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Note

Real-time *in vivo* imaging of surface-modified liposomes to evaluate their behavior after pulmonary administration

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ABSTRACT

Our previous study demonstrated that surface modification of liposomes using polyvinyl alcohol with a hydrophobic anchor (PVA-R) achieved sustained absorption from the lung after pulmonary administration and prolonged the pharmacological effects of the model peptide drug. In the present study, the behavior of PVA-R-modified liposomes in the lung and whole body was monitored using a real-time *in vivo* imaging system. Subsequently, the influence of surface modification with PVA-R on liposomal behavior in lung tissue was examined. Indocyanine green (ICG) was used as a near-infrared label of PVA-R-modified liposomes and was used to observe their dynamic behavior using non-invasive *in vivo* imaging (IVIS® imaging system) after pulmonary administration to rats. PVA-R-modified submicron-sized liposomes (ssLips) induced long-term retention in the lung compared with unmodified liposomes. Moreover, liposome association with alveolar macrophages (NR8383) was decreased by PVA-R modification *in vitro*. Therefore, PVA-R modification may prevent rapid elimination of ssLips by macrophages, thereby increasing retention in the lung.

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1. Introduction

Pulmonary drug delivery is one of the most promising non-invasive routes for both local and systemic treatments. Its advantages over other delivery routes include a large surface area, thin absorption barrier, low metabolic activity, evasion of first-pass metabolism, decreased side effects, and direct delivery of therapeutic agents to the site of action [1,2].

Local and systemic pulmonary delivery of several drugs, including small molecules, genes, and protein/peptide drugs, has been demonstrated [3–5]. In fact, many studies show applications of such drugs to the treatment of chronic respiratory diseases such as lung cancer, asthma, and chronic obstructive pulmonary disease [6–8].

In addition, pulmonary application of protein and peptide drugs offers great potential for systemic drug delivery [9,10]. Subsequent therapeutic outcomes and pharmacodynamic effects are related to pulmonary bioavailability and lung deposition of inhaled therapeutic drugs [11].

Liposomes are one of the most extensively investigated systems for controlled delivery of drugs to the lung [12–14]. In fact, inhaled liposomes protect drugs against enzymatic degradation and result in significantly higher relative bioavailability compared with plain drug solutions [15].

Our previous work demonstrated that modification of submicron-sized liposomes (ssLips) with polyvinyl alcohol conjugated hydrophobic anchors (PVA-R) feasibly improves peptide drug delivery through the lung [16]. Compared with peptide drug solutions, PVA-R-modified liposomes increased pharmacological availability after pulmonary administration. This observation suggested that long-term lung retention of PVA-R-modified liposomes may enhance the pharmacological effects of peptide drugs. However, the effects of PVA-R on the behavior of pulmonary liposome systems in the whole body require *in vivo* characterization; the fates of PVA-R-modified liposomes and the mechanisms behind their long-term lung retention after pulmonary administration have not been fully characterized.

Near-infrared (NIR) optical imaging is a powerful tool for *in vivo* observations of dynamic liposome behaviors because it is a minimally invasive, non-ionizing method that permits sensitive deep tissue imaging [17–19]. Therefore, to elucidate the mechanisms of enhanced peptide drug absorption, the effects of PVA-R modification on the pharmacodynamics and retention of liposomes in the

Abbreviations: DSPC, L-α-distearoylphosphatidylcholine; DCP, dicetyl phosphate; Chol, cholesterol; ssLips, submicron-sized liposomes; MLV, multilamellar vesicles; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; PVA-R, polyvinyl alcohol with a hydrophobic anchor; ICG, indocyanine green; MES, 2-morpholinoethanesulfonic acid monohydrate; HBSS, Hank's balanced salt solution; NR8383, rat alveolar macrophage cell line; IVIS, *in vivo* imaging system.

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lung were evaluated using *in vivo* imaging in rats. Indocyanine green (ICG), which has a fluorescence emission wavelength of approximately 820 nm, was used as a liposome tracer in rat bodies. Fluorescence was detected using an IVIS® imaging system with a hypersensitive charged coupled device camera *in vivo*. In this study, the effects of liposome particle size and PVA-R-surface modification on time-dependent changes in their fate in the lung were investigated using non-invasive *in vivo* imaging.

2. Material and methods

Surface-modified liposomes were prepared using a thin-film hydration method [16]. A mixture of L-α-distearoylphosphatidylcholine (DSPC; Nippon Oil and Fats Co. Ltd., Japan), dicetyl phosphate (DCP; Sigma, St. Louis, MO, USA), and cholesterol (Chol, Sigma) were dissolved at a molar ratio of 8:1:2 in chloroform containing indocyanine green (ICG; MP Biomedicals, LLC, Irvine, CA, USA) or 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI, Lambda Probes, Graz, Austria). The solvent was evaporated to dryness, and the lipid film was further dried overnight under vacuum. Multilamellar vesicles (MLVs) were produced by hydrating the lipid film in Hank's balanced salt solution (HBSS)–2-morpholinoethanesulfonic acid monohydrate (MES) buffer (pH 6.0), and ssLips were obtained by extrusion (LipoFast™-Pneumatic; Avestin, Inc., Ottawa, Canada). Final phospholipid, ICG, and DiI concentrations in the resulting liposomal suspensions were 8.06 mg/mL, 10 μg/mL, and 37.5 μg/mL, respectively. To prepare PVA-R (Kuraray Co., Ltd., Osaka, Japan)-modified liposomes, an aliquot of the liposomal suspension was mixed with the same volume of PVA-R polymer solution.

Particle sizes and zeta potentials were determined using a Zetasizer Nano ZS90 instrument (Malvern Instruments Ltd., Malvern, UK). To determine the efficiency of ICG loading into ssLips, ICG-loaded ssLips were separated from free ICG by ultracentrifugation (231,000*g*, 45 min) at 4 °C. ICG concentrations in the resulting supernatants were determined using a fluorescence spectrophotometer (Model FP-6600; Jasco, Japan).

All animal experiments were approved and monitored by the Institutional Animal Care and Use Committee of Gifu Pharmaceutical University (Gifu, Japan) in line with the Japanese legislation on animal studies. ICG-labeled liposomes were administrated to the lung and monitored using an *in vivo* imaging system (IVIS® imaging system, IVIS Lumina 2, Caliper Life Sciences, Cheshire, UK). After transiently anesthetizing male Wistar rats (6-weeks-old; Japan SLC, Inc., Shizuoka, Japan) by inhalation of isoflurane (approximately 0.1%), liposome suspensions were administered through tracheas at a volume of 0.2 mL/rat using a Microsprayer® (Model IA-C; Penn Century, Inc., Philadelphia, PA, USA). To detect ICG fluorescence, a 745-nm excitation filter and an 820-nm emission filter were used, and the exposure time was set at 1 s. During these measurements, rats were anesthetized with isoflurane on a stage maintained at 37 °C.

Uptake experiments were conducted *in vitro* using cultured NR8383 rat alveolar macrophages [American Type Culture Collection (ATCC), Manassas, VA, USA]. DiI-labeled liposome–cell associations were determined using a previously described method from this laboratory [16].

3. Results and discussion

Table 1 summarizes the physicochemical properties of several liposome preparations. Particle sizes of unmodified MLVs and ssLips were 883.4 and 102.2 nm, respectively. These liposomes exhibited negative zeta potential owing to the negatively charged lipid DCP. Liposome surfaces were modified by mixing with PVA-R polymer solutions. Hence, the effects of PVA-R modification may be explained by anchoring of the hydrophobic moiety of PVA-R to the lipid membranes of liposomes [20]. Indeed, particle sizes increased, and the zeta potentials shifted from negative to neutral after surface modification of MLVs and ssLips with PVA-R. Increased particle sizes indicated the formation of a thick PVA layer on the liposomal surface, and zeta potential shifts confirmed that modification of negatively charged liposomal surfaces involved interactions with PVA-R.

Table 1
Particle size and zeta potential of unmodified and surface-modified liposomes.

Surface modification polymer	Final polymer concentration (w/v%)	Particle size (nm)	Zeta potential (mV)
Unmodified MLVs	0	883.4	−97.0
PVA-R-modified MLVs	2.0	920.3	−13.6
Unmodified ssLips	0	102.2	−50.1
PVA-R-modified ssLips	2.0	154.8	−6.5

Hydrophobic ICG was embedded into the lipid bilayers of liposomes. Subsequently, the efficiency of ICG entrapment in liposomes was confirmed to be >90%, and almost all ICG was retained in the liposomes during storage in the HBSS–MES buffer (pH 6.0) for 48 h at 37 °C (data not shown). The positions and quantities of ICG can be monitored using fluorescent imaging. *In vivo* fluorescence can be detected more accurately from compounds with emission-wavelengths >620 nm because auto-fluorescence is minimized. ICG has an emission wavelength of about 820 nm, and was used as a liposome-labeling agent for pulmonary delivery. Indeed, ICG labeled liposomes have been previously used to image lymphatic flow and clearance in several mouse models [21]. To monitor pulmonary delivery of liposomes in rats, initially, ICG labeled MLVs with or without surface modification by PVA-R were administered, and fluorescence was observed in the whole body at specific time points (Fig. 1). Fluorescence intensities in the lungs did not differ greatly between animals treated with unmodified and PVA-R-modified MLVs, disappearing within 6 h post-administration in both cases. Hence, PVA-R modification of MLVs had negligible effects.

Chono et al. demonstrated the influence of particle size on the uptake of liposomes by alveolar macrophages. In their study, uptake of micron-sized particles by macrophages was significantly greater than that of submicron-sized particles [22]. Therefore, it was assumed that MLVs with or without surface modification are rapidly cleared by alveolar macrophages, potentially owing to their large particle sizes.

Fig. 2 illustrates IVIS® live imaging in rats during the 48 h after intratracheal administration of unmodified and PVA-R-modified ssLips. Changes in total radiant efficiency post-administration of unmodified and PVA-R-modified ssLips in the lung were calculated from these images (Fig. 3 and 4). In these experiments, ICG intensities of ssLips were higher than those of MLVs (Fig. 3). Moreover, fluorescence intensities of unmodified ssLips gradually decreased over 48 h post-administration (Fig. 4). ICG fluorescence from PVA-R-modified ssLips was similar to that of unmodified ssLips immediately after administration, but PVA-R-modified ssLips emitted sustained fluorescence in rats. More PVA-R-modified ssLips remained in the rat lungs after pulmonary administration than unmodified ssLips. It was expected that liposomal surface modification would improve the stability and retention of liposomes in the lung owing to the thick and flexible layer (ca. 20–30 nm) of PVA-R on liposome surfaces. Our previous study demonstrated that liposome surface modification using PVA-R improved drug circulation times and reduced uptake by the reticuloendothelial system (RES) after intravenous administration in rats [20]. These data

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