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Original Article

Extracellular vesicle-liposome hybrids via membrane fusion using cell-penetrating peptide-conjugated lipids

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ABSTRACT

Extracellular vesicles (EVs) are natural carriers for intercellular communication within the human body. Mimicking and utilizing EVs by combining them with artificial nanocarriers such as liposomes for drug delivery has garnered considerable attention. However, current technologies for manipulating EVs to facilitate their fusion with liposomes are limited; the existing technique of polyethylene glycol (PEG)-induced fusion is highly inefficient for fusion. In our previous study, we demonstrated that membrane fusion could be induced by Tat peptide (YGRKKRRQRRR)-conjugated poly(ethylene glycol)-phospholipids (Tat-PEG-lipids), in which the Tat peptide and lipid domain facilitate membrane attachment and subsequent fusion between cells and liposomes. This approach is promising for forming EV and liposomal hybrids. In this study, we aim to fuse EVs and liposomes using Tat-PEG-lipids. We isolated and characterized EVs derived from HEK293T cell culture medium and treated a mixture of EVs and liposomes composed of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine and cholesterol (1:1, molar ratio), with Tat-PEG-lipids with different lipid chain lengths. Here, we used nonanoyl (C9), dodecanoyl (C12), and myristoyl (C14) groups as lipid anchors with 5 kDa PEG chains. Dynamic light scattering analysis revealed a large increase in the apparent size of mixture of EVs and liposomes by adding Tat-PEG-lipids (especially C14, C12, followed by C9). Fluorescence resonance energy transfer, confocal laser scanning microscopy, and transmission electron microscopy, used to analyze the reaction process, revealed that the membrane fusion occurred between EVs and liposomes but not their aggregates. The short lipid domain of Tat-PEG-lipids effectively induced membrane fusion and the formation of hybrid EVs and liposomes. Thus, Tat-PEG-lipids (C9 and C12) could be promising candidates for inducing membrane fusion to fabricate EV-liposome hybrids.

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1. Introduction

Extracellular vesicles (EVs) are vital mediators of intercellular communication that facilitate the transfer of biomolecules between cells [1,2]. EVs are released by diverse cell types and play crucial roles in various physiological and pathological processes including immune response modulation, tissue regeneration, and cancer progression [3–5]. EVs have attracted significant attention as potential vehicles for drug delivery because of their ability to be transported [6]. EVs alone or in conjunction with artificial nanocarriers, such as liposomes, have demonstrated high efficacy in drug delivery and therapeutics [7–10]. In particular, liposomes are versatile and biocompatible, as they can encapsulate active pharmaceutical ingredients, such as small molecules and nucleic acids [11]. However, despite the potential benefits of EV-liposome hybrids, there are limitations in fabricating the hybrid nanoparticles using conventional methods. Although polyethylene glycol (PEG)-induced fusion is used conventionally, it is challenged by low efficiency and may cause alterations in EV surface properties and morphology [12]. Therefore, there is a need to develop alternative fusion methods that can generate EV-liposome hybrids with higher

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efficiency under milder conditions, without destroying the native characteristics of EVs.

We recently reported that membrane fusion mediated by Tat peptide (YGRKKRRQRRR)-conjugated polyethylene glycol (PEG)-lipids (Tat-PEG-lipids) is a promising approach to facilitate fusion of biological membranes [13]. The Tat peptide is a representative cell-penetrating peptide (CPP), and CPPs are known to penetrate cellular membranes and facilitate cargo delivery to cells [14] and have been used for drug delivery applications [15]. We found that combining the Tat peptide with the lipid domain could promote spontaneous membrane attachment and fusion between cells and liposomes when both domains were conjugated to PEG [16]. With our approach, membrane fusion can occur under mild conditions with higher efficiency than the conventional methods. Therefore, we hypothesized that a hybrid of EV and liposomes could be formed via membrane fusion induced by Tat-PEG-lipids.

In this study, we aim to investigate the formation of EV-liposome hybrids using Tat-PEG-lipids of different lipid chain lengths. EVs isolated from HEK293T cell culture medium and liposomes composed of an equimolar mixture of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and cholesterol (Chol) were used. We synthesized Tat peptide-conjugated PEG-lipids with different lipid chain lengths (nonanoyl (C9), dodecanoyl (C12), and myristoyl (C14) groups) and used amphiphilic polymers to induce membrane fusion between EVs and liposomes. The fusion process was analyzed using dynamic light scattering (DLS), fluorescence resonance energy transfer (FRET) measurements, confocal laser scanning microscopy (CLSM), and transmission electron microscopy (TEM). We examined the influence of the lipid domain of Tat-PEG-lipids on the efficient attachment and fusion of EVs with liposomes and optimized the molecular structure for the fusion.

2. Materials and methods

2.1. Materials

1,2-Dinonanoyl-*sn*-glycero-3-phosphocholine (DC9PC) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). 1,2-Dilauroyl-*sn*-glycero-3-phosphoethanolamine (lipid (C12)) was purchased from Bachem (Bubendorf, Switzerland). 1,2-Dimyristoyl-*sn*-glycero-3-phosphoethanolamine (lipid (C14)), α-3-[(3-maleimido-1-oxopropyl)aminopropyl-ω-(succinimidyloxy carboxy)] polyethylene glycol (Mal-PEG-NHS with a molecular weight of 5 kDa), and DPPC were purchased from NOF Corporation (Tokyo, Japan). Triethylamine (TEA), TLC plate silica gel, 1,6-diphenyl-1,3,5-hexatriene (DPH), bovine serum albumin (BSA), and a synthetic peptide YGRKKRRQRRRC (Tat peptide, cysteine conjugated at the C-terminal of the Tat sequence) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Micro BCA protein assay kit, goat anti-rabbit HRP, total exosome isolation reagent, FreeStyle™ 293 expression medium, DiI, DiO, and spin columns (Pierce™ polyacrylamide spin desalting columns, 7K MWCO) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). L-Cysteine, 2-aminoethanol (ethanolamine), iodine, 28% ammonia solution, diethyl ether, chloroform, methanol, chloroform-*d*, methanol-*d4*, acetone, dichloromethane (super dehydrated), Chol, sodium dodecyl sulfate (SDS), tetrahydrofuran (THF), sodium chloride (NaCl), sodium hydroxide (NaOH), dimethyl sulfoxide (DMSO), ethanol, Dulbecco's phosphate-buffered saline (PBS; pH 7.4), hydrochloric acid (HCl), 4% paraformaldehyde phosphate buffer solution, RIPA buffer, phosphotungstic acid hydrate, 2-mercaptoethanol, and a Chol assay kit (Cholesterol E-Test Wako) were purchased from Fujifilm Wako Pure Chemical (Osaka, Japan). Phospholipase D extracted from *Streptomyces* sp. (sPLD, T-39) was purchased from Asahi Kasei Pharma (Tokyo, Japan). Glass-bottomed dishes were purchased from Matsunami Glass Industries (Osaka, Japan). Tissue culture polystyrene (TCPS) dishes, 50 mL round-bottom flask, and 96-well plates were purchased from Iwaki (Tokyo, Japan). Extrusion filters (pore size: 1.0, 0.4, 0.2, and 0.1 μm, Nuclepore Track-Etch Membrane) were purchased from Whatman (Maidstone, UK). DMEM medium, fetal bovine serum (FBS), penicillin, streptomycin, and trypan blue solution were purchased from Invitrogen (Carlsbad, CA, USA). All synthetic peptides, polyvinylidene difluoride (PVDF) membrane, 0.22 μm Millex-GV syringe filter, protease inhibitor cocktail, and Amicon Ultra-15 centrifugal filters were purchased from MERCK (Burlington, MA, USA). The antibodies against EVs (anti-CD63, anti-CD81, and anti-CD9) were purchased from System Biosciences (Palo Alto, CA, USA). Protean tetra cell, clarity Western ECL substrate, and Gel Doc XR+ system were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA).

3. Methods

3.1. Synthesis of CPP-conjugated PEG-lipid

1,2-Dinonanoyl-*sn*-glycero-3-phosphatidylethanolamine (lipid C9) (Fig. 1 with m = 7) was synthesized from DC9PC, as previously reported [13]. In brief, DC9PC (28 mg) was dissolved in diethyl ether (10 mL) with ethanolamine-HCl aqueous solution (2 mL, 250 mM, pH 4.2) and sPLD aqueous solution (1 mL, 30 U/mL), and the mixture was stirred for 90 min at 35 °C. After evaporating the diethyl ether under a stream of nitrogen, a mixture of ultrapure water (0.2 mL), chloroform (4 mL), and methanol (8 mL) was added to the lipidic residue to obtain a homogeneous phase. Subsequently, chloroform (1 mL) and ultrapure water (1 mL) were added to induce phase separation, followed by centrifugation (650×*g*) for 15 min at room temperature to yield two clear phases. The collected chloroform phase was evaporated to obtain lipid C9.

Three different lipids (C9, C12, C14) were utilized to synthesize Mal-PEG-lipids (C9, C12, C14), as described in our previous papers [13,16,17]. In brief, lipid (C9), lipid (C12), and lipid (C14) (9.8 mg, 11 mg, and 13 mg, respectively, 1 equivalent each) were dissolved in dichloromethane (15 mL) with Mal-PEG-NHS (90 mg, 90 mg, and 90 mg, respectively, 1.1 equivalent each) and triethylamine (5.0 μL); the mixture was stirred for 48 h at room temperature. The resulting solution was precipitated with diethyl ether (3 L) to obtain Mal-PEG-lipids (80% yield). Cysteine solution (100 μL, 1 mg/mL, in PBS) was mixed with Mal-PEG-lipids (1 mg) for the inactivation of maleimide groups in the Mal-PEG-lipids, which were used as control PEG-lipids (10 mg/mL, in PBS). The Tat peptide and Mal-PEG-lipid were conjugated via a thiol-maleimide reaction between the maleimide and thiol groups at the C-terminus of the cysteine residue. They were mixed and stirred at room temperature for 24 h: Mal-PEG-lipids (100 μL, 10 mg/mL in PBS, 1 equivalent) and Tat peptide solution (33 μL, 33 μL, and 32 μL of 10 mg/mL in DMSO for Mal-PEG-lipids (C9, C12, and C14), respectively; 1.1 equivalent each). Thus, Tat-PEG-lipids with different lipid chain lengths (C9, C12, and C14) were obtained.

3.2. EV isolation

HEK293T cells, provided by Prof. Fukamizu (Life Science Center for Survival Dynamics, University of Tsukuba), were cultured in DMEM supplemented with 10% FBS, 50 U/mL penicillin, and 50 μg/mL streptomycin at 37 °C in a 5% CO_2 and 95% air atmosphere until 80% confluency was achieved. FreeStyle™ 293 Expression Medium was added after washing the cells twice with PBS to remove FBS from the culture dish. After 72 h, the culture medium was collected for EV isolation. The collected culture medium was subjected to centrifugation (200×*g*) for 3 min at 25 °C to remove the cells. The supernatant was then centrifuged (500×*g*) for 30 min at 4 °C,

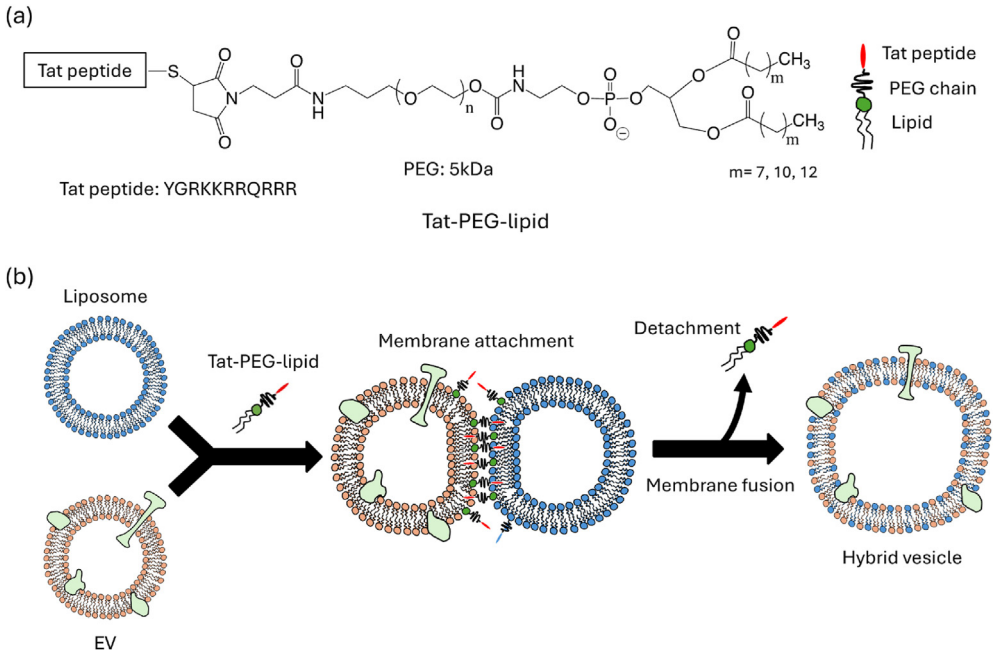

Fig. 1. Membrane fusion of EVs and liposomes facilitated by Tat-PEG-lipids. (a) Chemical structure of the Tat-peptide-conjugated polyethylene glycol-conjugated lipid (Tat-PEG-lipid) with different lipid chain lengths (C9 ($m = 7$), C12 ($m = 10$), or C14 ($m = 12$)). Molecular weight of PEG is 5 kDa. (b) Schematic of the membrane fusion of EV-liposome enabled by Tat-PEG-lipid. EVs and liposomes are anchored via Tat-PEG-lipid, and the Tat peptide and lipid domain interact with lipid membranes, inducing membrane attachment and subsequent membrane fusion.

followed by another centrifugation (2000×*g*) for 30 min at 4 °C to eliminate cell debris. Subsequently, the supernatant was filtered once using a 0.22 μm Millex-GV syringe filter to remove large vesicles. The filtrate was further centrifuged (5000×*g*) for 60 min at 4 °C using Amicon Ultra-15 centrifugal filters (molecular weight cutoff: 100 kDa; Merck Millipore). The upper solution in the centrifugal filters containing EVs was stored at −30 °C until further use. Protein concentration of the EV fraction was determined using microBCA ([protein] = 2 mg/mL).

3.3. Size analysis of EVs

The EV suspension ([protein] = 2 mg/mL, 5 μL) was diluted in PBS (45 μL), transferred to a measurement cell, and subjected to DLS using a Zetasizer Nano ZS (Malvern Instruments Co., Malvern, UK) to measure the particle size. Additionally, the size and particle number of EVs were determined by nanoparticle tracking analysis (NTA) using a NanoSight LM10-HS (Nanosight, Malvern, United Kingdom).

3.4. Western blotting

The collected EVs (18 μL/well, [protein] = 2 mg/mL) were lysed using RIPA buffer at 4 °C for 45 min. Subsequently, the lysate was combined with a sample loading buffer containing 2-mercaptoethanol and heated to 95 °C for 5 min. SDS-PAGE was performed on 12% gel in a mini-Protean tetra cell at 40 V for 40 min, followed by 60 V for 2 h. The gel was transferred to a PVDF membrane (5.5 cm × 8.5 cm, Merck). The PVDF membrane was blocked using Blocking One solution (5× dilution in TBS-T, Nacalai Tesque) at room temperature for 1 h. Primary antibodies (anti-CD9, anti-CD63, anti-CD81, diluted 1:1000 in TBS-T) were then incubated with the blocked PVDF membrane at 4 °C for 20 h. Following incubation, the membrane was washed three times with TBS-T at RT for 10 min each, and the membrane was incubated with the secondary antibody (goat anti-rabbit HRP, diluted 1:20,000 in TBS-T) at room temperature for 1 h, followed by three washes with TBS-T at room temperature for 10 min each. Finally, the membranes were treated with clarity Western ECL substrate, and images were captured using a Gel Doc XR+ system.

3.5. Liposome preparation

Liposomes were prepared as previously reported [13,17]. A solution of DPPC (1 mL, 10 mg/mL, in ethanol) and Chol (530 μL, 10 mg/mL, in ethanol) were mixed in a 1:1 M ratio and evaporated using a rotary evaporator to form a lipid film. The cholesterol effect can be seen at approximately 30 mol%, and the effect can be maintained up to equimolar to DPPC. Therefore, we used the liposome with 1:1 M ratio of DPPC and cholesterol. The lipid film was vacuum dried for 20 h in a desiccator, hydrated with PBS (1.5 mL), and vigorously stirred for 2 h at room temperature to prepare a liposome suspension. Finally, the liposome pellet was resuspended in PBS (1.5 mL). The resulting liposome suspension was extruded through membrane filters (pore sizes: 1.0, 0.4, 0.2, and 0.1 μm) using an extruder to achieve uniform liposome size. The liposome suspension was passed through each extrusion filter 21 times to ensure homogeneity. The liposome suspension ([Chol] = 4.6 mM, in PBS, 5 μL) was diluted in PBS (45 μL), transferred to a measurement cell, and subjected to DLS as described above.

3.6. Preparation of EV-liposome hybrid structures by adding Tat-PEG-lipids

3.6.1. Analysis of diameter

EV suspension (200 μL, [protein] = 2 mg/mL) and liposome suspension (20 μL, [Chol] = 4.6 mM, in PBS) were mixed with Total Exosome Isolation Reagent (500 μL) and centrifuged at 20,000×*g* for 70 min at 4 °C, and the supernatant was completely removed. Subsequently, a solution of Tat-PEG-lipid (C9, C12, or C14) (50 μL,

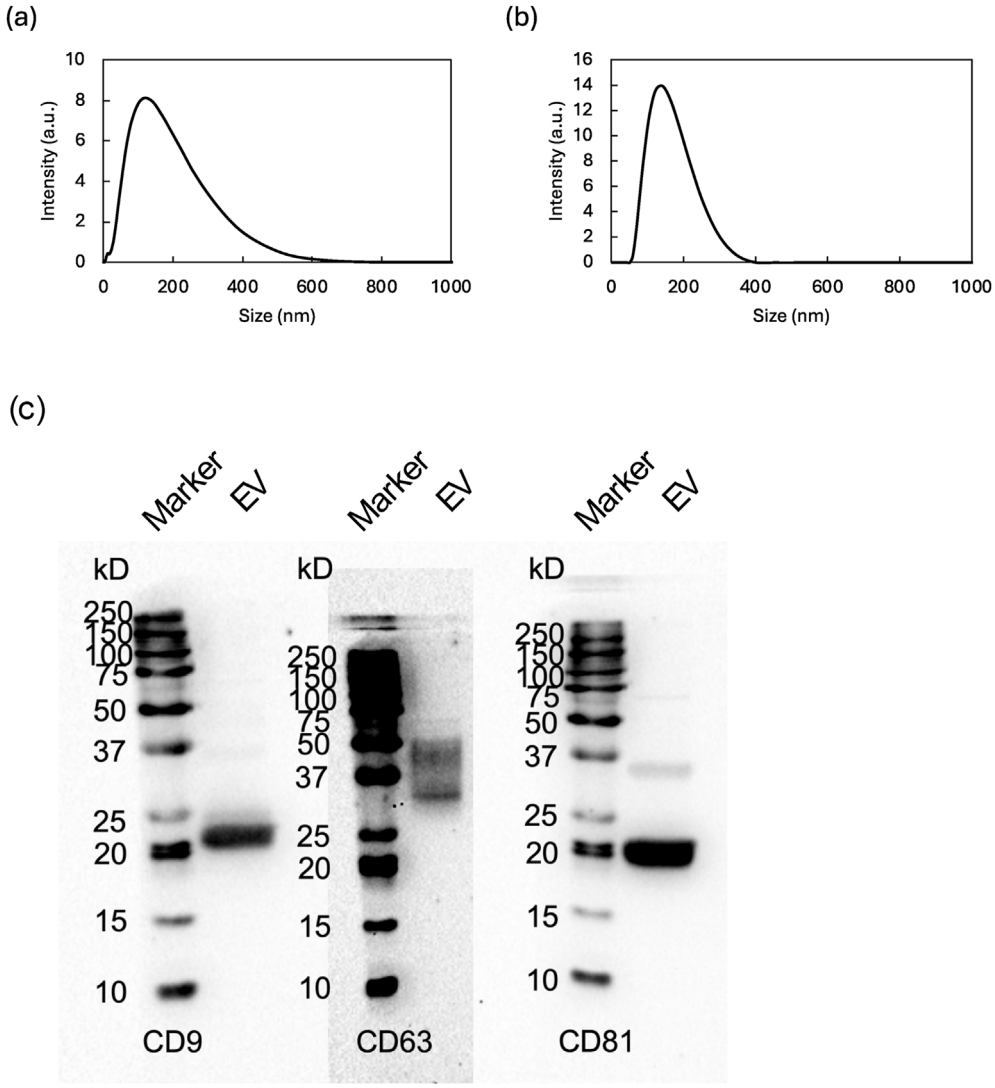

Fig. 2. Characterization of EVs derived from HEK293T cells. Size distribution of (a) EVs and (b) liposomes (DPPC/Chol (1:1, molar ratio)) analyzed using DLS. The intensity averaged sizes of EVs and liposomes were approximately 142 ± 96 nm and 149 ± 58 nm, respectively. (c) Western blotting of EVs using representative surface markers (CD9, CD63, and CD81).

1 mg/mL, in PBS) was mixed with the mixture pellet and incubated for 2 or 3 h at 37 °C. The diameter and zeta potential of the resulting mixture were measured using DLS as described above. We also analyzed EVs with Tat-PEG-lipids (C9, C12, and C14) as controls. A suspension of EVs (200 μL, [protein] = 2 mg/mL) was mixed with isolation solution (500 μL) and centrifuged at 20,000×*g* for 70 min at 4 °C, after which the supernatant was completely removed. Subsequently, a solution of Tat-PEG-lipid (C9, C12, or C14) (50 μL, 1 mg/mL, in PBS) was added to the EV pellet and incubated for 2 h at 37 °C. The diameters and zeta potentials were measured in the same manner.

3.6.2. CLSM

CLSM was used to observe the hybrids of EVs and liposomes after treatment with Tat-PEG-lipids. DiI and DiO were used for labeling. Briefly, EV suspension (1000 μL, [protein] = 2 mg/mL) was mixed with DiI (5 μL) and incubated for 30 min at 4 °C. After incubation, the mixed suspension was centrifuged at 20,000×*g* for 70 min at 4 °C, and the supernatant was removed. Similarly, liposome suspension (100 μL, [Chol] = 4.6 mM, in PBS) was treated with DiI (5 μL) using the same procedure. Thereafter, a hybrid of EVs and liposomes was prepared in the same way as described above. Hybrids diluted with PBS were seeded into glass-bottomed dishes and incubated for 30 min at room temperature. Subsequently, the dish was washed five times with PBS (1.0 mL) to remove the unbound EV-liposomes, and the glass substrate surface was observed using a CLSM (LSM700; Carl Zeiss Microscopy Co. Ltd., Jena, Germany).

3.6.3. FRET measurement of EV-liposome hybrid structures

Labeled EVs and liposomes were used here as well. EV suspension ([protein] = 2 mg/mL) and liposome suspension ([Chol] = 4.6 mM, in PBS) were mixed with Total Exosome Isolation Reagent and centrifuged at 20,000×*g* for 70 min at 4 °C, followed by the removal of the supernatant. A solution of Tat-PEG-lipid (1 mg/mL, in PBS) was then added to the pellet and incubated for 3 h at 37 °C. The resulting suspension was diluted 100 times with PBS. Fluorescence spectra (excitation: 488 nm, emission range: 500–600 nm) were obtained using a spectrofluorometer (FP-6500; JASCO Co., Tokyo, Japan). The FRET ratio for each sample was calculated using the following equations:

$$\text{FRET ratio} = \Delta R_{572\ nm} / \Delta G_{506\ nm} - \Delta R_{572\ nm}\,[PBS] / \Delta G_{506\ nm}\,[PBS] \quad (1)$$

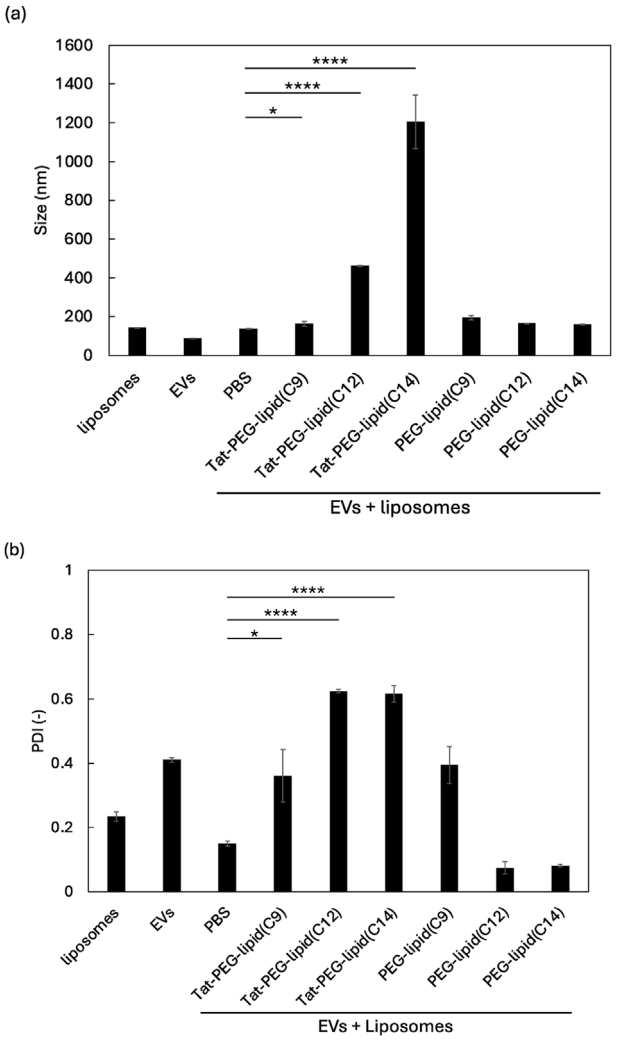

Fig. 3. Dynamic light scattering results for the (a) intensity averaged particle size (b) polydispersity index (PDI) of the mixture of EVs and liposomes (DPPC/Chol (1:1, molar ratio)) treated with Tat-PEG-lipids. Mixture of EVs and liposomes were mixed with Tat-PEG-lipids with different lipid chain lengths (C9, C12, or C14). The respective PEG-lipid without the Tat peptide was used as the respective control. Error bars indicate standard deviation; $n = 3$.

$$\Delta R_{572\ nm} = FI_{572\ nm}\,[2h] \,/\, FI_{572\ nm}\,[0h] \quad (2)$$

and

$$\Delta G_{506\ nm} = FI_{506\ nm}\,[2h] \,/\, FI_{506\ nm}\,[0h] \quad (3)$$

where $FI_{572\ nm}$ [0h] is the fluorescence intensity at 572 nm after incubation at 0 h, $FI_{572\ nm}$ [2h] is the fluorescence intensity at 572 nm after incubation for 2 h, $FI_{506\ nm}$ [0h] is the fluorescence intensity at 506 nm after incubation at 0 h, $FI_{506\ nm}$ [2h] is the fluorescence intensity at 506 nm after incubation for 2 h, and $\Delta R_{572\ nm}$ [PBS] and $\Delta G_{506\ nm}$ [PBS] are the respective terms derived from PBS. This equation enables calculation of the FRET ratio.

3.6.4. TEM

Samples were prepared for TEM using negative staining. The prepared sample suspension (10 μL) was placed on a thin-film mesh grid (Excel support film, Nisshin EM Co., Ltd., Tokyo, Japan) for 30 s, after which the excess solution was removed. The grid was then immersed in a sodium phosphotungstate solution (1% in pure water, pH~7) for 30 s. Excess solution was absorbed using filter paper, and the grid surface was air-dried. The prepared samples were observed using a Tecnai G2 F20 microscope (FEI Company Japan, Ltd., Tokyo, Japan) at an acceleration voltage of 120 kV.

3.7. Statistical analysis

All results were replicated at least three times and are presented as means ± standard deviations. Statistical analysis was performed using one-way ANOVA, followed by Dunnett's multiple comparison test, with significance set at $p < 0.05$. Levels of significance are denoted as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$. The calculated p values are shown in Figs. 3a, b and 5c. Statistical analyses were performed using GraphPad Prism 8 for MacOS version 8.4.2 (GraphPad Software, La Jolla, CA, USA).

4. Results and discussion

The Tat sequence is a representative CPP that is covalently attached to Mal-PEG-lipids of varying lipid chain lengths (C9, C12, and C14) (Fig. 1a). These conjugates were used to form hybrids of EVs and liposomes (Fig. 1b). We previously reported that Tat-PEG-lipid induced the membrane fusion of cells and liposomes [13]; thus, they could be used for the fusion of EVs and liposomes.

EVs isolated from the HEK293T culture medium were characterized by DLS to determine their size distribution (Fig. 2a). The size analysis revealed that the sizes of EVs ranged from approximately 2 nm to 600 nm, with an average of 142 ± 96 nm. Additionally, we analyzed the size and the number of EVs using NTA, which were 130 nm and 2.8 ± 0.2 × 10^9 particles/mL ([total protein] = 60 ng/μL), respectively (Supplementary Fig. S1), which was consistent with our previously published data [16]. Western blotting indicated a strong positive expression of CD9, CD63, and CD81, which are representative EV surface markers (Fig. 2c). Collectively, these findings revealed the successful isolation of EVs from HEK293T cells.

In this study, liposomes composed of DPPC and Chol were used for hybridization with EVs. A lipid composition of DPPC/Chol at a molar ratio of 1:1 is suitable for the current evaluation because liposomes are stable and do not spontaneously fuse with each other [13,17]. The sizes of liposomes ranged from approximately 50 nm to 400 nm, with the average size determined to be 149 ± 58 nm (Fig. 2b). Three types of Tat-PEG-lipids (C9, C12, and C14) were directly added to the mixed pellet of EVs and liposomes to induce membrane fusion. PEG-lipids without the Tat peptide were used as controls. Because we previously optimized the concentration of Tat-PEG-lipids for cell membrane fusion, we used 1 mg/mL for this experiment [13,16,17].

DLS was used to measure the size distribution and polydispersity index (PDI) of the hybrid EV-liposome structures after treatment with Tat-PEG-lipid (Fig. 3, Supplementary Fig. S4). No particle size changes were detected in EVs alone, liposomes alone, or a mixture of EVs and liposomes during incubation with PBS (Fig. 3a). In addition, the size of the liposomes remained unchanged after treatment with PEG-lipids (C9, C12, and C14) without the Tat peptide, in which the liposome surface was PEGylated, i.e., modified with PEG-lipids. Conversely, when Tat-PEG-lipids (C9, C12, and C14) were added to the mixture of EVs and liposomes, a significant increase in the size was detected for all three groups, and the sizes were 162 ± 14 nm (C9), 462 ± 4 nm (C12), and 1206 ± 41 nm, (C14) respectively. In particular, the liposomes treated with Tat-PEG-lipid (C14) exhibited a substantial increase in size, up to approximately 1.2 μm. Similar trends were observed for the PDI; the mixture of EVs and liposomes treated with Tat-PEG-lipids (C9, C12, and C14) showed an increase in the PDI. These results indicated that the aggregation and/or fusion of

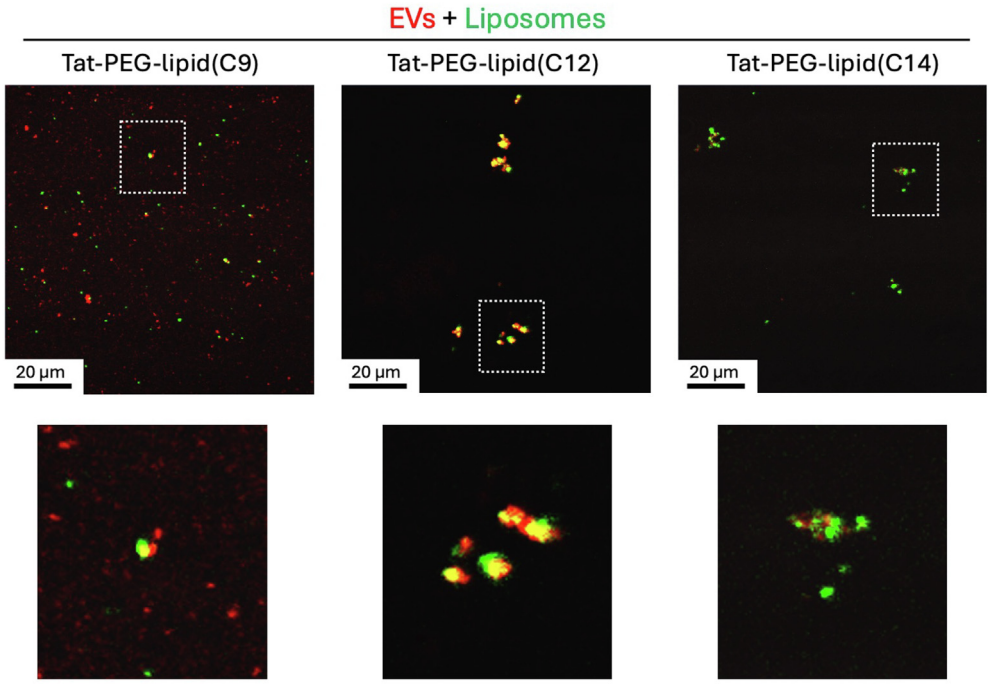

Fig. 4. Confocal microscopic images of the mixture of EVs and liposomes (DPPC/Chol (1:1, molar ratio)) treated with Tat-PEG-lipids with different lipid chain lengths. EVs and liposomes were labeled using DiI (red) and DiO (green) before treatment with Tat-PEG-lipids (C9, C12, and C14), followed by confocal laser scanning microscopy.

EVs and liposomes were induced by adding Tat-PEG-lipid (C9), Tat-PEG-lipid (C12), or Tat-PEG-lipid (C14).

We then used CLSM to observe the mixtures of EVs and liposomes treated with Tat-PEG-lipids (C9, C12, and C14). Before CLSM observation, both the EVs and liposomes were stained with membrane dyes (DiI for EVs and DiO for liposomes). Large conjugates of EVs and liposomes were visible for each Tat-PEG-lipid; yellow conjugates were detected for the Tat-PEG-lipid (C9) and Tat-PEG-lipid (C12) groups, whereas they were hardly detected for the Tat-PEG-lipid (C14) group, for which, only green and red conjugates were observed (Fig. 4). This implies that the fusion of EVs and liposomes could occur by adding Tat-PEG-lipid (C9) and Tat-PEG-lipid (C12). Time dependence investigation of the reaction by Tat-PEG-lipids (C9 and C12) using DLS revealed increased sizes of the particles of EV-liposome mixture, with a size of 347 ± 27 nm (Tat-PEG-lipid (C9)) and 2958 ± 43 nm (Tat-PEG-lipid (C12)) at 3 h (Supplementary Fig. S2). This result indicates that the fused particle size can be adjusted by the reaction time of Tat-PEG-lipids, EVs, and liposomes. As previously reported, the liposome surface became positively charged when treated with Tat-PEG-lipid while the surface of EV remained negative charge [13,16,17]. Consequently, the attachment between the EV and liposome proceeded via electrostatic interaction more easily, resulting in the formation of hybrid of EVs and liposomes. When adding Tat-PEG-lipids (C9, C12, or C14) to EVs alone, there were no noticeable changes in the size of EVs compared to those EVs mixed with liposomes (Supplementary Fig. S3), where the sizes of the EVs treated with Tat-PEG-lipids (C9, C12, or C14) were approximately 100 nm, 185 nm, and 150 nm, respectively. This suggests that the frequency of attachment between EVs was lower because the treated exosome surface was negatively charged and it did not occur the efficient attachment.

Next, we used FRET analysis to study membrane fusion between EVs and liposomes. DiI (red dye) and DiO (green dye) were used to label the EVs and liposomes, respectively, and FRET was studied by adding Tat-PEG-lipids (Fig. 5a). When PBS was added to a suspension containing both labeled EVs and liposomes as a control, no obvious change in fluorescence intensity was detected at approximately 572 nm, indicating that the mere mixing of EVs and liposomes did not induce membrane fusion or attachment (Fig. 5b). However, upon adding Tat-PEG-lipid (C14) to the suspension, a small increase in fluorescence intensity was detected at approximately 506 and 572 nm. In contrast, the addition of Tat-PEG-lipid (C9) or Tat-PEG-lipid (C12) to the mixture of EV and liposome suspension resulted in a large increase in fluorescence intensity at 506 and 572 nm, respectively, compared to that of the PBS and Tat-PEG-lipid (C14) groups (Fig. 5b). This indicates that FRET between DiI and DiO occurred in the mixture of EVs and liposomes by treatment with Tat-PEG-lipids. These findings imply that while Tat-PEG-lipid (C14) induces membrane attachment of EV-liposome (e.g., aggregation), Tat-PEG-lipid (C9) and Tat-PEG-lipid (C12) facilitate membrane fusion between EVs and liposomes. We computed the FRET ratio for each sample (Fig. 5c). We found a significantly higher FRET ratio for the mixture of EVs and liposomes when treated with Tat-PEG-lipid (C9) and Tat-PEG-lipid (C12) than when treated with Tat-PEG-lipid (C14). This result was consistent with the CLSM results (Fig. 4). Based on these results, we conclude that membrane fusion between EVs and liposomes was induced by Tat-PEG-lipid (C9) and Tat-PEG-lipid (C12). Finally, we observed a hybrid of EVs and liposomes using TEM (Fig. 6). The liposomes treated with PBS were approximately 100 nm in size. In contrast, the hybrids of EVs and liposomes treated with Tat-PEG-lipid (C9) and Tat-PEG-lipid (C12) were larger than 100 nm, indicating fusion, as there was no clear boundary between the EVs and liposomes in those aggregates. These TEM findings are consistent with the size data obtained from the DLS analysis (Fig. 3) and CLSM images (Fig. 4). These data suggest that membrane fusion occurred in the EV-liposome hybrid structures on adding Tat-PEG-lipid (C9) or Tat-PEG-lipid (C12).

Membrane fusion between EVs and liposomes induced by Tat-PEG-lipids is facilitated by the membrane-penetrating ability of

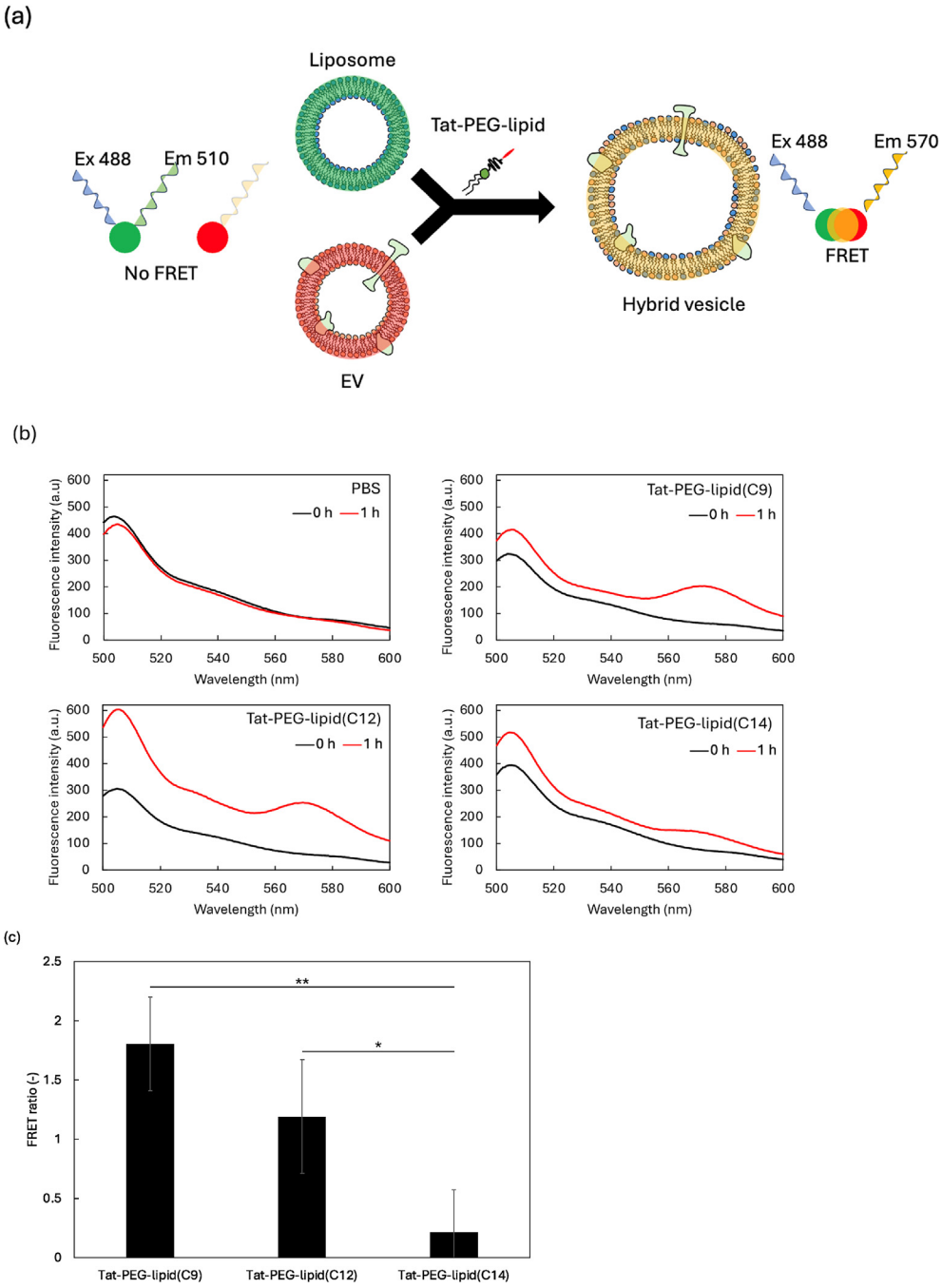

Fig. 5. Fluorescence resonance energy transfer (FRET) measurement of the mixture of EVs and liposomes (DPPC/Chol (1:1, molar ratio)) treated with Tat-PEG-lipids. EVs and liposomes were labeled using DiI (red) and DiO (green) before treatment with Tat-PEG-lipids (C9, C12, or C14). PBS was used as a control. (a) The fluorescence spectra of the treated mixture of EVs and liposomes (Excitation: 488 nm) observed using a fluorophotometer and (b) FRET efficiency calculated based on the ratio of fluorescence intensity at 518 nm and 552 nm. Error bars indicate standard deviation; $n = 3$.

the Tat peptide and membrane insertion of the lipid anchor. Both the Tat peptide and the lipid anchor spontaneously insert into cellular membranes, bringing closer contact between EVs and liposomes. In particular, the shorter lipid anchors (C9 and C12) were more effective. Lipid exchange occurs easily and induces membrane fusion during close contact between lipid membranes and detachment of Tat-PEG-lipids (C9 and C12). Detachment through weak hydrophobic interactions with the lipid membrane can result in spontaneous membrane fusion [13].

This approach enabled the successful easy and mild fusion of EVs and liposomes, allowing the retention of the native functionalities of EVs while imparting the advantages of liposomes, such as drug encapsulation and surface modification capabilities.

The delivery of nucleic acids such as siRNAs by EVs often suffers from low transfection efficiency. However, upon fusion with cationic liposomes, the resulting EV-liposome hybrids substantially enhanced gene expression levels, ensuring up to 4 times higher transfection efficiencies [18]. This improvement in nucleic acid delivery was attributed to the synergistic effects of the innate targeting abilities of EVs and the efficient cargo encapsulation and release mechanisms of liposomes. We speculate that the EV-liposome hybrid system employing this approach can deliver a

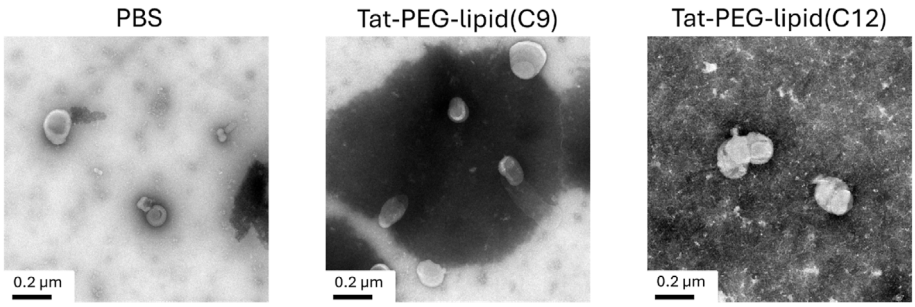

Fig. 6. Representative TEM images of the mixture of EVs and liposomes (DPPC/Chol (1:1, molar ratio)) treated with Tat-PEG-lipids. The samples were negatively stained using 1% sodium phosphotungstate solution (pH~7). The samples were treated with PBS (for control) or Tat-PEG-lipids (C9 or C12).

wide range of therapeutic cargoes, including small-molecule drugs, proteins, imaging agents, plasmid DNAs for gene therapy, mRNA and siRNA therapeutics, and emerging gene-editing tools such as CRISPR/Cas9 [19–22]. Furthermore, by combining targeting ligands or antibodies with EVs, selective delivery to specific cells or tissues can be achieved while mitigating off-target side effects [23]. The versatility of liposomal formulations enables encapsulation and protection of these labile cargoes, whereas the EV component facilitates targeted delivery and cellular uptake. Such multifunctional EV-liposome hybrids leverage the inherent biodistribution and signaling properties of EVs, while integrating the versatile functionalities of liposomes. This EV-liposome fusion approach significantly boosts the delivery of nucleic acids and other therapeutic cargoes by synergizing the advantages of both delivery vehicles.

5. Conclusion

We successfully fabricated hybrid vesicles consisting of EVs and liposomes via membrane fusion using Tat-PEG-lipids (C9, 12). Tat-PEG-lipids with shorter lipid chains are presumed to more effectively induce membrane fusion and formation of hybrid vesicles. Thus, Tat-PEG-lipids (C9 and C12) could be promising candidates for inducing membrane fusion to fabricate EV-liposome hybrids.

Declaration of competing interest

The authors (Yuya Sato, Weixu Zhang, Teruhiko Baba, Ung-il Chung, and Yuji Teramura) declare no conflict of interest for this manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2024.07.006.

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