Effect of exosome isolation methods on physicochemical properties of exosomes and clearance of exosomes from the blood circulation

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ABSTRACT

Exosomes, which are expected to be delivery systems for biomolecules such as nucleic acids, are collected by several methods. However, the effect of exosome isolation methods on the characteristics of exosomes as drug carriers, such as recovery efficiency after sterile filtration and pharmacokinetics, has not been investigated despite the importance of these characteristics for the development of exosome-based delivery systems. In the present study, exosomes collected from murine melanoma B16-BL6 cells by several methods were compared with respect to dispersibility, recovery rate after filtering, and clearance from the blood circulation in mice. The exosomes were collected by three ultracentrifugation-based methods: simple ultracentrifugation/pelleting (pelleting method), ultracentrifugation with an iodixanol cushion (cushion method), and ultracentrifugation on an iodixanol density gradient (gradient method). The isolation methods had little effect on the particle number of exosomes. In contrast, transmission electron microscopy observation and size distribution measurement using tunable resistive pulse sensing indicated that the exosomes of the gradient method were more dispersed than the others. The exosomes were labeled with *Gaussia* luciferase and intravenously injected into mice. Clearance of injected exosomes from the blood circulation did not significantly change with isolation methods. When the exosomes were filtered using a 0.2-μm filter, the recovery rate was 82% for the exosomes of the gradient method, whereas it was less than 50% for the others. These results indicate that the exosome isolation method markedly affects the dispersibility and filtration efficiency of the exosomes.

Introduction

Exosomes are extracellular vesicles with a diameter of 30–150 nm that contain biomolecules including RNAs and proteins [1-3]. Exosomes are secreted from most types of cells and play roles in intercellular communication. Exosomes derived from specific cells have therapeutic potential. For example, exosomes derived from mesenchymal stem cells exert protective effects on myocardial ischemia/reperfusion injury [4]. Thus, exosomes themselves are expected to be used in disease treatments. They are also expected to be used as a biocompatible and efficient delivery carrier given that they are endogenous intercellular delivery carriers that transfer biomolecules including RNAs and proteins [5-7]. Exosomes as well as exosome-mimetic vesicles may be used to deliver therapeutic molecules such as small interfering RNAs, microRNAs (miRNAs), and small-molecule drugs including curcumin, doxorubicin, and porphyrin to target cells [8-12].

Exosomes are usually collected from cell culture medium in such applications as delivery carrier and therapeutic treatment. There are several methods for isolating exosomes from the medium such as ultracentrifugation-based, solvent precipitation, column chromatography, and immunoaffinity methods [13]. The most commonly used method of isolation is the ultracentrifugation-based method. This method has a low cost, a low risk of contamination with separation reagent, and allows collection of large numbers of exosomes [4, 11]. Several ultracentrifugation-based methods have been developed for isolating exosomes. Another frequently used method is based on solvent precipitation [14]. This method is performed by incubation of samples with solvent reagent followed by centrifugation at a relatively low speed, and it does not require ultracentrifugation. The isolation methods influence the composition of miRNA and protein of exosomes [15, 16] as well as exosome morphology [17]. However, little is known about the effect of isolation

methods on the yield and physicochemical properties of exosomes, such as size, zeta potential, and dispersibility, despite the fact that these parameters affect the characteristics of exosomes as a delivery carrier and may change the therapeutic effect and delivery efficiency of exosome-based treatments. Although a few studies have compared the yield and physicochemical properties of exosomes obtained by different methods [18, 19], whether or not differences in physicochemical properties influence the characteristics of exosomes as drug carriers has not been investigated.

In the present study, we selected the solvent precipitation method and three ultracentrifugation-based methods, namely, simple ultracentrifugation/pelleting (pelleting method), ultracentrifugation with a cushion at the bottom of the tubes (cushion method), and density gradient ultracentrifugation (gradient method) as exosome isolation methods and investigated the effects of these isolation methods on the characteristics of the collected exosomes. We used B16-BL6 murine melanoma cells to obtain exosomes because these cells produce large numbers of exosomes and B16 cell line-derived exosomes have frequently been characterized [2, 20]. The fusion protein of *Gaussia* luciferase (gLuc) and lactadherin (gLuc-LA) was then used as a probe to evaluate the effects of the methods on the clearance of exosomes from the blood circulation [21, 22].

Materials and Methods

Cell culture and transfection

B16-BL6 murine melanoma cells were obtained and cultured as described previously [21]. Plasmid DNA encoding gLuc-LA was constructed in the previous study [21]. To obtain gLuc-LA-labeled exosomes, plasmid DNA encoding gLuc-LA was transfected to B16-BL6 cells with polyethyleneimine (PEI) "max" (Polysciences, Warrington, PA, USA). Briefly, 128 μL PEI "max" solutions of 0.323 mg/mL, pH 8.0, and 16 μg plasmid DNA were

individually diluted to 500 μL with 150 mM NaCl. They were mixed and incubated at 15 min to make a complex. The solution was added to the cells. The medium was changed to Opti-MEM 4 h after transfection, and cells were incubated for additional 24 h.

Exosome collection

Cells were seeded in 150-mm dishes at a cell number of 5.0×10^6 cells per dish and cultured for 24 h. Then, the cell culture medium was removed and cells were washed thrice with phosphate-buffered saline (PBS). Next, 30 mL of Opti-MEM was added to the dish and the cells were incubated for additional 24 h. The culture medium was processed before ultracentrifugation by differential centrifugation at 300×g for 10 min, 2,000×g for 20 min, and 10,000×g for 30 min to remove cell debris and large vesicles. In addition, the medium was filtered with a 0.2-μm syringe filter. All the ultracentrifugation steps described below were performed using a Himac CP80WX ultracentrifuge (Hitachi Koki, Tokyo, Japan).

In the pelleting method, the processed cell culture medium was spun at 100,000×g in an angle rotor (P50AT2, Hitachi Koki) for 1 h to obtain pellets containing exosomes. The pellet was then resuspended in PBS and spun again at 100,000×g in the angle rotor for 1 h. The exosomes obtained were resuspended in PBS.

In the cushion method, 9 mL of the processed cell culture medium was overlaid onto 40% OptiPrep (Axis-Shield PoC, Oslo, Norway) in 0.25 M sucrose/10 mM Tris-HCl, pH 7.5 and was spun at 100,000×g in a swinging-bucket rotor (P40ST, Hitachi Koki) for 1 h. An aliquot from the border zone of the medium and the OptiPrep was collected and resuspended in PBS and was spun at 100,000×g in the angle rotor for 1 h to obtain pellets containing exosomes. The exosomes were resuspended in PBS.

In the gradient method, 40%, 20%, 10%, and 5% OptiPrep solutions were prepared by dilution of a stock solution of OptiPrep with 0.25 M sucrose/10 mM Tris-HCl, pH 7.5. The

gradient was formed by sequentially layering 2.5 mL of each solution in descending order of density. The processed cell culture medium was concentrated to 1 mL using ultrafiltration (100k), layered on the top of the gradient layer, and spun at 100,000×g in the swinging bucket rotor for 18 h. Then, 1 mL of each fraction was collected sequentially from the top and numbered by collection order. Thus, the density of the collected fraction increased with fraction number. It was expected that exosomes would be recovered in fraction 9, based on the density of this fraction. After confirmation by a preliminary experiment that exosome marker proteins were detected mostly in this fraction, the fraction was used without additional treatment. In preliminary experiments, we confirmed that iodixanol remaining in the sample did not influence the assays, including filtration and pharmacokinetic analysis (unpublished results).

In solvent precipitation, exosomes were collected from the processed cell culture medium using Exoquick-TC (System Biosciences, Mountain View, CA, USA) according to the manufacturer's instruction.

Western blotting

Exosome samples (0.05 μg protein) and cell lysate of B16BL6 (1 μg protein) were reduced by addition of 100 mM dithiothreitol and heat treatment at 95°C for 3 min. The samples were then electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gel. Proteins were transferred from the gel to polyvinylidene fluoride membrane. The membrane was soaked with Blocking One (Nacalai Tesque, Kyoto, Japan) for 30 min. Then the membrane was soaked with antibody solutions to detect Alix and heat shock protein (HSP) 70, both of which are exosome marker proteins. To detect Alix, monoclonal mouse anti-Alix antibody (BD Biosciences, San Jose, CA, USA) was used at 1:1000 dilution, followed by anti-mouse IgG1 horseradish peroxidase-conjugated antibody (Life Technologies, Grand Island, NY, USA) at

1:1000 dilution. To detect HSP70, monoclonal mouse anti-HSP70 antibody (Cell Signaling Technology, Danvers, MA, USA) was used at 1:2000 dilution, followed by goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:5000 dilution. To detect CD63, polyclonal rabbit anti-CD63 antibody (Santa Cruz Biotechnology) was used at 1:200 dilution, followed by goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:5000 dilution. To detect calnexin, polyclonal rabbit anti-calnexin antibody (Santa Cruz Biotechnology) was used at 1:200 dilution, followed by goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology) at 1:5000 dilution. The membrane was soaked with Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore, Billerica, MA, USA), and chemiluminescence was observed with a LAS3000 instrument (Fujifilm, Tokyo, Japan).

Transmission electron microscopy (TEM)

Exosomes were fixed in 4% paraformaldehyde and layered on a carbon/Formvar film-coated TEM grid (Okenshoji Co., Ltd., Tokyo, Japan) for 20 min. The grids were treated with 1% glutaraldehyde and washed eight times with dH_2O for 2 min. They were then stained with 1% uranyl acetate for 10 min. Observation was performed with a transmission electron microscope (Hitachi H-7650, Hitachi High-Technologies Corporation, Tokyo, Japan). To measure Feret's diameter of the particles, TEM images were analyzed using ImageJ software (Rasband, W.S., U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2014).

Protein quantification

Estimation of exosome amounts was performed with the Quick Start Bradford protein assay (BioRad, Hercules, CA, USA) according to the manufacturer's instructions.

Tunable resistive pulse sensing (TRPS)

Particle number and particle size of exosomes were measured with a qNano instrument (Izon Science Ltd, Christchurch, New Zealand) with the NP100 nanopore according to the manufacturer's instructions. For the measurements, PBS was used as a buffer. Voltage was set to achieve approximately 100 nA current. Using a variable pressure module, 0.7 kPa of pressure was applied. Collected data were processed by Izon Control Suite software version 3.2. Calibration was performed using data obtained by measuring control reagent (CPC100B; Izon Science) diluted 1:1000 with buffer.

Measurement of zeta potential

Zeta potential of the collected exosomes was determined at 25 °C with a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) and DTS 1070 disposable capillary cell. For measurement, exosomes were diluted with distilled water (1:100).

Confirmation of labeling of exosomes with gLuc-LA

Labeled exosome samples (1×10^9 RLU/s) were electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gel. The gel was washed with 2.5 % Triton X-100 for 30 min twice and PBS for 30 min. Then the gel was soaked with a substrate in a sea pansy luciferase assay system (Picagene Dual; Toyo Ink, Tokyo, Japan). Chemiluminescence was observed with a LAS3000 instrument. To confirm linear relationship between the amount of exosomes and the signal intensity of gLuc, labeled exosomes were serially diluted and gLuc activity was measured. Labeling of exosomes by gLuc-LA had little effect on size and zeta potential of

exosomes [21, 22]. It was also confirmed in a previous study that the label is stable when incubated in PBS containing 20% fetal bovine serum [22].

Evaluation of exosome recovery rate after filtration sterilization

gLuc-LA-labeled exosomes in 200 μL of PBS or PBS containing 2% bovine serum albumin (BSA) (2×10^8 RLU/s/mL) were filtered through a 0.2-μm Minisart RC4 (Sartorius Stedim Biotech GmbH, Goettingen, Germany). Filtered samples were mixed with a sea pansy luciferase assay system (Picagene Dual), and the emitted chemiluminescence was measured with a luminometer (Lumat LB 9507; EG&G Berthhold, Bad Wildbad, Germany). Recovery rate of exosomes was calculated from gLuc activity. For estimation by Western blotting, exosomes in 200 μL of PBS or PBS containing 2% BSA (1 μg protein/mL) were filtered through a 0.2-μm Minisart RC4. Then, 16 μL of each sample before and after filtration was used for the analysis.

Animals

Five-week-old male C57BL6/J mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). All protocols for the animal experiments were approved by the Animal Experimentation Committee of the Graduate School of Pharmaceutical Science of Kyoto University. The animal experiments were carried out in accordance with EC Directive 86/609/EEC.

Analysis of clearance from blood circulation

gLuc-LA-labeled and unlabeled exosomes were mixed to adjust the gLuc activity and protein amount of the exosome samples (5×10^8 RLU/s and 1 μg protein/200 μL; corresponding to the injection of 2.0×10^9, 2.9×10^9, and 3.1×10^9 particles of the sample collected by the pelleting, cushion, and gradient methods, respectively). We confirmed that the clearance of

labeled exosomes was not affected by mixing unlabeled exosomes. In our previous study, the pharmacokinetics of unlabeled exosomes was comparable to that of gLuc-LA-labeled exosomes [21]. Then the exosomes were intravenously injected to C57BL6/J mice via tail vein. Approximately 30 μL of blood was collected at 5, 10, 30, 60, 120, and 240 min after administration. Serum was obtained by centrifuging clotted whole blood at 8,000×g for 20 min at 4°C. The gLuc activity in serum was measured as described above. The amount of exosomes in samples was normalized to the injected dose based on the gLuc activity and expressed as a percent of the injected dose/mL (% ID/mL). The half-life in the early stage ($T_{1/2\alpha}$), area under the curve (AUC), mean residence time (MRT), and clearance (CL) were calculated as described previously [14].

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA). Differences were evaluated by Tukey–Kramer test, and $p < 0.05$ was considered statistically significant.

Results

B16-BL6-derived exosomes were isolated by three different ultracentrifugation-based methods

Samples collected by ultracentrifugation methods or solvent precipitation were observed under TEM to check the morphology of exosomes (Fig. 1A). Many large aggregates and nonspherical lumps with diameter >1 μm were observed in the exosome sample collected by solvent precipitation. Figure 1B shows that these large lumps occupied a large volume, although their number was relatively small. As it was expected that the pharmacokinetics of aggregated exosomes of diameter over 1 μm would be different from that of dispersed

exosomes, the solvent precipitation method was excluded from further experiments. In contrast, vesicles of diameter approximately 100 nm were observed in the samples collected by all the ultracentrifugation methods. Some aggregated vesicles were observed in the samples collected by the pelleting and the cushion methods, although the degree of aggregation was much smaller in these samples than in the solvent precipitation samples. Figure 1C shows the particle size distribution of exosomes evaluated by the TRPS method. The mean diameters of Exo-pellet, Exo-cushion and Exo-gradient were 111, 109, and 85.3 nm, respectively. Exo-pellet and Exo-cushion contained more particles over 100 nm in diameter than Exo-gradient. The zeta potentials of Exo-pellet, Exo-cushion, and Exo-gradient were −35.8, −37.5, and −36.2 mV, respectively.

Figure 2 shows the immunoblotting of Alix, HSP70, CD63, and calnexin. Alix, HSP70, and CD63 are exosome marker proteins. Calnexin is a negative marker. Positive markers were detected in the exosomes collected by the pelleting (Exo-pellet) and the cushion method (Exo-cushion). Fraction 9 collected by the gradient method (Exo-gradient) also contained positive markers. Calnexin was not detected in the three exosome samples.

The numbers of exosomes collected by each method were estimated by measurement of protein amount and particle number (Fig. 3). The protein amount of Exo-cushion and Exo-gradient was lower than that of Exo-pellet, whereas particle numbers were almost identical between the three groups.

Exosomes disappeared rapidly from the blood circulation after intravenous injection

Figure 4A shows the image of gLuc zymography of labeled exosomes. All samples displayed a band at the expected molecular mass of gLuc-LA (55 kDa). This result suggests that exosomes in all samples were labeled with gLuc-LA irrespective of the collection method. gLuc activity of labeled exosome was linearly related to the protein amount of exosomes

(Figure 4B). Figure 4C shows the time course of serum concentrations of gLuc-LA labeled exosomes after intravenous injection. gLuc activity disappeared from the blood circulation immediately, irrespective of the exosome collection method. Table I shows the pharmacokinetic parameters of injected exosomes. These parameters were not markedly different among the three methods.

Recovery rate of Exo-gradient after filtration was higher than those of the other methods

In practical use of exosomes, they may require sterilization before use. We accordingly investigated recovery rates of exosomes after sterile filtration, using gLuc-LA labeled exosomes suspended in PBS. After sterile filtration, approximately 9%, 7%, and 25% of gLuc activity were recovered for Exo-pellet, Exo-cushion, and Exo-gradient, respectively (Fig. 5A). When exosomes suspended in PBS containing 2% BSA were filtered, approximately 48%, 41%, and 82% of gLuc activity were recovered for Exo-pellet, Exo-cushion, and Exo-gradient, respectively (Fig. 5B). The recovery rate estimated from Western blotting was similar to that estimated from gLuc activity (Fig. 5C and D).

Discussion

Although several isolation methods for exosomes have been developed, the effects of isolation method on exosome characteristics as drug formulations have not been investigated. In this study, we demonstrated that the exosome isolation method affects the dispersibility of exosomes and their recovery rate after sterile filtration, but that the aggregation caused by the pelleting and cushion methods has little effect on the clearance of exosomes from the blood circulation after intravenous injection in mice.

Solvent precipitation offers the advantages of simplicity and no requirement of ultracentrifugation. However, we found that the sample collected by solvent precipitation using the ExoQuick reagent contained many large aggregates, a result in agreement with previous reports [19, 23]. These aggregates greater than 1 μm were much larger than those in exosomes collected by the centrifugation methods. In addition, as shown in figure 1B, the volume of nonaggregated exosomes was much lower than that collected by the other methods. As such large and frequent aggregations are not desirable in use of exosomes as a delivery carrier, the solvent precipitation method seems unsuitable for isolation of exosomes as a delivery carrier.

Protein amount of the sample collected by the cushion method and gradient method was lower than that of the sample collected by the pelleting method. However, there was little difference in particle number between the pelleting and cushion methods. This disparity between protein amount and particle number may result from contamination with free proteins in the Exo-pellet samples. As shown by Lamparski et al., aggregated protein may be co-collected by the pelleting method, as the method cannot separate exosomes and high-density protein aggregates [24]. Although protein quantification is the method most frequently used to estimate the number of exosomes, it may overestimate this number by detecting contaminating proteins that are not associated with exosomes [25]. Our analyses of Western blotting showed that amounts of exosome marker proteins for a given amount of protein were different between the isolation methods. Although this difference may result from a difference in protein composition of exosomes, this result also suggests different degrees of contamination of aggregated protein for exosome samples collected by different methods. Thus, particle number seems to be appropriate for the evaluation of exosome yield.

Size distribution as evaluated by tunable resistive pulse sensing and TEM images suggested that the pelleting and cushion methods cause aggregation of exosomes. Tauro et al.

and Jeppesen et al. showed that the pelleting method causes aggregation of exosomes [15, 18]. Our finding is in good agreement with these previous studies. These results, including ours, suggest that exosomes aggregate by pelleting because of the pressure against the ultracentrifuge tube and localized high concentration of exosomes. Exo-gradient resulted in more dispersibility than the other methods as the pressure and localized high concentration of exosomes may not occur with this method. The results of this study suggest that the high dispersibility of Exo-gradient results in high recovery after sterile filtration. Thus, the gradient method seems to be suitable for applications of exosomes to drug delivery.

To evaluate clearance from blood circulation and recovery rate after sterile filtration, we used gLuc-LA labeled exosomes. The results shown in Figure 4A and 4B suggest that gLuc-LA can be used to label exosomes irrespective of the isolation method. Calculated gLuc signal per particle was 0.98, 0.73, and 0.72 RLU/s gLuc activity per particle in the samples collected by the pelleting, cushion, and gradient methods, respectively. Our previous study using a radioactive probe [20] showed that a single exosome was labeled with approximately 43 molecules of streptavidin–lactadherin fusion protein when the plasmid encoding streptavidin–lactadherin was introduced to exosome-producing cells. As both gLuc-LA and streptavidin–lactadherin bind to exosome by the interaction of lactadherin with exosomes, it is expected that the labeling efficiency of gLuc-LA is similar to that of streptavidin–lactadheirn. This assumption strongly suggests that almost all of the exosomes were labeled with gLuc-LA, although detailed investigation is needed in future.

The clearance rates of the exosomes from the blood circulation were comparable with those observed in our previous study [21]. We previously showed that intravenously injected exosomes accumulate in the liver, spleen, and lung [20]. We also reported that uptake of exosomes by macrophages is an important factor in rapid disappearance from the blood circulation [22]. Given that exosomes have phosphatidylserine and many membrane proteins

that could be recognized by the reticuloendothelial system [26, 27], clearance of exosomes from the blood circulation may be dependent on these molecules rather than the dispersibility of exosomes relative to that of exosomes collected by ultracentrifugation methods.

The aggregation of exosomes may be the reason for the low recovery rate of exosomes after sterile filtration. gLuc activity measurement and Western blotting for Alix similarly indicated that the recovery rate of Exo-gradient was higher than that of the other samples. This finding is reasonable, based on the consideration that more large than small particles are trapped by filters. The recovery rates were lower than 30% when exosomes were suspended in PBS. The recovery rate may be influenced by factors such as the pore size and materials of the filter, similar to the case for the filtration of BSA and polysaccharides [28, 29]. This low recovery rate after sterile filtration is in accord with a previous study [24], in which at least 80% exosomes collected by differential centrifugation were trapped by filtration. Given that the recovery rate was improved by addition of 2% BSA, BSA may inhibit nonspecific adsorption of exosomes to the cellulose filter. Given that cellulose is an uncharged material that hardly adsorbs electronegative viruses [30], most of the adsorption was probably caused by hydrophobic and not electrostatic interaction. However, BSA did not change the tendency of Exo-gradient to pass through the filter more efficiently than the other exosomes. This result supports the speculation that trapping of aggregated exosomes by the filter decreased the recovery rate after sterile filtration. Approximately, 50% of Exo-pellet and Exo-cushion and 20% of Exo-gradient were trapped on the filter even in the presence of BSA, although these samples contained few aggregates over 200 nm and thus larger than the pore size of the filter. The difference in particle counting between size measurement and sterile filtration experiments may account for this discrepancy. In the size distribution measurement, an exosome and an aggregate of exosomes were counted as one particle. In contrast, gLuc activity of an aggregate is much higher than that of dispersed exosomes. This

result also implies that exosomes whose diameter was under 200 nm were also trapped by the filter. Liposomes smaller than the pore size of the ultrafiltration membrane caused pore constriction or blockage and decreased flux [31]. A similar event may occur during sterile filtration of exosomes. In particular, aggregated exosomes are more likely to cause blockage, given that most aggregates are not sphere shaped, and the greatest diameter of the aggregates may exceed the measured value.

Conclusion

In the present study, we showed that isolation methods of exosomes affect the dispersibility of exosomes and the recovery rate of exosomes after sterile filtration, whereas they cause little difference in the clearance from the blood circulation. These results provide useful information for the development of exosome-based therapeutics.

Acknowledgment

References

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Figure legends

Figure 1. (A) Transmission electron microscopy (TEM) images of collected exosomes. TEM images of samples collected by pelleting, cushion, and gradient methods and by solvent precipitation. Scale bar in the image of the sample from the precipitation method represents 500 nm, and scale bars in the other images represent 100 nm. (B) Histograms of particle size

distribution obtained by analyzing the TEM images. Vertical axis of larger histograms shows % population by number (by count) and that of the smaller one shows % population by occupied area (by area). (C) Histograms of particle size distribution determined by TRPS.

Figure 2. Confirmation of marker proteins in exosome preparations. Western blotting of samples by anti-Alix, anti-HSP70, anti-CD63, and anti-calnexin antibody.

Figure 3. Yield of exosomes by each method. Yield of exosomes was estimated by (A) protein amount quantification and (B) particle number determined by tunable resistive pulse sensing. These results are expressed as means ± standard deviations (SDs) (n = 4). *, $p < 0.05$

Figure 4. (A, B) Confirmation of gLuc-LA labeling of each sample. (A) gLuc zymography and (B) dilution curve of gLuc-LA labeled exosomes collected by the three ultracentrifugation-based methods. (C) Time course of serum concentrations in each sample in mice after intravenous injection of Exo-pellet (circles), Exo-cushion (squares), and Exo-gradient (triangles). These results are expressed as means ± standard deviations (SDs) (n = 3).

Figure 5. Recovery rate after sterile filtration. Recovery rate of Exo-pellet (closed column), Exo-cushion (gray column), and Exo-gradient (white column) suspended in (A) phosphate-buffered saline (PBS) or (B) PBS containing 2% BSA after sterile filtration by 0.2-μm syringe filter estimated by measuring gLuc activity. These results are expressed as means ± standard deviations (SDs) (n = 3). *, $p < 0.05$. (C) and (D) Western blotting of samples diluted in (C) PBS or (D) PBS containing 2% BSA before and after filtration.
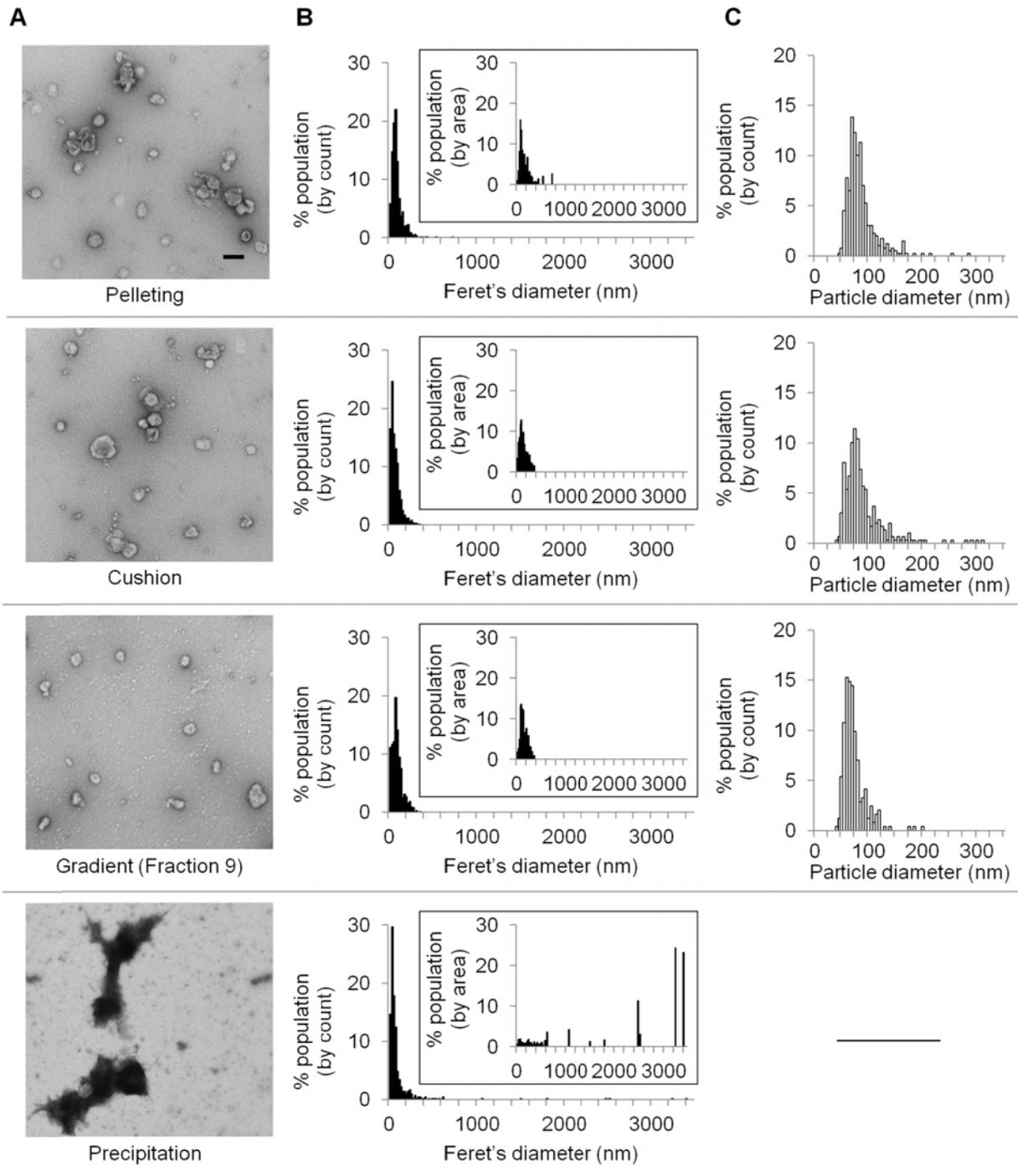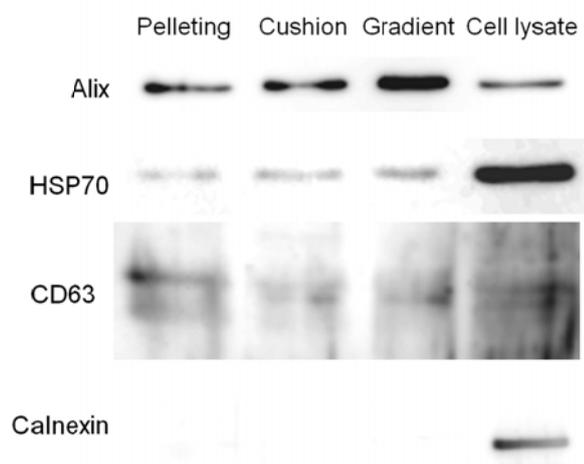
Figure 1

Figure 2

Figure 3

Figure 4

A

	Pelleting	Cushion	Gradient
75 kDa			
63 kDa			
48 kDa			
35 kDa			
25 kDa			
17 kDa			

B

RLU/s/µL vs Exosome amount (ng protein/µL)

- ● Pelleting
- ■ Cushion
- △ Gradient

C

%ID/mL vs Time (min)

- ● Pelleting
- ■ Cushion
- △ Gradient

Figure 5

A

Recovery rate (%)

Pelleting   Cushion   Gradient

B

Recovery rate (%)

Pelleting   Cushion   Gradient

C

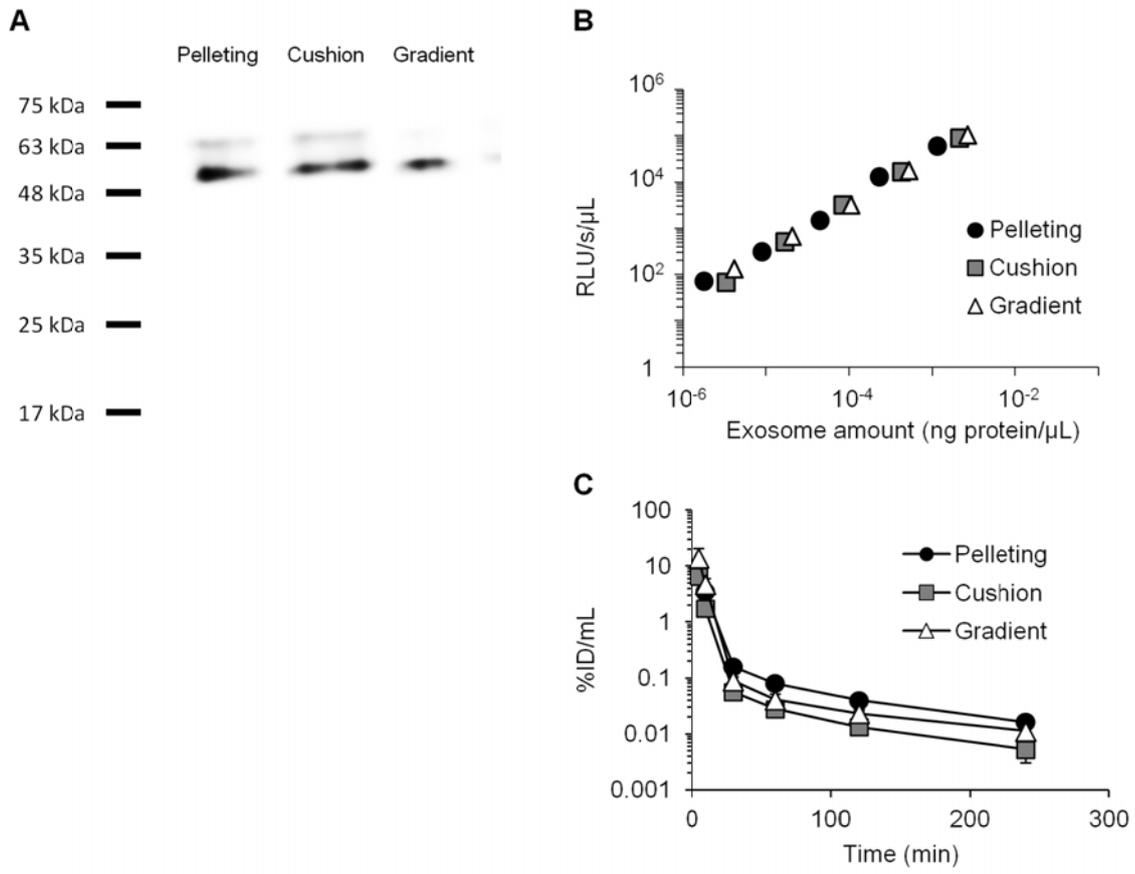|	Pelleting		Cushion		Gradient	
Filtration	-	+	-	+	-	+
Relative intensity	1.0	0.12	3.4	0.30	3.9	1.5
Recovery rate	12%		9.0%		39%	

D

	Pelleting		Cushion		Gradient	
Filtration	-	+	-	+	-	+
Relative intensity	1.0	0.52	1.4	0.85	2.9	2.5
Recovery rate	52%		60%		88%	