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Original Article

Surface modulation of extracellular vesicles with cell-penetrating peptide-conjugated lipids for improvement of intracellular delivery to endothelial cells

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ABSTRACT

Exosomes (diameter 30–200 nm) are a subtype of extracellular vesicles secreted by cells containing DNA, microRNA (miRNA), and proteins. Exosomes are expected to be valuable as a means of delivering drugs or functional miRNAs in treatment of diseases. However, the delivery of exosomes is not sufficiently effective, even though exosomes have intrinsic delivery functions. Cell-penetrating peptides (CPPs) are short peptide families that facilitate cellular intake of molecules and vesicles. We previously reported that the modification of cells, and liposomes with CPP-conjugated-lipids, CPPs conjugated with poly (ethylene glycol)-conjugated phospholipids (PEG-lipid), that induce adhesion by CPPs, can be useful for cell-based assays and harvesting liposomes. In this study, we aimed to modulate the exosome surface using Tat peptide (YGRKKRRQRRR)-PEG-lipids to improve intracellular delivery to endothelial cells. We isolated and characterized exosomes from the medium of HEK 293 T cell cultures. Tat conjugated PEG-lipids with different spacer molecular weights and lipid types were incorporated into exosomes using fluorescein isothiocyanate labeling to optimize the number of Tat-PEG-lipids immobilized on the exosome surface. The exosomes modified with Tat-PEG-lipids were incubated with human umbilical vein endothelial cells (HUVECs) to study the interaction. Tat conjugated with 5 kDa PEG and C16 lipids incorporated on the exosome surface were highly detected inside HUVECs by flow cytometry. Fluorescence was negligible in HUVECs for control groups. Thus, Tat-PEG-lipids can be modified on the exosome surface, improving the intracellular delivery of exosomes.

1. Introduction

Exosomes are a subtype of extracellular vesicles 30–200 nm in diameter with a lipid bilayer formed via the endosomal route [1]. Extracellular vesicles, including exosomes, are secreted by most

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cells. Exosomes are abundantly in bodily fluids, such as blood, urine, saliva, and tears [2]. Since microRNAs (miRNAs), lipids, metabolites, and some functional proteins are included in exosomes, exosomes play an important role in cell regulation [3] and cell–cell interactions in immune responses [4]. In addition, exosomes reflect the physiological status of the cells of origin. Some exosomes have been used as biomarkers. For example, exosomes expressing CD63 and Caveolin-1 in plasma, and exosomes from urine have been identified as tumor markers [5,6]. Exosomes from saliva were identified as Sjogren's syndrome markers [7]. In addition, exosomes have recently been used for therapeutic purposes, such as cancer treatment and stroke. Breast cancer cell proliferation was reportedly inhibited by exosomes encapsulating miRNA-134 [8]. Mesenchymal stromal cell-derived exosomes protected the fetal brain from post-hypoxia ischemia [9]. Treatment with exosomes attenuated ischemia in a mouse model [10]. Thus, exosomes are expected to function as carriers of functional miRNAs for disease treatment.

Hindering this potential, the delivery of exosomes is passive and has a short clearance time, even though exosomes have intrinsic delivery functions and minimal reactions to the innate immune system [11,12]. In addition, intracellular delivery is passive, and therefore the therapeutic effect is limited. It will be beneficial to promote the intracellular delivery of exosomes for use as drug carriers.

Surface modification of exosomes with functional peptides may improve the efficiency of intracellular delivery of exosomes. The modification involves cell-penetrating peptides (CPPs), which can induce penetration into the cellular membrane [13,14]. We previously reported the noncytotoxic surface modification of living cells and liposomes with CPP-conjugated poly (ethylene glycol)-phospholipids (CPP-PEG-lipids) [15]. In the present study, we aimed to investigate the surface modification of exosomes with CPP-PEG-lipids. We focused on a representative CPP, the Tat peptide conjugate (Tat-PEG-lipid). We examined the different lengths of PEG and lipids of Tat-PEG-lipid to optimize the surface modification of exosomes and studied the influence of PEG chain length on the intracellular delivery of modified exosomes into human umbilical vein endothelial cells (HUVECs).

2. Materials and methods

2.1. Materials

The following chemicals were purchased from NOF (Tokyo, Japan):1,2-dipalmitoyl-*sn*-glycerol-3-phosphatidylethanolamine (DPPE), 1,2-dimyristoyl-*sn*-glycerol-3-phosphatidylethanolamine (DMPE), and the following four PEG derivatives: α-3-[(3-maleimido-1-oxopropyl)aminopropyl-ω-(succinimidyloxy carboxy)] (Mal-PEG (40 k)-NHS, molecular weight (MW) 40 kDa), α-3-[(3-maleimido-1-oxopropyl)aminopropyl-ω-(succinimidyloxy carboxy)] (Mal-PEG (20 k)-NHS, MW 20 kDa), and α-3-[(3-maleimido-1-oxopropyl)aminopropyl-ω-(succinimidyloxy carboxy)] (Mal-PEG (5 k)-NHS, MW 5.0 kDa). Protein desalting spin column, Micro BCA Protein Assay Kit, goat anti-rabbit horseradish peroxidase (HRP), FreeStyle™ Expression Medium, and Total Exosome Isolation Reagent were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Dichloromethane, dichloroethane, diethyl ether, and triethylamine were purchased from Kanto Chemical Co., Inc (Tokyo, Japan). DMEM, Dulbecco's phosphate-buffered saline (PBS; pH 7.4), fetal bovine serum (FBS), EDTA (0.5 M), and trypan blue solution were purchased from Invitrogen (Carlsbad, CA, USA). EBM™-2 endothelial cell basal medium and HUVECs were purchased from Lonza (Walkersville, MD, USA). Tissue culture polystyrene and glass-bottom culture dishes were purchased from IWAKI (Tokyo, Japan). All synthetic peptides, 1-dodecanthiol, 11-mercaptoundecanoic acid, bovine serum albumin (BSA), protease inhibitor cocktail, polyvinylidene difluoride (PVDF) membranes, 0.22 μm Millex-GV syringe filters, and Amicon Ultra-15 centrifugal filters were purchased from MERCK (Burlington, MA, USA). RIPA buffer (50 mM Tris–HCl, pH 8.0, 150 mM sodium chloride, 0.5% w/v sodium deoxycholate, 0.1% w/v sodium dodecyl sulfate, 1.0% w/v NP-40 substitute), penicillin, streptomycin, phosphotungstic acid hydrate, and β-mercaptoethanol were purchased from Wako Pure Chemicals (Osaka, Japan). The blocking solution was purchased from Nacalai Tesque (Kyoto, Japan). Antibodies against exosomes (anti-CD63, anti-CD81, anti-CD9, anti-HSP70) were purchased from System Biosciences (Palo Alto, CA, USA). The enhanced Chemiluminescence kit was purchased from Abcam (Cambridge, UK).

2.2. Methods

2.2.1. Synthetic peptide-conjugated PEG-lipid

Maleimide-PEG-lipids with PEG chains of different lengths (PEG:5, 20, and 40 kDa) and lipids (lipid: DPPE and DMPE) were synthesized as previously reported [16–18]. Briefly, three different molecular weights of Mal-PEG (5, 20, and 40 k)-NHS (100, 101, and 100 mg, respectively) were mixed with DPPE (15, 4, and 2 mg, respectively), triethylamine (50 μL), and dichloromethane (1.5 mL). Two different molecular weights of Mal-PEG (5 and 40 kDa)-NHS (100 and 100 mg, respectively) were mixed with DMPE (11 and 2 mg, respectively), triethylamine (50 μL), and dichloromethane (1.5 mL). The mixed solution was stirred for 24 h at room temperature. Precipitation with diethyl ether yielded Mal-PEG (5, 20, and 40 k)-DPPE and Mal-PEG (5 k and 40 k)-DMPE powders (78%, 90%, and 89%, respectively). The following synthetic oligopeptides were conjugated to Mal-PEG-lipids (Tat: YGRKKRRQRRRC, FITC-Tat: FITC-YGRKKRRQRRRC, fluorescein isothiocyanate [FITC]: FITC-GC), and a cysteine residue was added to the C-terminus of all peptides to allow conjugation to Mal-PEG-lipid via a thiol–maleimide reaction.

Mal-PEG (5, 20, and 40 k)-DPPE (2 mg) was mixed with FITC-Tat (850, 230, and 120 μg, respectively) and PBS (115, 177, and 188 μL, respectively). After thorough mixing, the solution was maintained at room temperature for 12 h and purified using spin columns (equilibrium with PBS) to obtain FITC-Tat-PEG-DPPE.

Mal-PEG (5 and 40 k)-DMPE (2 mg) was mixed with FITC-Tat (860 μg and 120 μg, respectively) and PBS (114 μL and 188 μL, respectively). After thorough mixing, the solutions were kept at room temperature for 12 h and then purified using spin columns (equilibrium with PBS) to obtain FITC-Tat-PEG-DMPE.

Mal-PEG (5 k)-DPPE (2 mg) was mixed with Tat (690 μg) and PBS (131 μl). After thorough mixing, the solutions were kept at room temperature for 12 h and then purified using spin columns (equilibrium with PBS) to obtain Tat-PEG-DPPE.

Mal-PEG (5 k)-DPPE (2 mg) was mixed with FITC-GC (253 μg) and PBS (175 μl). After thorough mixing, the solutions were kept at room temperature for 12 h and then purified using spin columns (equilibrium with PBS) to obtain FITC-PEG-DPPE.

2.2.2. Cell culture

HEK 293 T cells were kindly gifted by Professor Akiyoshi Fukamizu, Life Science Center for Survival Dynamics, University of Tsukuba. The cells were cultured in DMEM (10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin) at 37 °C in 5% CO_2 and 95% air until 80% confluent. FreeStyle™ Expression Medium for exosome harvest was added after washing twice with PBS to remove FBS in the dish. After 72 h, culture media were collected for exosome isolation.

HUVECs were cultured in EBM-2 supplemented with serum and all additives at 37 °C in 5% CO_2 and 95% air until confluence.

2.2.3. Exosome isolation

The collected culture media from HEK 293 T cells was centrifuged (200×*g*) for 3 min at 25 °C to remove the cells. The supernatant was centrifuged (500×*g*) for 30 min at 4 °C and again (2000×*g*) for 30 min at 4 °C to remove cell debris. The supernatant was filtered a 0.22 μm Millex-GV syringe filter to remove larger vesicles. The filtrate was then centrifuged (5000×*g*) for 60 min at 4 °C using Amicon Ultra-15 centrifugal filters (molecular weight cutoff:100 kDa). The upper solution in the centrifugal filters was stored at −30 °C before use as an exosome-containing fraction. The protein concentration of the exosome fraction was determined using a Micro BCA Protein Assay Kit ([protein] = 2 μg/μL).

2.2.4. Size analysis of exosome

The exosome fraction was diluted with PBS (filtered through a 0.22 μm pore size filter) to adjust the protein concentration to 20 μg/mL. The size and particle numbers of exosomes were measured using NanoSight LM10-HS (Nanosight, Malvern, UK) according to the manufacturer's instructions.

2.2.5. Transmission electron microscopy (TEM)

Exosomes were isolated as described above. For TEM observation, the samples were prepared by negative staining technique. The exosome suspension (2 μg/μL, 10 μL) was first placed on a thin film mesh grids (Excel support film, Nisshin EM Co., Ltd., Tokyo, Japan) for 30 s. The solution was then rinsed and replaced by phosphotungstic acid hydrate solution (1% in pure water, 150 μL). The mesh grid was exposed to the acid solution for 30 s. The remaining solution was finally removed by filter paper and the surface was dried in air. The samples thus prepared were observed using Tecnai G2 F20 (FEI Company Japan Ltd., Tokyo, Japan) at an acceleration voltage of 120 kV.

2.2.6. Western blot

Exosome solution ([protein] = 2 μg/μL) was lysed using RIPA buffer (50 mM Tris−HCl, pH 8.0, 150 mM sodium chloride, 0.5w/v% sodium deoxycholate, 0.1% w/v sodium dodecyl sulfate, 1.0% w/v NP-40 substitute) supplemented with a protease inhibitor cocktail (1 tablet in 50 mL of RIPA buffer) for 40 min at 4 °C. The lysed samples were incubated with loading buffer containing beta-mercaptoethanol for 10 min at 95 °C and then subjected to electrophoresis via 12% SDS-PAGE (40 V for 20 min, 60 V for 2.5 h) using a Mini-PROTEAN Tetra Cell (BioRad). The gel was then transferred to a PVDF membrane (Merck) and blocked with blocking solution (5 × dilution in TBS-T, Nacalai Tesque) for 30 min at room temperature. The blocked PVDF membrane was then treated with primary antibodies (anti-CD63, anti-CD81, anti-CD9, anti-HSP70, 1:1000 dilution in TBS-T, containing 5% BSA; System Biosciences, Palo Alto, CA, USA) for 16 h at 4 °C. After washing with TBS-T three times for 10 min each time at room temperature, the PVDF membrane was incubated with a secondary antibody (goat anti-rabbit HRP, 1:20000 in TBS-T) for 1 h at room temperature. The membrane was treated with an Enhanced Chemiluminescence kit and imaged using a Gel Doc XR + system (BioRad, Hercules, CA, USA).

2.2.7. Quantification of the number of Tat-PEG-lipid modified onto exosomes

FITC-Tat-PEG-lipid was used for fluorophotometer detection. For the surface modification of exosomes, FITC-Tat-PEG-lipid (final concentrations of 0.1, 0.5, 1, 2.5, and 5 mg/mL) was mixed with exosomes (60 μg in PBS) and incubated for 30 min at 37 °C. After washing with PBS, Total Exosome Isolation Reagent (Invitrogen, Carlsbad, CA, USA) was added to the mixture and incubated for 60 min at 4 °C. The mixture was centrifuged (10000×*g*) for 60 min at 4 °C to obtain the pellets. The pellet was suspended in PBS (800 μL) and the fluorescence intensity (excitation wavelength of 488 nm, emission wavelength of 528 nm) was measured using a fluorophotometer (FP-6500, JASCO Corporation, Tokyo, Japan).

PBS were used as negative controls using a fluorophotometer. FITC-Tat-PEG-lipid (final concentrations of 0.1, 0.5, 1, 2.5, and 5 mg/mL) was mixed with 30 μL PBS (same volume as exosomes) and incubated for 30 min at 37 °C. After washing with PBS, Total Exosome Isolation Reagent was added to the mixture and incubated for 60 min at 4 °C. The mixture was centrifuged (10000×*g*) for 60 min at 4 °C. PBS (800 μL) was added to measure the fluorescence intensity (excitation wavelength of 488 nm, emission wavelength of 528 nm) using the aforementioned fluorophotometer.

2.2.8. Zeta potential of modified exosomes

The zeta potential of the exosomes was determined using a Zetasizer Nano ZS (Malvern Instruments Co., Ltd. Worcestershire, UK). Exosomes (60 μg) were incubated with Tat-PEG-lipids (final concentration 1 mg/mL in PBS) for 30 min at 37 °C. After washing with PBS, Total Exosome Isolation Reagent was added to the mixture and incubated for 60 min at 4 °C. The mixture was centrifuged (10000×*g*) for 60 min at 4 °C to obtain the pellets. Pellets were resuspended in PBS (1 mL) for analysis.

2.2.9. Analysis of interaction between Tat-PEG-lipid modified exosomes and charged surface by quartz crystal microbalance with energy dissipation (QCM-D)

QCM-D measurements were performed using a Q-sense instrument (Biolin Scientific AB, Stockholm, Sweden). The instrument simultaneously monitors the shifts in the oscillator frequency (Δf) and energy dissipation (ΔD) at 4.95 MHz a gold-coated QCM-D sensor at different overtones [19]. Δf decreases when any substance is adsorbed on the sensor surface. Thus, the amount of adsorbed material can be measured from the change in f. Here, we used the change in f to estimate the amount of adsorbed material using the Sauerbrey equation, as follows:

Adsorbed mass (ng/cm^2) = −17.7 × Δf at the 7th overtone (Hz)

Tat-PEG (5 k)-DPPE-modified exosomes were prepared as described above using 1 mg/mL Tat-PEG (5 k)-DPPE. In the final step, the exosome pellet was resuspended in 320 μL PBS ([protein] = 0.3 μg/μL). The gold-coated QCM-D sensor chips were immersed in 1 mM ethanol solution of 11-mercaptoundecanoic acid or 1-dodecanethiol for 18 h at RT. This allowed the formation of SAM that carried carboxyl or methyl groups (COOH−C_{10}-SAM and CH_3−C_{11}-SAM, respectively). The chips were washed three times with ethanol and dried in air. The sensor chip was first exposed to PBS until a stable baseline was obtained. The exosome solution was then injected for 2 min, followed by washing with PBS for 10 min to remove the unbound exosomes. All measurements were performed at 25 °C and three independent experimental data points were gathered.

2.2.10. Analysis of retention time of Tat-PEG-DPPE on exosome

FITC-Tat-PEG (5, 20, and 40 k)-DPPE (1 mg/mL) was mixed with exosomes (30 μg in PBS) and incubated for 30 min at 37 °C. Total Exosome Isolation Reagent (Invitrogen) was added to the mixture and incubated for 60 min at 4 °C. The mixture was centrifuged (10000×*g*) for 60 min at 4 °C to obtain the pellets. The pellet was suspended in PBS (100 μL) and preserved at RT for 0, 6, 24 and 48 h. Then, after the pellet was collect in the same way, the pellet was resuspended in PBS (800 μL) and the fluorescence intensity (excitation wavelength of 488 nm, emission wavelength of 528 nm) was measured using a fluorophotometer (FP-6500, JASCO Corporation, Tokyo, Japan).

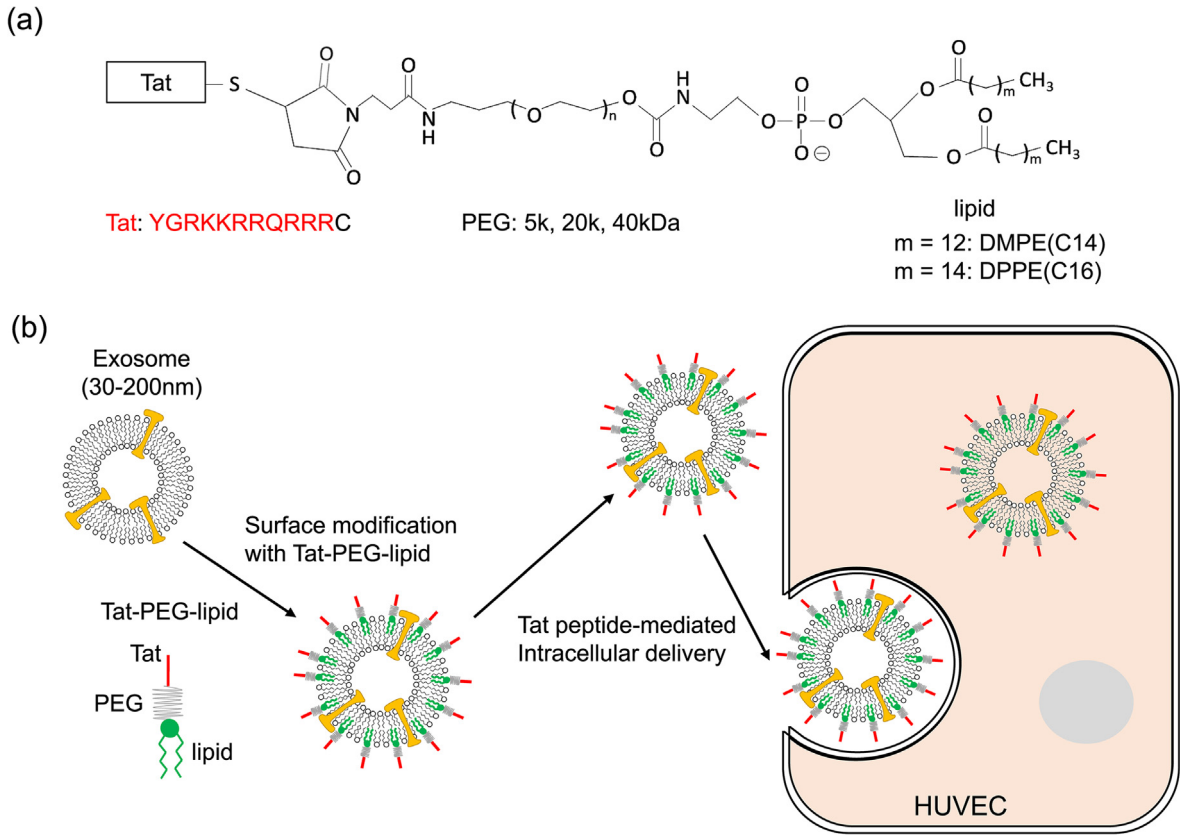

Fig. 1. Schematic illustration of exosome modification with Tat-PEG-lipid for intracellular delivery. (a) Chemical structure of Tat-PEG-lipid and (b) schematic illustration of intracellular delivery of modified exosomes.

2.2.11. Confocal microscopy

HUVECs (4×10^4) were seeded onto a 35 mm glass-bottom dish and cultured for 24 h at 37 °C in an atmosphere of 5% CO_2. The culture dish was incubated for 10 min at 4 °C before incubation with exosomes.

Bovine carotid artery normal endothelial cells (8×10^4) were seeded onto a 35 mm glass-bottom dish and cultured for 24 h at 37 °C in an atmosphere of 5% CO_2. The culture dish was incubated for 10 min at 4 °C before incubation with exosomes.

HEK 293 T (8×10^4) were seeded onto a 35 mm glass-bottom dish and cultured for 24 h at 37 °C in an atmosphere of 5% CO_2. The culture dish was incubated for 10 min at 4 °C before incubation with exosomes.

Exosomes (100 μg) were mixed with FITC-Tat-PEG-lipids (final concentration:1 mg/mL in PBS) and incubated for 30 min at 37 °C. After washing with PBS, Total Exosome Isolation Reagent was added to the mixture and incubated for 60 min at 4 °C. The mixture was centrifuged (10000×*g*) for 60 min at 4 °C to obtain the pellets.

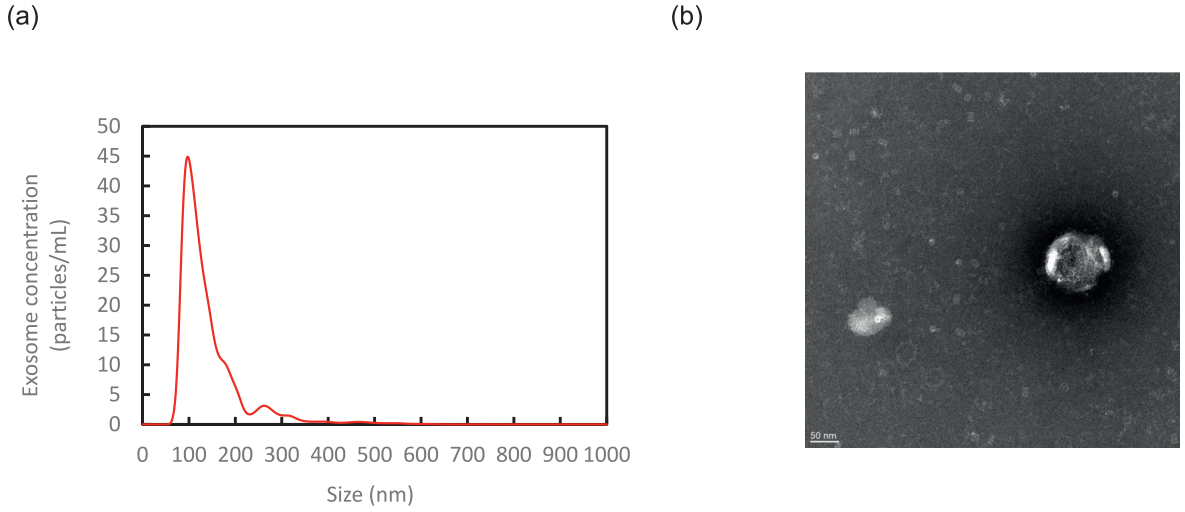

Fig. 2. Characterization of exosomes. (a) Analysis of exosome size distribution and particle number by NanoSight. The average size of exosomes was approximately 98 ± 3 nm. There were $7.53 \pm 0.24 \times 10^{10}$ exosome particles/mL. (b) Representative transmission electron microscopy (TEM) images of exosomes. The sample was treated with 1% phosphotungstic acid hydrate solution for negative staining and TEM observation.

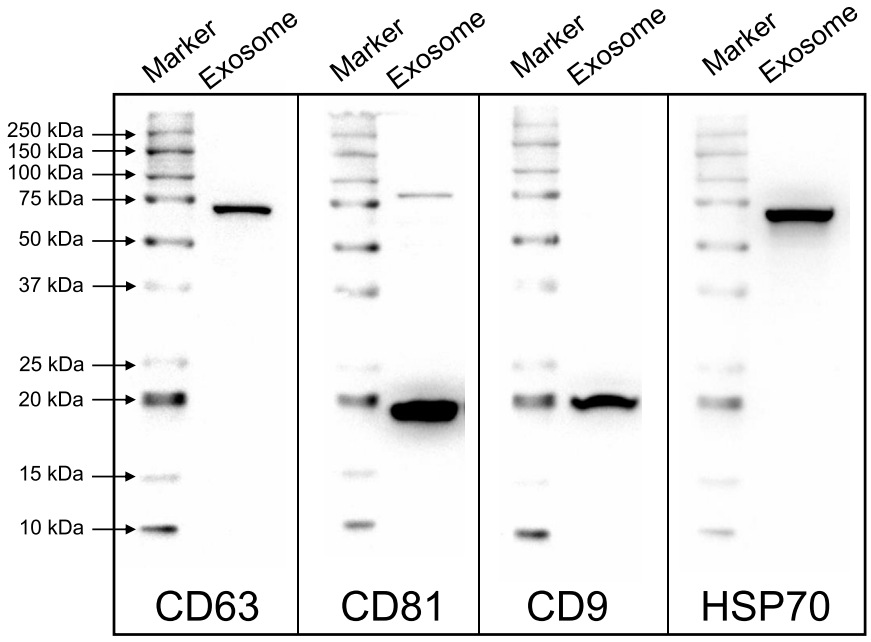

Fig. 3. Characterization of exosomes by western blot using representative surface markers (CD63, CD81, CD9 and HSP70). Isolated exosomes (25 μg) were loaded for the analysis of each marker.

The pellets were resuspended in EBM-2 (250 μL) and incubated with HUVEC for 30 min at 4 °C, or EMEM (250 μL) and incubated with Bovine carotid artery normal endothelial cells for 30 min at 4 °C or DMEM (250 μL) and incubated with HEK 293 T for 30 min at 4 °C. After washing twice with PBS, the cells were observed by a confocal laser scanning microscope (LSM510; Carl Zeiss Microscopy Co. Ltd., Jena, Germany). FITC-PEG-lipid was used as a control.

2.2.12. Quantification of exosomes in HUVECs by flow cytometry

HUVECs (6×10^5) were seeded in wells of a 6-well microplate and cultured for 24 h at 37 °C in an atmosphere of 5% CO_2. The culture dish was incubated for 10 min at 4 °C before incubation with the exosomes. Exosomes (100 μg) were mixed with FITC-Tat-PEG (5, 20, or 40 k)-lipids (final concentration 1 mg/mL in PBS). The cells were incubated for 30 min at 37 °C. After washing with PBS, Total Exosome Isolation Reagent (Invitrogen) was added to the mixture and incubated for 60 min at 4 °C. The mixture was centrifuged (10000×*g*) for 60 min at 4 °C to obtain the pellets. The pellets were resuspended in EBM-2 (1.3 mL) and the resultant solution was added to a HUVEC culture dish for 30 min at 4 °C. After washing twice with PBS, the HUVECs were incubated with EBM-2 for 24 h at 37 °C in an atmosphere of 5% CO_2. The cells were observed under a confocal laser scanning microscope (LSM510; Carl Zeiss Microscopy). After incubation, cells were collected following

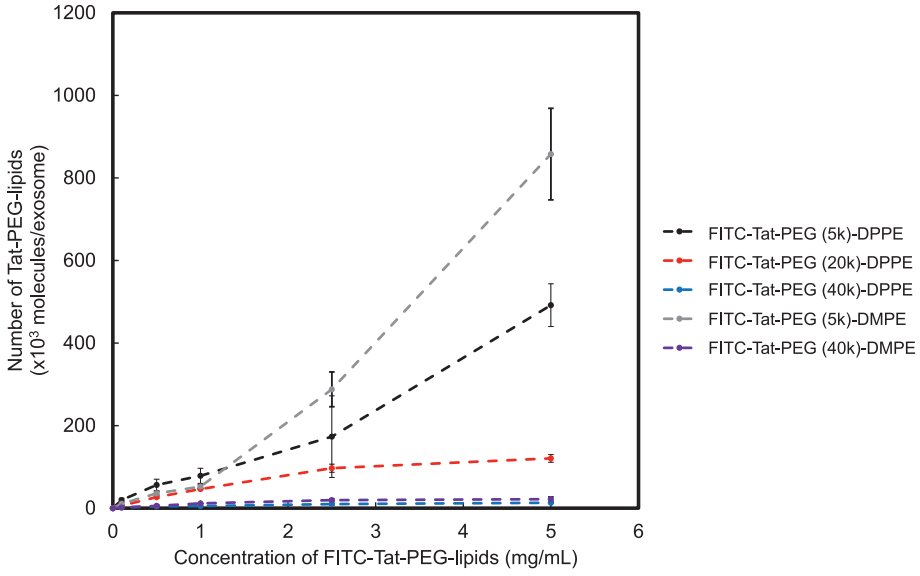

Fig. 4. Influence of the molecular weight of PEG and acyl chain on the surface modification of exosome by Tat-PEG-lipids. The number of incorporated FITC-Tat-PEG-lipids into exosome as a function of the PEG-lipid concentration. (a) Three different molecular weights of PEG (5 kDa, 20 kDa and 40 kDa) and (b) two different acyl chains (DMPE and DPPE) were used. Data are presented as mean values ± SD. N = 3.

trypsin treatment. The cells were then resuspended in 400 μL of EBM-2 for flow cytometry (Gallios, Beckman Coulter, Inc., Palo Alto, CA, US).

3. Statistical analysis

All experiments were repeated at least three times. Data are presented as mean ± standard deviation or representative images. Statistical analysis was conducted using one-way analysis of variance, followed by Dunnett's multiple comparison tests with a single pooled variance using GraphPad Prism 9 for macOS version 9.0.0 (GraphPad Software, La Jolla, California, USA). The calculated p-values are indicated in Fig. 5.

4. Results and discussion

The Tat peptide was covalently attached to PEG-lipids with various MWs of PEG chains and lipids and then used to modify the exosome surface (Fig. 1). Exosomes isolated from HEK 293 T culture medium were measured using NanoSight to measure size, particle number, and TEM for morphology analysis (Fig. 2). The size analysis revealed a size distribution from approximately 60 nm–500 nm. The majority of the exosomes were 98 ± 3 nm in diameter (Fig. 2a). The determined particle number of exosomes was $7.53 \pm 0.24 \times 10^{10}$ particles/mL ([total protein] = 2 μg/μL). We also observed exosomes using TEM (Fig. 2b). The observed size of the particles ranged from 50 nm to 200 nm in diameter, with an average size of approximately 100 nm, which is consistent with NanoSight data. The findings indicated that exosomes isolated from HEK 293 T cells were similar in size to those reported by other groups [20,21]. Western blotting indicated that the exosome markers CD63, CD81, CD9, and HSP70 were positively expressed (Fig. 3). Collectively, these results indicated the successful isolation of exosomes from HEK 293 T cells.

We previously reported that surface modification of cells and liposomes with PEG-lipids was maximum at approximately 30 min [22]. Therefore, 30 min was used as the incubation time in the present experiment using FITC-Tat-PEG-lipid at different concentrations for exosome modification (Fig. 4). Three different molecular weights of PEG (5, 20, and 40 kDa) were used (Fig. 4a). Two different acyl chains (DMPE and DPPE) were used (Fig. 4b). The number of incorporated Tat peptides on the exosomes depended on the feed concentration of each Tat-PEG-lipid. However, the number that were incorporated differed between the PEG molecular weights. The number of immobilized Tat peptides reached 4.9×10^5, 1.2×10^5, and 1.3×10^4 molecules per an exosome in for 5, 20, or 40 kDa PEG, respectively (Fig. 4a). Because the viscosity of PEG-lipid increases with concentration, it was impossible to increase the concentration further. The number of immobilized Tat peptides on the exosome decreased with an increased PEG molecular weight, presumably due to an increase in the steric hindrance of the PEG chain. We also analyzed Tat-PEG-DMPE, which has a shorter acyl chain (C14) than that of DPPE (C16). The number of incorporated Tat peptides increased with increased feed

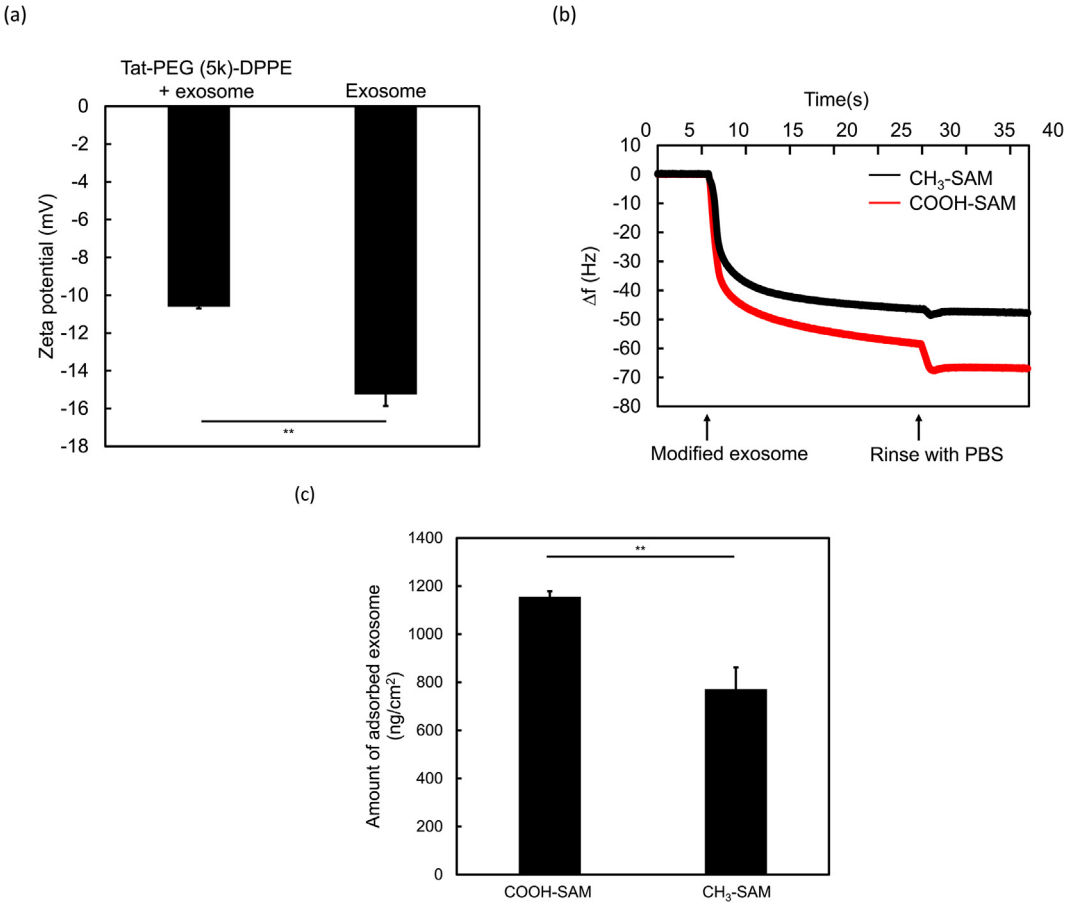

Fig. 5. Surface charge of exosome modified with Tat-PEG (5 k)-DPPE. (a) Zeta potential of treated exosomes determined by dynamic light scattering. Nontreated exosomes were used as the control. (b) QCM-D analysis of the interaction between the modified exosomes and SAM surface with different surface properties carrying methyl (uncharged) and carboxyl (negative charge) functional groups. Representative QCM-D profiles of modified exosomes adsorption on each SAM surface. After the exosome solution was flowed, PBS was used for washing. (c) Amounts of modified and non-modified exosomes adsorbed onto the SAM surfaces. Data are presented as mean values ± SD. N = 3.

concentration for both Tat-PEG-DMPE and Tat-PEG-DPPE and also showed a higher number of incorporated Tat peptides for Tat-PEG-DMPE when we compared 5 kDa PEG and 40 kDa PEG chain. However, we decided to use Tat-PEG-DPPE for exosome modification because PEG-DMPE rapidly detached from the cellular surface, although the initial incorporation number was higher than that of PEG-DPPE [23]. Therefore, Tat-PEG-DPPE was used for further experiments. The retention time of Tat-PEG-DPPE on exosome was measured (Supplementary Fig. S1). The number of Tat peptides immobilized on the exosome surface could be controlled using Tat-PEG-lipids with different molecular weights of PEG and lipids.

Next, we studied the surface charge of the exosomes before and after modification with Tat-PEG (5 k)-DPPE (Fig. 5). Zeta potential measurements showed that Tat-PEG (5 k)-DPPE-modified exosome was -10.6 ± 0.1 mV, whereas intact exosome was -15.3 ± 0.6 mV (Fig. 5a). This result indicated that the surface charge of modified exosome shifted to positive side by +4.7 mV, indicating the incorporation of positively charged Tat peptide on the exosome surface. We expected that the surface charge of the modified exosomes would be positive due to the incorporation of the Tat peptide. However, the surface charge was still negative even though it shifted from the original value to a more positive value. Similar results were observed for modified cells after treatment with Tat-PEG-lipid [24]. Cationic Tat peptides are surrounded by sialic acid and membrane proteins, resulting in a slight positive shift in the zeta potential of the total charge. In addition, because the Tat peptide shows cell-penetrating properties, it is possible that it was transiently located inside the cellular membrane. However, an actual positive shift was detected on the modified cellular surface, which strongly suggested that most Tat peptides were located outside the exosome. We previously reported that Tat peptides could be detected on the cellular surface using biotin labeling and detection of Alexa 488-streptavidin [25]. Therefore, we concluded that the Tat peptide in the PEG-lipid was able to locate inside and outside of the cellular membrane, which was supported by the zeta potential measurements.

We also examined the interaction between the modified exosomes and negatively charged surfaces using QCM-D (Fig. 5b). COOH-SAM was used as a negatively charged surface and CH_3-SAM as a non-charged surface to study the interactions with modified exosomes. We detected greater binding of modified exosomes onto the COOH-SAM surface (1156 ± 22 ng/cm^2) than the CH_3-SAM surface (772 ± 90 ng/cm^2), suggesting that the modified exosomes had a positively charged surface. Although there was nonspecific binding of the modified exosome onto the CH_3-SAM surface, we observed a significant difference in the binding of the modified exosomes, which was deemed to be due to electrostatic interactions.

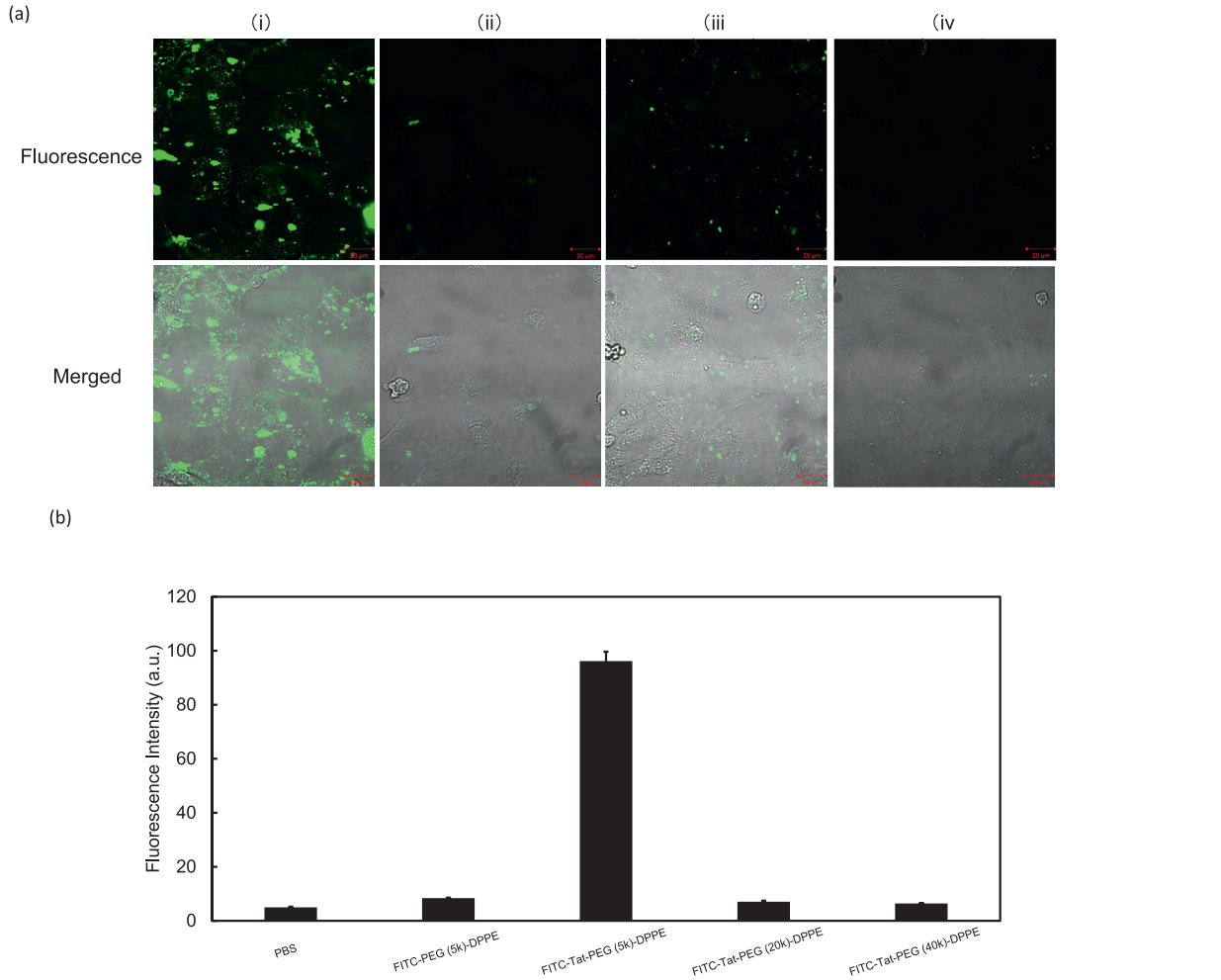

Fig. 6. Analysis of interaction between Tat-PEG-lipid-modified exosomes and HUVEC observed by a confocal laser scanning microscope. (a) HUVEC cells were incubated with: (i) exosome modified with Tat-PEG (5 k)-DPPE and (ii) exosome modified with FITC-PEG (5 k)-DPPE at 4 °C for 30 min. Also, we used (iii) the sample obtained from isolation procedure without exosome when adding Tat-PEG (5 k)-DPPE and (iv) PBS as a control. (b) Flow cytometry analysis of HUVECs treated with modified exosomes. Here we used four different FITC-Tat-PEG-DPPE with different molecular weight of PEG (5 kDa, 20 kDa and 40 kDa) and FITC- PEG-DPPE. **$p < 0.01$. Data are presented as mean values ± SD. N = 3.

Next, we incubated FITC-Tat-PEG (5 k)-DPPE-modified exosomes with HUVECs to study the interaction (Fig. 6). Primary HUVECs were used as a model of endothelial cells. We did not use direct fluorescence labeling of the exosome membrane for observation, but did use FITC-labeled Tat peptide. This was because the fluorescence disappeared from the exosomes after modification with Tat-PEG (5 k)-DPPE (data not shown). We considered that the fluorescence molecule in the membrane competed with Tat-PEG (5 k)-DPPE and was expelled from the exosome surface. Therefore, we decided to use FITC-Tat-PEG (5 k)-lipids to trace exosomes in HUVECs. Here, we used FITC-PEG-DPPE and PBS as controls, and we used the sample obtained from the isolation procedure without exosomes. We needed to ensure that we completely isolated the modified exosomes from free FITC-Tat-PEG (5 k)-DPPE. Therefore, we performed an isolation procedure without exosomes after adding FITC-Tat-PEG (5 k)-DPPE. When we treated HUVECs with FITC-Tat-PEG (5 k)-DPPE modified exosomes, we observed strong fluorescence in the cells, with many modified exosomes detected inside the cells (Fig. 6a). In contrast, fluorescence was negligible in HUVECs in other control groups. Since the average size of exosomes was 116 nm, the detected fluorescence was attributed to the clustering of exosomes inside the HUVEC. In addition, we performed cell experiments using FITC-Tat-PEG (20 k)-DPPE and FITC-Tat-PEG (40 k)-DPPE to compare the different lengths of the PEG chains for quantitative analysis by flow cytometry (Fig. 6b and Supplementary Fig. S2). The analysis revealed that the highest fluorescence intensity was detected in HUVECs treated with exosomes, which were treated with FITC-Tat-PEG (5 k)-DPPE, among other Tat-PEG (20, 40 k)-DPPEs. The number of Tat peptides incorporated was different among Tat-PEG-DPPEs, where the number of Tat peptides on exosome was 7.8×10^4 (for FITC-Tat-PEG (5 k)-DPPE), 4.6×10^4 (for FITC-Tat-PEG (20 k)-DPPE), and 5.1×10^3 (for FITC-Tat-PEG (40 k)-DPPE) molecules per exosome. In addition, steric hindrance of the PEG chain affected the interaction between Tat and the HUVEC surface. Therefore, the 5 kDa PEG spacer is suitable for achieving higher incorporation of the Tat peptide into exosomes. The similar results were observed for other cells such as bovine carotid artery normal endothelial cells and HEK 293 T cells (Supplementary Fig. S3). Proteoglycans on the cellular surface play an important role in the intracellular delivery of Tat-modified exosomes via cell-penetrating peptides [26]. We also used the Tat peptide for the modification of exosomes, so that proteoglycans are highly involved in the intracellular delivery of modified exosomes. Thus, we found that the number of incorporated Tat peptides was crucial for the intracellular delivery of modified exosomes.

Other arginine-rich CPPs have also been studied for the modification of exosomes via covalent binding to membrane proteins, and the conjugation of R16 onto exosomes was most efficient for intracellular delivery [27]. Covalent conjugation of exosomes with CPPs might induce random modification of proteins, resulting in loss of function. On the other hand, our modification is achieved through hydrophobic interactions between the exosome lipid domain and the lipid part of Tat-PEG-lipid. Therefore, there is no risk for the loss of function of proteins. In addition, a PEG spacer can be added to functionalize Tat and control the surface density. Therefore, our approach using a PEG-lipid construct has some advantages over covalent conjugation.

5. Conclusion

Tat peptide-conjugated lipids can modify the exosome surface, and the number of Tat peptides immobilized on the exosome surface can be controlled using different concentrations and molecular weights of Tat-PEG-lipids. Modification with Tat-PEG (5 k)-DPPE improves the intracellular delivery of exosomes to HUVEC in vitro. Tat peptide-conjugated lipids can improve the delivery function of exosomes and we anticipate that this material will be useful for specific exosome delivery for disease treatment.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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References

[1] Zaborowski MP, Balaj L, Breakefield XO, Lai CP. Extracellular vesicles: composition, biological relevance, and methods of study. Bioscience 2015;65:783–97.
[2] Vlassov AV, Magdaleno S, Setterquist R, Conrad R. Exosomes: current knowledge of their composition, biological functions, and diagnostic and therapeutic potentials. Biochim Biophys Acta 2012;1820:940–8.
[3] Kapustin AN, Chatrou ML, Drozdov I, Zheng Y, Davidson SM, Soong D, et al. Vascular smooth muscle cell calcification is mediated by regulated exosome secretion. Circ Res 2015;116:1312–23.
[4] Gao K, Jin J, Huang C, Li J, Luo H, Li L, et al. Exosomes derived from septic mouse serum modulate immune responses via exosome-associated cytokines. Front Immunol 2019;10:1560.
[5] Logozzi M, De Milito A, Lugini L, Borghi M, Calabro L, Spada M, et al. High levels of exosomes expressing CD63 and caveolin-1 in plasma of melanoma patients. PLoS One 2009;4:e5219.
[6] Nyalwidhe JO, Betesh LR, Powers TW, Jones EE, White KY, Burch TC, et al. Increased bisecting N-acetylglucosamine and decreased branched chain glycans of N-linked glycoproteins in expressed prostatic secretions associated with prostate cancer progression. Proteonomics Clin Appl 2013;7:677–89.
[7] Michael A, Bajracharya SD, Yuen PS, Zhou H, Star RA, Illei GG, et al. Exosomes from human saliva as a source of microRNA biomarkers. Oral Dis 2010;16:34–8.
[8] O'Brien K, Lowry MC, Corcoran C, Martinez VG, Daly M, Rani S, et al. miR-134 in extracellular vesicles reduces triple-negative breast cancer aggression and increases drug sensitivity. Oncotarget 2015;6:32774–89.
[9] Ophelders DR, Wolfs TG, Jellema RK, Zwanenburg A, Andriessen P, Delhaas T, et al. Mesenchymal stromal cell-derived extracellular vesicles protect the fetal brain after hypoxia-ischemia. Stem Cells Transl Med 2016;5:754–63.
[10] Hu GW, Li Q, Niu X, Hu B, Liu J, Zhou SM, et al. Exosomes secreted by human-induced pluripotent stem cell-derived mesenchymal stem cells attenuate limb ischemia by promoting angiogenesis in mice. Stem Cell Res Ther 2015;6:10.
[11] Clayton A, Harris CL, Court J, Mason MD, Morgan BP. Antigen-presenting cell exosomes are protected from complement-mediated lysis by expression of CD55 and CD59. Eur J Immunol 2003;33:522–31.
[12] Ridder K, Keller S, Dams M, Rupp AK, Schlaudraff J, Del Turco D, et al. Extracellular vesicle-mediated transfer of genetic information between the hematopoietic system and the brain in response to inflammation. PLoS Biol 2014;12:e1001874.
[13] Futaki S, Suzuki T, Ohashi W, Yagami T, Tanaka S, Ueda K, et al. Arginine-rich peptides. An abundant source of membrane-permeable peptides having potential as carriers for intracellular protein delivery. J Biol Chem 2001;276:5836–40.
[14] Futaki S. Membrane-permeable arginine-rich peptides and the translocation mechanisms. Adv Drug Deliv Rev 2005;57:547–58.
[15] Teramura Y, Asif S, Ekdahl KN, Gustafson E, Nilsson B. Cell adhesion induced using surface modification with cell-penetrating peptide-conjugated poly(-ethylene glycol)-lipid: a new cell glue for 3D cell-based structures. Acs Appl Mater Inter 2017;9:244–54.
[16] Teramura Y, Chen H, Kawamoto T, Iwata H. Control of cell attachment through polyDNA hybridization. Biomaterials 2010;31:2229–35.

[17] Teramura Y. Cell surface modification with ssDNA-PEG-lipid for analysing intercellular interactions between different cells. Biomaterials 2015;48:119–28.
[18] Sakurai K, Teramura Y, Iwata H. Cells immobilized on patterns printed in DNA by an inkjet printer. Biomaterials 2011;32:3596–602.
[19] Hook F, Kasemo B, Nylander T, Fant C, Sott K, Elwing H. Variations in coupled water, viscoelastic properties, and film thickness of a Mefp-1 protein film during adsorption and cross-linking: a quartz crystal microbalance with dissipation monitoring, ellipsometry, and surface plasmon resonance study. Anal Chem 2001;73:5796–804.
[20] Wang C, Li N, Li Y, Hou S, Zhang W, Meng Z, et al. Engineering a HEK-293T exosome-based delivery platform for efficient tumor-targeting chemotherapy/internal irradiation combination therapy. J Nanobiotechnol 2022;20:247.
[21] Song Z, Mao J, Barrero RA, Wang P, Zhang F, Wang T. Development of a CD63 aptamer for efficient cancer immunochemistry and immunoaffinity-based exosome isolation. Molecules 2020;25.
[22] Ohgaki R, Teramura Y, Hayashi D, Quan L, Okuda S, Nagamori S, et al. Ratiometric fluorescence imaging of cell surface pH by poly(ethylene glycol)-phospholipid conjugated with fluorescein isothiocyanate. Sci Rep 2017;7:17484.
[23] Inui O, Teramura Y, Iwata H. Retention dynamics of amphiphilic polymers PEG-lipids and PVA-Alkyl on the cell surface. ACS Appl Mater Interfaces 2010;2:1514–20.
[24] Noiri M, Goto Y, Sato Y, Nakamura N, Ishihara K, Teramura Y. Exogenous cell surface modification with cell penetrating peptide-conjugated lipids causes spontaneous cell adhesion. ACS Appl Bio Mater 2021;4:4598–606.
[25] Teramura Y, Asif S, Ekdahl KN, Gustafson E, Nilsson B. Cell adhesion induced using surface modification with cell-penetrating peptide-conjugated poly(-ethylene glycol)-lipid: a new cell glue for 3D cell-based structures. ACS Appl Mater Interfaces 2017;9:244–54.
[26] Nakase I, Tadokoro A, Kawabata N, Takeuchi T, Katoh H, Hiramoto K, et al. Interaction of arginine-rich peptides with membrane-associated proteoglycans is crucial for induction of actin organization and macropinocytosis. Biochemistry 2007;46:492–501.
[27] Nakase I, Noguchi K, Aoki A, Takatani-Nakase T, Fujii I, Futaki S. Arginine-rich cell-penetrating peptide-modified extracellular vesicles for active macropinocytosis induction and efficient intracellular delivery. Sci Rep 2017;7:1991.