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Research paper

Pulmonary delivery of elcatonin using surface-modified liposomes to improve systemic absorption: Polyvinyl alcohol with a hydrophobic anchor and chitosan oligosaccharide as effective surface modifiers

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ABSTRACT

The aim of this study was to investigate the feasibility of surface-modified liposomes for pulmonary delivery of a peptide. Chitosan oligosaccharide (oligoCS) and polyvinyl alcohol with a hydrophobic anchor (PVA-R) were used as surface modifiers. The effect of liposomal surface modification on the behavior of the liposomes on pulmonary administration and potential toxicity were evaluated *in vitro* and *in vivo*. In an association study with A549 cells, PVA-R modification reduced interaction with A549 cells, whereas oligoCS modification electrostatically enhanced cellular interaction. The therapeutic efficacy of elcatonin (eCT) after pulmonary administration to rats was significantly enhanced and prolonged for 48 h after separate administration with oligoCS- or PVA-R-modified liposomes. oligoCS-modified liposomes adhered to lung tissues and caused opening of tight junctions, which enhanced eCT absorption. On the other hand, PVA-R-modified liposomes induced long-term retention of eCT in the lung fluid, leading to sustained absorption. Consequently, surface modification of liposomes with oligoCS or PVA-R has potential for effective peptide drug delivery through pulmonary administration.

1. Introduction

Pulmonary drug delivery has many advantages over other delivery routes because the lungs have a large absorptive area, extensive vasculature, easily permeable membranes, and low extracellular and intracellular enzyme activity [1,2]. The alveolar epithelia have been reported to be thin and permeable, thereby possibly allowing systemic absorption of higher molecular weight drugs (e.g., peptide and protein drugs) through the alveolar region of the lungs [3,4].

Liposomes are attractive drug carriers for designing drug delivery systems because liposomes can control drug release and provide selective drug targeting [5,6]. Liposomes as drug carriers have the advantage of safety because they consist of phospholipids, which are biocomponents.

Another advantage of liposomal carriers is the relative ease of liposomal surface modification [7,8]. We previously reported the feasibility of modifying the surface of liposomes using polyvinyl alcohol with hydrophobic anchors (PVA-R) to improve the drug circulation time, with less uptake by the reticuloendothelial system (RES) after intravenous administration in rats, compared with unmodified liposomes [9]. The steric hindrance caused by a PVA layer formed on the surface of liposomes can account for this phenomenon, which is similar to the stealth function of polyethylene glycol-modified liposomes [10,11]. The flexible layer (ca. 20–30 nm) of PVA-R on the liposomal surface was responsible for the stability of surface-modified liposomes in the presence of serum, as well as in the bloodstream due to a reduction in RES uptake; in contrast, a layer of PVA without hydrophobic anchor was not found to be effective under the same experimental conditions. In addition, we have demonstrated that it is feasible to design a mucoadhesive liposomal system for oral peptide delivery by modification with chitosan (CS) [12,13]. CS is generally recognized as a biocompatible and biodegradable polysaccharide that exhibits low toxicity [14]. Therefore, we expected that PVA-R or CS modification would also be effective for pulmonary delivery of peptides using liposomes. In our previous study, a high molecular weight CS was used as a surface

Abbreviations: CS, chitosan; oligoCS, chitosan oligosaccharide; PVA-R, polyvinyl alcohol with a hydrophobic anchor; eCT, elcatonin; RES, the reticuloendothelial system; DSPC, L-α-distearoylphosphatidylcholine; DCP, dicetyl phosphate; CHOL, cholesterol; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; MES, 2-morpholinoethanesulfonic acid monohydrate; HBSS, Hank's balanced salt solution; A549, human lung cancer epithelial cell line; Calu-3, human airway epithelial cell line; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; Triton X-100, 1.0% polyoxyethylene octylphenyl ether; BAL, bronchoalveolar lavage; BALF, bronchoalveolar lavage fluid.

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modifier; however, this type of CS is not suitable for surface modifier to induce the aggregated liposomal shapes and has poor physical properties, such as low solubility at neutral pH and high viscosity at concentrations used *in vivo*. We speculated that these drawbacks could be surmounted if a lower molecular weight CS was used. In this study, we selected a CS oligosaccharide (oligoCS) and PVA-R to modify liposomes and investigated the usefulness of these surface-modified liposomes for pulmonary drug delivery systems. The safety and efficacy of surface-modified liposomes were tested in cell culture models and rodents. The pharmacological effect of surface-modified liposomes for pulmonary delivery was evaluated using elcatonin (eCT) as a model peptide drug. eCT consists of 31 amino acids and is a drug for the treatment of osteoporosis. It is calcitonin derivative that is transformed from eel's calcitonin by changing the S–S bond into the stable C–N bond, resulted in being stable physically and biologically. The biological activity of eCT is equal to that of eel's calcitonin several times stronger than that of human's [15].

2. Materials and methods

2.1. Materials

L-α-Distearoylphosphatidylcholine (DSPC; COATSOME MC-8080) was purchased from Nippon Oil and Fats Co., Ltd. (Tokyo, Japan). Dicetyl phosphate (DCP) and cholesterol (CHOL) were procured from Sigma Chemical Co. (St. Louis, MO, USA). The fluorescent lipid reagent 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) was purchased from Lambda Probes (Graz, Austria). OligoCS (MW around 1000 [16]) was supplied by Katakura Chikkarin Co., Ltd. (Tokyo, Japan). PVA-R with a hydrophobic anchor ($C_{16}H_{33}S$–) at the terminal end of the polymer (MW 20 000) was supplied by Kuraray Co., Ltd. (Osaka, Japan). eCT was kindly supplied by Asahi Chemical Co., Ltd. (Osaka, Japan). 2-Morpholinoethanesulfonic acid monohydrate (MES) was purchased from Nakalai Tesque (Kyoto, Japan). Hank's balanced salt solution (HBSS) was purchased from Gibco BRL (Grand Island, NY, USA). All reagents were of analytical grade.

2.2. Cell lines

Human lung A549 epithelial cells (Riken Gene Bank, Ibaraki, Japan) were maintained as adherent monolayer cultures in Dulbecco's modified Eagle's medium supplemented with 10% v/v fetal bovine serum, 100 IU/mL penicillin, and 100 IU/mL streptomycin. Calu-3 human airway epithelial cells (American Type Culture Collection, Manassas, VA, USA) were cultured in Eagle's minimum essential medium supplemented with 10% v/v fetal bovine serum, 100 IU/mL penicillin, 100 IU/mL streptomycin, 2 mM L-glutamine, and 1× MEM nonessential amino acid solution. All reagents utilized in cell cultivate were obtained from GIBCO, USA. The medium was changed every 2 days and subcultured after achieving 85–90% confluence.

2.3. Preparation of surface-modified liposomes

Submicron-sized liposomes composed of DSPC, CHOL, and DCP with a molar ratio of 8:1:2 were prepared by a hydration method followed by extrusion (LipoFast™-Pneumatic; Avestin, Inc., Ottawa, Canada) with a size-controlled polycarbonate membrane (membrane filter pore size 0.1 μm; Whatman Japan KK, Tokyo, Japan). The lipid mixture containing DiI was dissolved in a small amount of chloroform in a round-bottom flask and dried in a rotary evaporator under reduced pressure at 40 °C to form a thin lipid film. The film was dried in a vacuum oven overnight to ensure complete removal of the solvent. After addition of HBSS/MES buffer, the lipid film was incubated in a water bath at 70 °C for 30 s and then vortexed for about 30 s. This cycle was repeated five times. The resultant multilamellar liposomal suspension was incubated at 10 °C for 30 min and then extruded 41 times under nitrogen pressure (200 psi). The final phospholipid and DiI concentrations in the resultant liposomal suspension were 8.06 mg/mL and 37.5 μg/mL, respectively.

For the preparation of surface-modified liposomes, an appropriate amount of PVA-R or oligoCS was dissolved in an acetate buffer solution (100 mM, pH 4.4). An aliquot of the liposomal suspension was mixed with the same volume of polymer solution of various concentrations (0–2% w/v for PVA-R or 0–0.3% w/v for oligoCS). The mixed liposomal suspension with the polymer solution was incubated at 10 °C for 60 min. Particle size was measured for an aliquot of the particulate suspension diluted with a large amount of distilled water using the dynamic laser scattering method (Zetasizer Nano ZS, Malvern, Worcestershire, UK). The zeta potential of surface-modified liposomes was quantified using a laser Doppler method (Zetasizer Nano ZS). For *in vivo* studies, eCT-containing liposomes were prepared in a similar manner as described earlier. eCT was loaded into different liposomal carriers during the lipid film hydration step. Instead of using buffer only, hydration was performed with 66.67 mM phosphate buffer (pH 6.8)-containing eCT. eCT-loaded liposomes were mixed with an aliquot of 0.6% w/v oligoCS or 4% w/v PVA-R solution in 100 mM acetate buffer solution (pH 4.4) (the final eCT concentration of surface-modified liposomes was 20 μg/mL). As a control, an eCT solution (20 μg/mL) in 66.67 mM phosphate buffer (pH 6.8) was prepared.eCT-loaded liposomes were ultracentrifuged for 45 min at 231 000*g* at 4 °C, and the eCT concentration of the supernatant was determined using the Micro BCA protein assay kit (Bio-Rad, CA, USA). The efficiency of eCT entrapment in each liposome was around 100%.

2.4. Cytotoxicity test using an A549 cell monolayer

The cytotoxicity of surface-modified liposomes was determined by measuring the production of the colored formazan product upon cleavage of the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) reagent by mitochondrial dehydrogenases in viable cells. A549 cells were seeded at a density of 1.58×10^5 cells/mL on 96-well plates. The culture medium was removed and washed twice with 200 μL of HBSS. The cells were then exposed to 150 μL of different liposomal formulations (0.01–0.06% w/v oligoCS-modified liposomes and 0.2–1.0% w/v PVA-R-modified liposomes). After 120 min of incubation, the cells were washed three times with 150 μL of HBSS. The cells were incubated with 20 μL of CellTiter 96® AQueous One Solution Reagent (Promega, Madison, WI, USA) composed of 317 μg/mL MTS in 100 μL of culture medium. After incubation in a CO_2 incubator for 2 h, absorbance values were measured using a microplate reader (MTP 120; Corona Electric, Tokyo, Japan) at a wavelength of 492 nm. The background absorbance in cell-free wells was measured and subtracted from the measurement absorbance. A solution of 1.0% polyoxyethylene octylphenyl ether (Triton X-100) in HBSS/MES buffer served as a positive control, and HBSS/MES buffer served as a negative control. The percentage of cell viability was calculated by the following equation: % Cell viability = $(ABS_{sample}/ABS_{control}) \times 100$, where ABS_{sample} is the absorbance value of a well exposed to polymer-coated liposomes and $ABS_{control}$ is the absorbance value of a well treated with HBSS/MES buffer.

2.5. Cellular association experiment

A549 cells were plated in 24-well plates at a density of 1.0×10^6 cells/mL 24 h prior to studying the liposomal cellular

uptake. The cells were washed three times with HBSS/MES buffer (pH 6.0) and then incubated for 2 h with DiI-labeled liposomes or surface-modified DiI-labeled liposomes. After incubation, the cells were washed three times with ice-cold HBSS/MES buffer (pH 6.0) and then lysed with 0.2 mL of 1.0 N NaOH. The fluorescent dye taken up by the cells was extracted from the cell lysate using an appropriate amount of chloroform–methanol mixture (1:1 v/v); the sample was vigorously mixed using a reciprocal shaker (SR-2; Taitec, Tokyo, Japan). The organic phase was analyzed for DiI using a fluorescence spectrophotometer (F3010; Hitachi, Tokyo, Japan). The amount of cellular uptake of DiI was normalized to that of the protein determined using the Micro BCA protein assay kit (Bio-Rad).

2.6. Observation of DiI fluorescence using a confocal laser scanning microscope (CLSM)

A549 cells were seeded on a Lab-Tek chamber slide system (Nunc, Copenhagen, Denmark) at a density of 1.0×10^6 cells/mL 24 h prior to the CLSM study. The cells were washed three times with HBSS/MES buffer (pH 6.0) and then added to DiI-labeled liposomes or surface-modified DiI-labeled liposomes. After incubation at 37 °C for 2 h, the cells were washed three times with ice-cold HBSS/MES buffer and then fixed with paraformaldehyde at 4 °C for 10 min. The coverslips were placed on a drop of SlowFade® Light Antifade (Molecular Probes, Carlsbad, CA, USA). Each cell sample was placed on CLSM (Zeiss, Thornwood, NY, USA) and observed with an excitation wavelength of 550 nm and an emission wavelength of 570 nm.

2.7. Transepithelial electrical resistance (TEER) study across Calu-3 cell line

Calu-3 cells were seeded at a density of 5.0×10^5 cells/well onto polycarbonate Transwells® (pore size 0.4 μm, area 1 cm^2; Corning International KK, Tokyo, Japan). After the cells had attached to Transwells®, the medium was removed from the apical chamber to allow the monolayer to grow under the air-interface condition. It has been reported that air-interface conditions stimulate differentiation of the Calu-3 cell monolayers to form a polarized, bioelectrically tight epithelial monolayer and that the air-interface cultured cell layers are a more suitable model of the tracheobronchial epithelium than liquid-covered cultured cells [17]. The medium in the basolateral chamber was changed, and days 11–14 monolayers were used in the experiments. TEER measurements were taken using the EVOM® epithelial voltohmmeter and handheld STX-2 electrodes (World Precision Instruments, Inc., Hertfordshire, UK). On the day of the experiment, the medium was aspirated and the cell layers were washed twice with warm HBSS (37 °C; apical chamber, 0.1 mL; basolateral chamber, 0.6 mL). Next, 0.5 mL and 1.5 mL of HBSS was introduced into the apical and basolateral chambers, respectively, and the cells were returned to the incubator at 37 °C for 1 h to equilibrate. Following equilibration, HBSS in the apical chamber was replaced with 0.5 mL of different liposomal formulations (HBSS/MES buffer as a negative control, liposomes without modification, and 0.015% w/v oligoCS-modified liposomes). The change in TEER of the monolayers after exposure to each sample was monitored over 2 h.

2.8. Toxicity study on surface-modified liposomes

All animal experiments were approved and monitored by the Institutional Animal Care and Use Committee of Gifu Pharmaceutical University. The toxicity study on surface-modified liposomes was conducted using male Wistar rats (6 weeks old; Japan SLC, Inc., Shizuoka, Japan). After the rats were transiently anesthetized with inhalation of isoflurane (approximately 0.1%), phosphate-buffered saline (PBS), 1.0% v/v Triton X-100, and surface-modified liposome suspensions were administered through rat tracheas at a volume of 0.2 mL/rat according to the method by Ho and Furst [18] or Yamada et al. [19]. In brief, each rat was placed on a retainer with the upper incisors hooked onto a wire frame and the body positioned in such a way that its head, neck, and thorax hung down vertically. Samples were administered to the lungs of the rats using Microsprayer® (Model IA-C; PennCentury, Inc., Philadelphia, PA, USA) gently inserted through the glottis. After perfusion of the lungs with PBS through the pulmonary artery, bronchoalveolar lavage (BAL) was performed using PBS at 5 h after administration and the BAL fluids (BALFs) were obtained. The protein concentrations in the BALF supernatant were determined to evaluate the acute toxicity of surface-modified liposomes using the Micro BCA protein assay kit with bovine serum albumin as the standard.

2.9. Evaluation of the association of surface-modified liposomes with lung tissue

DiI-labeled liposomes or surface-modified DiI-labeled liposomes were administered through rat tracheas at a volume of 1.61 mg DSPC/7.5 μg DiI/0.2 mL/rat according to the same procedure as described earlier. After perfusion of the lungs with physiological saline through the pulmonary artery, BAL was performed with PBS at 5 h after administration. BALFs were obtained and then centrifuged at 4620*g* for 5 min. Quantitative analysis of DiI in lung tissue and in the BALF supernatant was performed using the fluorescence spectrophotometer. For measuring the DiI concentrations, 1 mL of tissue homogenate or BALF supernatant was extracted with an appropriate amount of the chloroform–methanol mixture (1:1 v/v); the sample was then vigorously mixed for 40 min using the reciprocal shaker, followed by centrifugation (9240*g*, 10 min). The organic phase was subjected to DiI measurements using the fluorescence spectrophotometer.

2.10. Pulmonary administration of eCT-loaded liposomes

The absorption test was performed using rats fasted for 24 h before intratracheal administration of eCT-loaded liposomes (125 IU/kg rat). As a reference, an equivalent amount of eCT solution in 66.67 mM phosphate buffer (pH 6.8) was administered. The rats had free access to water during the experiment. The fasting conditions were maintained for 24 h after drug administration. A 200-μL blood sample was obtained from the jugular vein at an appropriate interval to determine the calcium level. The plasma calcium level was measured using a commercially available kit (Calcium E-test Wako; Wako Pure Chemical, Osaka, Japan). The relative pharmacological efficacies of different formulations were determined by calculating the area above the blood calcium concentration–time curve (AAC_{0-48h}) by a trapezoidal method.

2.11. Statistical calculation

All results were expressed as mean ± SD. The one-way analysis of variance (ANOVA) followed by Tukey–Kramer test was used in case of multiple comparison.

3. Results

3.1. Physicochemical properties of surface-modified liposomes

Surface modification of liposomes was performed with two different polymers (oligoCS and PVA-R) by mixing the liposomes with

a polymer solution. Different concentrations of oligoCS (0.03%, 0.06%, and 0.3% w/v) and PVA-R (0.2%, 1.0%, and 2.0% w/v) were used. The particle size and zeta potential of the resultant liposomes are shown in Table 1. The particle size of unmodified liposomes was 111.8 nm, and the zeta potential was negative (−60 mV) because of the negatively charged lipid (DCP)-containing liposomes. The particle size increased depending on the concentrations of oligoCS; the liposomes aggregated after adding 0.3% of oligoCS (particle size, 317 nm). The zeta potential of oligoCS-modified liposomes shifted to neutral with increasing oligoCS concentration. The particle size increased to approximately 150 nm, and the zeta potential shifted from negative to neutral after surface modification with PVA-R.

3.2. Effects of surface-modified liposomes on the viability of A549 cells

The potential toxic effects of oligoCS- or PVA-R-modified liposomes were evaluated *in vitro* using A549 cell lines by an MTS assay, which is a well-established technique for assessing toxicity (Fig. 1). The viability of A549 cells was almost unchanged by contact with oligoCS- or PVA-R-modified liposomal suspensions in the range used in the cell studies that followed, as shown in Fig. 1A and B, respectively. This confirmed the very low cytotoxicity of these surface-modified liposomes. In contrast, the positive control of Triton X-100, a solution that contained 1.0% w/v of a nonionic water-soluble surfactant, dramatically decreased cell viability.

3.3. Cellular association of surface-modified liposomes with A549 cells

The cellular associations of different liposomal formulations labeled with DiI as a fluorescence marker of liposomes were evaluated by quantifying the amount of DiI normalized to that of the cell protein (Fig. 2) and by confocal fluorescence imaging (Fig. 3).

oligoCS modification enhanced the liposomal association with A549 cells by approximately twofold compared with unmodified liposomes. In contrast, PVA-R-modified liposomes exhibited a significant decrease in cellular association compared with unmodified liposomes. The confocal image showed that A549 cells treated with oligoCS-modified liposomes were characterized by clear fluorescence on the periphery of the cells (Fig. 3B), whereas a relatively low degree of red fluorescence was observed in the A549 cell monolayers incubated with unmodified (Fig. 3A) and PVA-R-modified liposomes (Fig. 3C). These imaging results correlated with the quantitative assessments of cellular association with liposomes.

3.4. Effect of oligoCS-modified liposomes on opening of intercellular tight junctions in a Calu-3 cell monolayer

CS is a well-known mucoadhesive excipient that causes transient opening of intercellular tight junctions, thereby increasing the permeability of an epithelium [20]. The ability of CS to decrease TEER and then act as an absorption enhancer was demonstrated

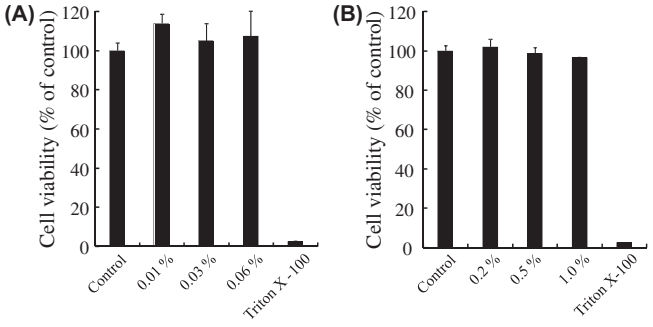

Fig. 1. Cell viability of A549 cells after exposure to (A) 0.01–0.06% w/v oligoCS-modified liposomes and (B) 0.2–1.0% w/v PVA-R-modified liposomes as measured by the MTS assay ($n = 8$). Control: HBSS/MES buffer (pH 6.0). Each point represents mean ± SD.

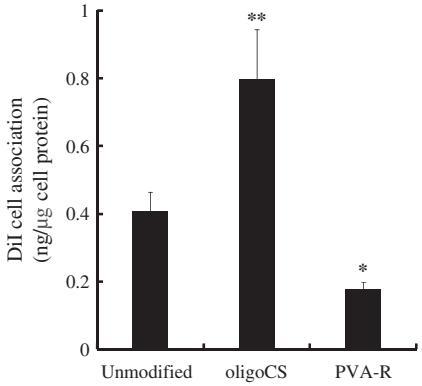

Fig. 2. Effect of surface modification of liposomes on the association with the A549 cell monolayers. Unmodified liposomes, liposomes without surface modification; oligoCS, 0.03% w/v oligoCS-modified liposomes; PVA-R, 0.5% w/v PVA-R-modified liposomes. Data are shown as mean ± SD ($n = 3$). ** $p < 0.01$, * $p < 0.05$ compared with unmodified liposomes.

using several cell lines representative of mucosa [21]. The Calu-3 cell formed layers interconnected by intercellular tight junctions. Therefore, this cell line has been used recently as a model for the airway epithelium in a number of drug transport studies. Changes in TEER across the Calu-3 cell monolayers were assessed to investigate the potential effect of oligoCS-modified liposomes on the opening of intercellular tight junctions (Fig. 4). Addition of oligoCS-modified liposomes into the apical chamber resulted in a gradual reduction in the TEER values of the Calu-3 cell monolayers over 120 min, while no significant change was found in the TEER values from incubation with unmodified liposomes during the entire measurement period, compared with those incubated with the control buffer. We confirmed that changes in TEER by oligoCS were not mediated through damage to cell functions because the change in TEER was completely reversible within 24 h (data not shown).

Table 1
Particle size and zeta potential of surface-modified liposomes. Data on each particle size show the average particle size based on light scattering intensity. PDI – polydispersity index.

Surface modification polymer	Polymer concentration (w/v %)	Particle size (nm)	PDI	Zeta potential (mV)
Unmodified liposomes	0	111.8 ± 0.8	0.14 ± 0.011	−60.0 ± 9.5
OligoCS-modified liposomes	0.03	110.2 ± 0.6	0.16 ± 0.011	−35.3 ± 8.6
	0.06	109.1 ± 2.3	0.15 ± 0.014	−29.2 ± 9.0
	0.3	317.0 ± 9.3	0.33 ± 0.042	−11.5 ± 3.8
PVA-R-modified liposomes	0.2	151.1 ± 1.2	0.09 ± 0.020	−13.9 ± 7.5
	1	155.5 ± 2.9	0.11 ± 0.004	−6.5 ± 5.8
	2	149.7 ± 1.9	0.12 ± 0.016	−7.4 ± 5.8

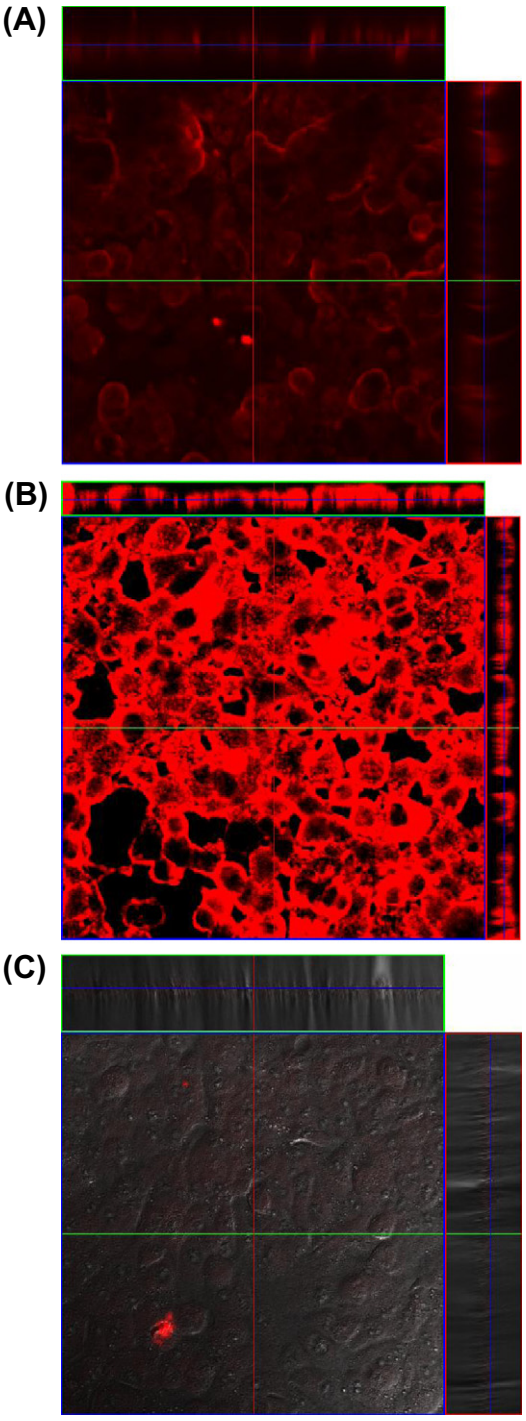

Fig. 3. CLSM microphotographs of the A549 cell monolayers after 2 h of incubation with DiI-labeled liposomes: (A) liposomes without surface modification, (B) 0.03% w/v oligoCS-modified liposomes, and (C) 0.5% w/v PVA-R-modified liposomes.

3.5. In vivo toxicity study on surface-modified liposomes after pulmonary administration

Henderson reported that the total protein concentration in BALF could be used as a marker of acute injury [22]. Therefore, we evaluated the protein concentration in BALF after pulmonary administration of different liposomal formulations (Fig. 5). The total protein concentration in BALF after Triton X-100 (positive control)

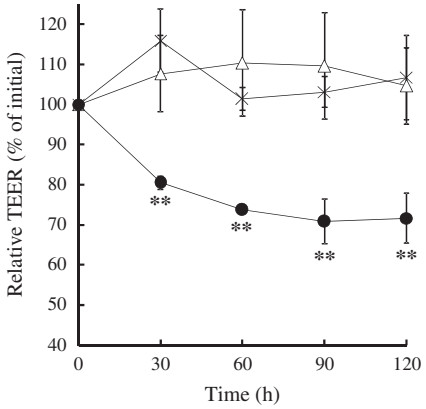

Fig. 4. Time-course changes in TEER of Calu-3 cells in the presence of oligoCS-modified liposomes: (×) HBSS/MES buffer (pH 6.0), (△) liposomes without surface modification, and (●) 0.015% w/v oligoCS-modified liposomes. Data are shown as mean ± SD ($n = 3$). ** $p < 0.01$ compared with liposomes without surface modification.

administration was markedly elevated compared with that after PBS administration. All liposome formulations did not show significant increases in protein concentration and had protein concentrations similar to that of the PBS solution (negative control). The very low toxicity of these liposomes showed promise for safe formulations *in vivo*.

3.6. Association of surface-modified liposomes with lung tissue and BALF after pulmonary administration

The association of surface-modified liposomes with lung tissue following pulmonary administration in rats was evaluated as shown in Fig. 6. The remaining DiI-labeled liposomes were quantitated in BALF collected by buffer perfusion at 5 h after administration and in BALF collected from lung tissue. The DiI recovery in lung tissue and BALF was shown almost 70% irrespective of the liposomal formulations. The remaining liposomes in BALF and lung tissue were increased by oligoCS modification compared with unmodified liposomes. In contrast, PVA-R modification prevented the association of liposomes with lung tissue. However, the remaining PVA-R liposomes in BALF were highest among different liposomal formulations.

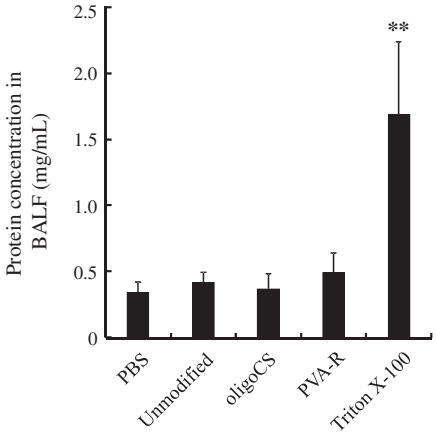

Fig. 5. The amount of total protein in bronchoalveolar lavage fluid at 5 h after pulmonary administration of surface-modified liposomes. PBS, phosphate-buffered saline; unmodified, liposomes without surface modification; oligoCS, 0.3% w/v oligoCS-modified liposomes; PVA-R, 2.0% w/v PVA-R-modified liposomes. Data are shown as mean ± SD ($n = 3$). ** $p < 0.01$ compared with PBS.

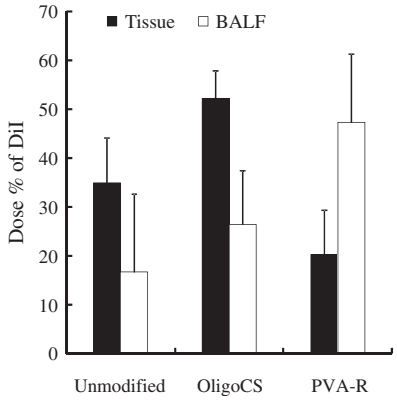

Fig. 6. Effect of surface modification on the behavior of liposomes in the lungs after pulmonary administration. Dose: 1.61 mg DSPC/7.5 μg DiI/0.2 mL/rat. Closed bar, lung tissue; open bar, BALF; unmodified liposomes, liposomes without surface modification; oligoCS, 0.3% w/v oligoCS-modified liposomes; PVA-R, 2.0% w/v PVA-R-modified liposomes. Data are shown as mean ± SD (n = 3).

3.7. Pharmacological effects of eCT-loaded liposomes after pulmonary administration

The therapeutic efficacy of different liposomal carriers loaded with eCT was evaluated after pulmonary administration to rats. Fig. 7 and Table 2 show the percent reductions in blood calcemia and AAC values, respectively, following pulmonary administration of eCT-loaded liposomes (125 IU/kg rat). The drug solution temporarily decreased the blood calcium level at the initial stage. Unmodified liposomes also temporarily decreased the blood calcium level and tended to prolong the reduced blood calcium level profile compared with the drug solution. oligoCS and PVA-R modifications resulted in even stronger reductions in the blood calcium levels at the initial stage and a prolonged reduction (after 48 h) in the blood calcium level after pulmonary administration compared with unmodified liposomes.

4. Discussion

In the present study, surface-modified liposomes were evaluated as a pulmonary delivery carrier of peptides to enhance systemic absorption. To improve pulmonary delivery of peptides, we modified liposomal surfaces using oligoCS, a mucoadhesive cationic polymer, and PVA-R, a nonionic hydrophilic polymer. The modifier layer on the liposomal surface increased the particle size compared with that of unmodified liposomes, irrespective of the surface modifier (Table 1). Zeta potential is one of the important surface properties of liposomes. Modifications of negatively charged liposomes with oligoCS or PVA-R shifted the zeta potentials from negative to neutral. This shift proved that negative liposomal surfaces could be modified by an interaction with oligoCS or PVA-R. The proposed mechanism of oligoCS modification assumes that liposomal polyelectrolyte complexes are formed between negatively charged liposomes and cationic polymers. The effect of PVA-R modification can be explained by anchoring of the hydrophobic moiety of PVA-R to the lipid membrane of liposomes [9]. Our studies indicated that at the concentrations used, these surface-modified liposomes did not induce significant cytotoxicity (Fig. 1).

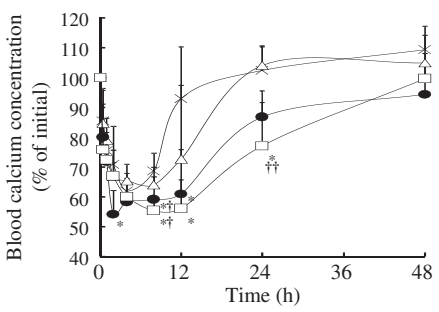

Fig. 7. Profiles of the blood calcium concentration after pulmonary administration of eCT-loaded liposomes (125 IU/kg) to male rats (6 weeks): (×) eCT solution; (△) liposomes without surface modification; (●) 0.3% w/v oligoCS-modified liposomes; (□) 0.2% w/v PVA-R-modified liposomes. Data are shown as mean ± SD of at least three experiments. ** $p < 0.01$, * $p < 0.05$ compared with the eCT solution, †† $p < 0.01$, † $p < 0.05$ compared with liposomes without surface modification.

Table 2
Area above the blood calcium concentration–time curves (AAC) after pulmonary administration of eCT-loaded liposomes to male rats. Unmodified, liposomes without surface modification. Each point represents mean ± SD of at least three experiments.

	AAC_{0-48h} (μg/mL*h)
Elcatonin solution	423.6 ± 177
Unmodified liposomes	484.9 ± 2.5
OligoCS-modified liposomes	1075.5 ± 337*†
PVA-R-modified liposomes	1181.2 ± 204**††

* $p < 0.05$ compared with the eCT solution.
** $p < 0.01$.
† $p < 0.05$ compared with liposomes without surface modification.
†† $p < 0.01$.

We evaluated the interactions between surface-modified liposomes and lung epithelial cells (A549), as shown in Figs. 2 and 3. Fluorescence from surface-modified liposomes was observed in the cytoplasm or around the nucleus by analysis using confocal imaging after the association experiments (Fig. 3). oligoCS modification increased the cellular association of liposomes. It was assumed that the cationic groups of oligoCS on the surface electrostatically interacted with the negatively charged cell membrane. In contrast, the cellular association of liposomes was decreased by PVA-R modification. This suppression of the interaction of PVA-R-modified liposomes with A549 cells was induced by the thick flexible layer of PVA-R on the liposomal surface.

The *in vivo* liposomal association study showed that the association of liposomes with lung tissue was increased by oligoCS compared with that of unmodified liposomes (Fig. 6), which correlated with the *in vitro* study using A549 cells (Fig. 2). The slower elimination of oligoCS-modified liposomes from the lungs may be explained by the adhesion of liposomes because of their mucoadhesive properties to the mucus and the epithelial cells of the trachea and lungs [14,23]. On the other hand, PVA-R modification interrupted the direct association of liposomes with lung tissues (Fig. 6), similar to the results from the *in vitro* cell study (Fig. 2). However, the remaining PVA-R-modified liposomes in BALF increased after pulmonary administration compared with other liposomes (Fig. 6; white bar). We previously demonstrated that PVA-R modification of liposomes reduced the uptake by the RES after injection in rats and interaction with J774.1 macrophages because of the thick and flexible layer of PVA-R on the liposomal surface [24]. Therefore, this steric hindrance caused by the PVA-R layer might suppress phagocytosis by alveolar macrophages and interaction with lung tissues. PVA-R modification may possibly prevent the rapid elimination of liposomes from the lung by ciliary movement and from the macrophages. As a result, the remaining level of liposomes in BALF collected at 5 h after administration was increased by PVA-R modification.

We examined the *in vivo* pharmacological effect of surface-modified liposomes after pulmonary administration using eCT as a model peptide drug (Fig. 7). In this study, eCT was protected from the enzymatic attack by encapsulation with liposomes, leading to a

slightly enhanced pharmacological effect compared with the eCT solution alone. Surface modification of liposomes by oligoCS and PVA-R may enhance peptide drug activity through pulmonary administration. In addition, the AAC values showed that the pharmacological efficacy of surface-modified liposomes was significantly increased by more than twofold compared with that of the eCT solution alone and unmodified liposomes (Table 2). The prolonged effect of oligoCS-modified liposomes may be because of the interaction with lung tissue due to the mucoadhesive property (Fig. 6) and the drug-absorption enhancing functionality that opens the tight junctions between cells (Fig. 4). On the other hand, the mechanism of absorption improvement by PVA-R modification could be different from that by oligoCS modification. PVA-R-modified liposomes delivered by pulmonary administration may remain for a longer period in the lung fluids because of the steric hindrance of the PVA-R layer against macrophages and ciliary movement (Fig. 6), leading to sustained systemic absorption of eCT.

We have successfully developed a pulmonary delivery system for peptides by surface modification of liposomes with oligoCS and PVA-R. Further improvement of liposomes as pulmonary carriers of peptides may be achieved by combining these surface modifiers, which may result in different physicochemical properties.

5. Conclusions

Surface modification of liposomes with polymers led to the control of their behavior in the lungs. Both oligoCS- and PVA-R-modified liposomes significantly enhanced and prolonged the pharmacological effects of eCT after pulmonary administration. Furthermore, surface-modified liposomes have negligible toxicity in pulmonary tissue. These findings suggested that surface-modified liposome can be applied to the therapeutics for various lung diseases to control the peptide absorption mechanism using different surface modifiers.

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