ± 0.01

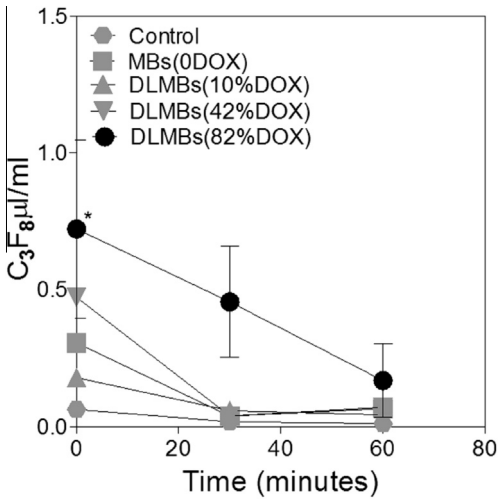

Fig. 1. Time course study of DLMBs with different doxorubicin concentrations presented as percentage equivalent to anionic phospholipids. Mean ± SEM. $^*P < 0.05$ versus the corresponding control group (saline with PFP gas).