Dependence of release of liposome included in giant cluster vesicles on microbubble concentration

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1. Introduction

Intraoperative localization of main tumors, as well as estimation of metastatic status of the regional lymph nodes, especially sentinel lymph nodes (SLNs; the first draining lymph nodes from the main tumor), is desired for surgical treatments of malignant diseases. A multifunctional contrast agent, which has two contradictory properties i.e. retention in tissue for lesion-marking and migration to lymphatics, has been proposed. The agent named Giant Cluster Vesicles (GCVs) can stay in the injected area for a long time and includes liposomally formulated indocyanine green (ICG) derivatives as a tracer particle for SLN identification. We aim to instantaneously release the encapsulated tracer particles via the destruction of GCVs caused by the interaction between ultrasound and microbubbles (MBs). The previous studies have shown the possibility of controlling the amount of tracer particles released by adjusting the sound pressure conditions. To maximize the release of tracer particles while avoiding harmful damage to living tissue, it is necessary to investigate the effect of the number density of MBs. In this report, the amount of released tracer particles was quantitatively evaluated at various conditions for the number density of MBs.

2. Materials and methods

2.1 Preparation of Giant Cluster Vesicles

The GCV, which is an aggregate of vesicles with a particle size larger than 1 μm, is composed of a lipid bilayer with polyglycerol polyricinoleate (PGPR) as the membrane. We encapsulated LP-ICG-C18 in GCV as liposomally formulated ICG derivatives. LP-ICG-C18 incorporates ICG-C18 into the liposome membrane composed of 1,2-dioleoyl-3-sn-glycero-phosphocholine (DOPC) as the main component and *N*-(methylpolyoxyethylene oxycarbonyl)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine sodium salt (DSPE-PEG5000) and cholesterol. ICG-C18 is an ICG derivative synthesized by substitution of one sulfonate groups of the ICG with an 18-carbon alkyl chain [1][2].

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To examine the dependence of the release of LP-ICG-C18 on the number density of MBs, MBs suspensions with three different conditions for the number densities (n) were prepared and mixed with GCVs suspension at a volume ratio of 1:1. The commercial ultrasound contrast agent named Sonazoid®, dispersed in 1 mol/L sucrose solution, was used as MBs suspensions. The mean particle diameter of MBs was 2.5 μm, and the number densities were 1.0×10^{14}, 1.0×10^{13}, and 1.0×10^{12} m^{-3}, respectively. The GCVs suspension without MBs was mixed with 1 mol/L sucrose solution at a volume ratio of 1:1 as control.

2.2 Procedure for releasing tracer particles

A silicone tube with an inner diameter of 2 mm was placed in an experimental cell filled with degassed water, and 1 mL of sample suspension was flowed at 0.35 mL/min using a syringe pump. The sample so in the tube was irradiated with ultrasound from above using the shear wave elastography mode of the ultrasonic equipment (LOGIQ S8, GE Healthcare) and linear array probe (9L-D, GE Healthcare). The peak frequency of the transmitted ultrasound was 4.2 MHz. The lateral direction of the probe and the long axis direction of the tube were made orthogonal. The focus point of the ultrasound beam was positioned at the center of the tube.

In this experiment, the ultrasound with a peak negative sound pressure (P_{np}) of 1.5, 1.8, and 6.9 MPa was irradiated, and the reference experiments were also conducted without ultrasound exposure. The ultrasonic equipment for this study was adjusted for preclinical experiments, and the settings for ultrasound irradiation differed from those used in clinical.

2.3 Quantification of the amount of released tracer particles

The sample suspension (0.80 mL) collected after the ultrasound irradiation was centrifuged and sedimented at 6,000 rpm for 30 min using a small centrifuge. The supernatant liquid was collected to recover LP-ICG-C18 released from GCVs. The supernatant liquid was diluted, and the absorbance was measured using a UV-visible spectrophotometer (UV-1800, SHIMADZU). Six data were obtained per each experimental condition to check the reproducibility.

The normalized release amount (R_N) of LP-ICG-C18 under the same conditions for the number density of MBs was calculated from the dilution factor (n_0) and the concentration of LP-ICG-C18 in the supernatant liquid (c_0) without ultrasound exposure, the dilution factor (n_r) and the concentration of LP-ICG-C18 in the supernatant liquid (c_r) under all experimental conditions.

$$R_N = \frac{n_r \cdot c_r - n_0 \cdot c_0}{n_0 \cdot c_0} \quad (1)$$

The concentration of ICG-C18 was calculated from the preliminarily-obtained calibration curve of absorbance and ICG-C18 concentration.

3. Results

Figure 1 shows R_N of LP-ICG-C18 under each ultrasound pressure condition. There was no significant difference in R_N of LP-ICG-C18 between without ultrasound exposure and P_{np} = 1.5 MPa ($p \geq 0.05$), confirming that the release of LP-ICG-C18 was not achieved in each condition for the number density of MBs. On the other hand, there was a significant difference in R_N without ultrasound exposure and $P_{np} \geq 1.8$ MPa for all conditions ($p < 0.05$), indicating that the release of LP-ICG-C18 was achieved. This additionally suggested that the release of LP-ICG-C18 had sound pressure threshold at 1.5 < $P_{np} \leq 1.8$ MPa, which was independent of the number density of MBs.

Figure 2 shows R_N of LP-ICG-C18 versus the number density of MBs. At $P_{np} \geq 1.8$ MPa, there was no significant difference between R_N at $n = 1.0 \times 10^{14}$ m^{-3} and that at $n = 1.0 \times 10^{13}$ m^{-3} in the same sound pressure conditions ($p \geq 0.05$). However, compared to these two conditions, R_N at $n = 1.0 \times 10^{12}$ m^{-3} was lower and significantly different ($p < 0.05$). This indicated that the release of LP-ICG-C18 increased with an increase in the number density of MBs for $n < 1.0 \times 10^{13}$ m^{-3}. In contrast, the release enhancement effect was saturated for $n > 1.0 \times 10^{13}$ m^{-3}.

4. Conclusions

In this report, the dependence of LP-ICG-C18 release on the number density of MBs was quantitatively investigated under different sound pressure conditions, and it was confirmed that 1) the sound pressure threshold for the release of LP-ICG-C18 was independent of the number density of MBs and 2) the enhancement of LP-ICG-C18 release saturated at a certain number density of MBs.

Acknowledgment

This work was partly supported by JSPS Core-to-Core Program JPJSCCA20170004 and JSPS Grant-in-Aid for Scientific Research 19H04436. We would also like to thank Dr. Naohisa Kamiyama and Dr. Takuma Oguri (GE Healthcare Japan) for their cooperation in the experiments using the ultrasonic equipment.

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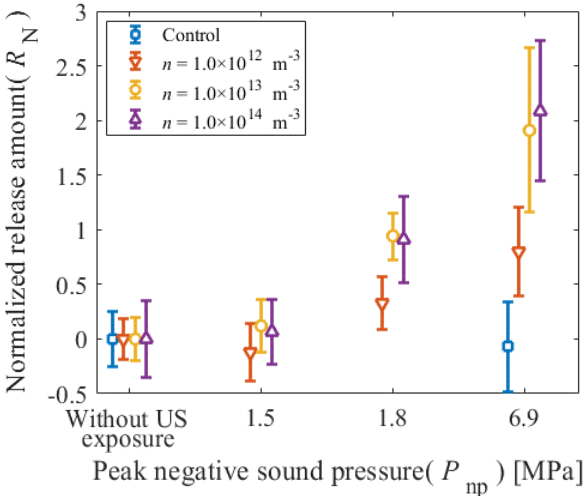

Fig. 1 Normalized release amount of tracer particles as a function of sound pressure.

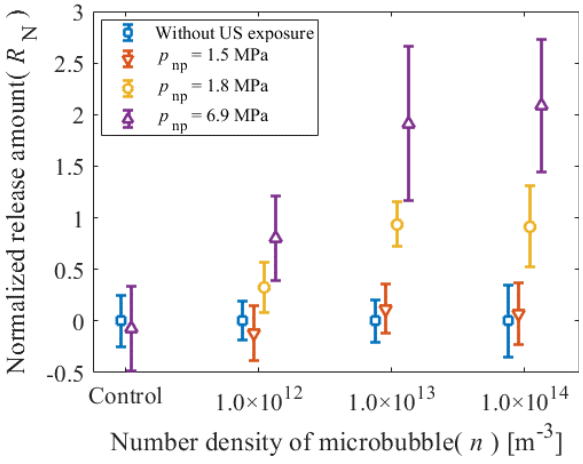

Fig. 2 Normalized release amount of tracer particles as a function of the number density of microbubbles.