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Research Article

Exosome enrichment of human serum using multiple cycles of centrifugation

In this work, we compared the use of repeated cycles of centrifugation at conventional speeds for enrichment of exosomes from human serum compared to the use of ultracentrifugation (UC). After removal of cells and cell debris, a speed of 110 000 × *g* or 40 000 × *g* was used for the UC or centrifugation enrichment process, respectively. The enriched exosomes were analyzed using the bicinchoninic acid assay, 1D gel separation, transmission electron microscopy, Western blotting, and high-resolution LC-MS/MS analysis. It was found that a five-cycle repetition of UC or centrifugation is necessary for successful removal of nonexosomal proteins in the enrichment of exosomes from human serum. More significantly, 5× centrifugation enrichment was found to provide similar or better performance than 5× UC enrichment in terms of enriched exosome protein amount, Western blot band intensity for detection of CD-63, and numbers of identified exosome-related proteins and cluster of differentiation (CD) proteins. A total of 478 proteins were identified in the LC-MS/MS analyses of exosome proteins obtained from 5× UCs and 5× centrifugations including many important CD membrane proteins. The presence of previously reported exosome-related proteins including key exosome protein markers demonstrates the utility of this method for analysis of proteins in human serum.

Keywords:
Centrifugation / Exosomes / Human serum / Mass spectrometry / Ultracentrifugation
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1 Introduction

Exosomes are small endosomal derived membrane microvesicles (~30–100 nm in diameter) secreted by most cell types. Exosomes are found in many biological fluids, such as blood [1, 2], urine [3–7], saliva [8, 9], and breast milk [10]. Exosomes have received much attention recently since exosomes are believed to have important roles in intercellular communications [11]. There are several recent review papers in the literature providing an overview of the current status of exosome research [12–16], among which Simpson and his co-workers [12] provided proteomic insights and diagnostic potentials of exosomes and reported 24 proteins commonly identified in most exosome studies.

The most common exosome enrichment method involves using ultracentrifugation (UC) at high speeds such as 110 000 × *g*. Prior to the UC, whole cells and large cell debris are removed by low-speed centrifugations or by filtration using a 0.22 μm filter. UC is performed one [17, 18], two [19–22], or three times [23], where the supernatant is removed followed by addition of a buffer solution after each UC. After the initial UC, density-gradient UC using sucrose [22] or iodixanol [2, 23] is often applied to improve the purity of exosomes. UC on a sucrose cushion has also been performed to isolate exosomes [24, 25].

There are several commercially available kits used to enrich exosomes such as the Total Exosome Isolation kit (Life Technologies) [26], ExoQuick (System Bioscience) [10, 21, 27], Exo-spin (Cell Guidance System) [28], and PureEXO (101Bio). An immunoaffinity pull-down method can also be performed where an exosome-specific antibody is used to selectively enrich exosomes [7]. Recently, two types of antibodies on photosensitizer beads were utilized to perform a rapid and sensitive

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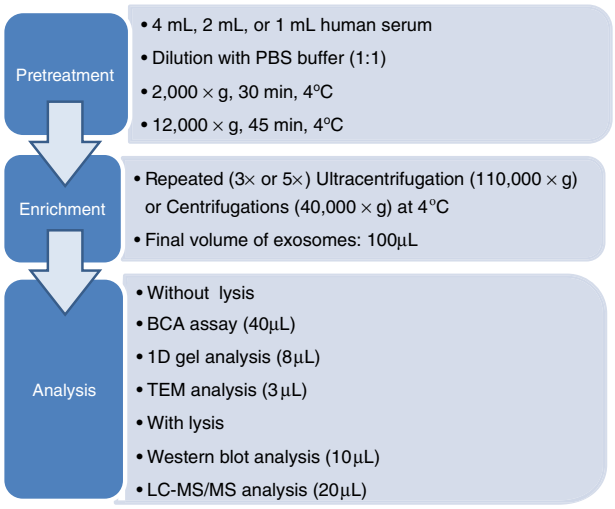
Abbreviations: **BCA**, bicinchoninic acid; **CD**, cluster of differentiation; **DG**, density gradient; **EI**, EpCAM-based immunoaffinity pull-down; **FA**, formic acid; **FASP**, filter-aided sample preparation; **IPA**, Ingenuity Pathway Analysis; **MW**, molecular weight; **PBST**, PBS with Tween-20; **TEM**, transmission electron microscopy; **UC**, ultracentrifugation

Colour Online: See the article online to view Scheme 1 and Fig. 2 in colour.



Scheme 1. Summary of the current investigation.

detection of extracellular vesicles including exosomes [29]. A filtration device has also been used to enrich exosomes where ultrafiltration devices with 10 000 Da molecular weight (MW) cut-off membranes were used [30]. Field-free fractionation [31] or size-exclusion chromatography where particles are separated based on their size has also been applied to enrich exosomes.

A combination of two or more enrichment methods has often been used to isolate exosomes with varying degrees of success. These may include UC with ExoQuick precipitation [10], size exclusion chromatography with immunoaffinity [32], filtration using a 100 000 MW cut-off filter with UC [33, 34], filtration using a 100 000 MW cut-off filter with the application of a commercial enrichment kit [35], filtration using 100 000 MW cut-off filter with immunoaffinity and UC [18], filtration using 100 000 MW cut-off filter with sucrose density-gradient UC, or UC followed by a commercial kit [36]. However, exosome enrichment from human serum using UC or a commercial kit often suffers from impurities [24, 25], due to the presence of high abundant proteins such as albumin and immunoglobulin G in human serum. Although density-gradient (DG) UC is often used to improve the purity, it is a relatively long (~18 h) process [2, 10].

Recently, three different isolation methods (DG, UC, and EpCAM-based immunoaffinity pull-down (EI)) were compared for isolating exosomes from normal human plasma, where a total of 213 exosome proteins were identified [2]. The authors mentioned that DG was superior in isolating pure exosomes since it successfully removed highly abundant plasma proteins compared to the other two techniques. The LC-MS/MS analyses revealed 148 (69.5%), 78 (36.6%), and 39 (18.3%) exosome proteins from DG, UC, and EI, respectively.

In the current study, we have explored the application of centrifugation at a speed of 40 000 × *g*, which is much more affordable and accessible to most scientists than that of UC and whether it can enrich exosomes from a human serum sample comparable to that obtained by UC. Centrifugation at 40 000 × *g* was applied three and five times to study the effect of multiple cycles of centrifugation. The results from centrifugation were compared with those from conventional UC based on several methods including 1D gel analysis, transmission electron microscopy (TEM), Western blotting, and LC-MS/MS analysis on a high-resolution Orbitrap mass spectrometer (see Scheme 1). It was found that centrifugation at 40 000 × *g* could provide comparable or improved results relative to UC by using multiple cycles of centrifugation. The current results show that the exosome enrichment can be successfully achieved in a rather inexpensive centrifuge instrument.

2 Materials and methods

2.1 Materials

Pooled normal human serum samples were obtained from Innovative Research (Novi, MI, USA). Anti-CD63 antibody (ab59479, Mouse monoclonal to CD63), goat anti-mouse IgG H&L (horseradish peroxidase) preadsorbed (ab97040) were from Abcam (Cambridge, MA). PBS (P-5368) was obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Exosome enrichment—pretreatment of serum

The serum sample was first diluted with an equal volume of PBS buffer solution to decrease viscosity. The diluted serum sample was then centrifuged at 2000 × *g* for 30 min at 4°C. The supernatant was transferred into 1 mL tubes and centrifuged at 12 000 × *g* for 45 min at 4°C. The supernatant was filtered through a 0.22 μm filter to remove any remaining cell particles or cell debris.

2.3 Exosome enrichment—UC

UC was performed using a Beckman Optima XL-70 Ultracentrifuge with a speed of 110 000 × *g* at 4°C for 120 min (the first UC step) or 70 min (the subsequent UC steps). Ultra-Clear™ tubes (catalog number: 344057, from Beckman Coulter) were used with an SW 55 Ti rotor in the process of UC. The total volume of each tube was limited to 4.0 mL to avoid any overflowing or contamination during sample preparation steps.

Five consecutive UC steps were performed to improve the purity of exosomes obtained. For the exosomes obtained starting from 4.0 mL serum (corresponding to 8.0 mL of 2× diluted serum), the two pellets were combined after the first UC. For the exosomes obtained starting from 1.0 mL serum (2.0 mL of 2× diluted serum), 2.0 mL PBS buffer was added to the tube containing 2.0 mL of 2× diluted serum prior to the first UC step. After each UC step, supernatant was removed, followed by addition of 4 mL PBS buffer. After the fifth UC

step, the pellet was resuspended in 100 μL PBS buffer after the supernatant was removed.

2.4 Exosome enrichment—centrifugation

Centrifugation to enrich exosomes was performed using a Sorvall Stratos Centrifuge from Thermo at a speed of 40 000 × *g* (20 762 rpm) at 4°C for 120 min (first run) or 70 min (for subsequent runs) with a microcentrifuge tube (Axygen MCT-175-L-C) from Axygen.

For exosomes prepared from 1.0 mL serum (2.0 mL of 2× diluted serum), two tubes were used where each tube contains 1.0 mL of 2× diluted serum. For exosomes prepared from 2.0 mL serum, three tubes were used where each tube contains 1.33 mL of 2× diluted serum. For exosomes prepared from 4.0 mL serum, six tubes were used where each tube contains 1.33 mL of 2× diluted serum. After the first UC, the pellets were combined. After each centrifugation, 1.2 mL PBS buffer was added to the pellet after removing the supernatant. After the fifth centrifugation step, the pellet was resuspended in 100 μL PBS buffer.

2.5 Quantitation and gel analysis

The total protein concentration in the supernatants and in the final pellet was determined using the bicinchoninic acid (BCA) assay (Pierce Biotechnology, Pittsburgh, PA, USA), where two of 20 μL for each sample were used. For the 1D gel analysis, sample (8 μL) was mixed with a lane marker nonreducing sample buffer (5×) from Thermo Scientific Pierce (catalog number: PI-39001), followed by incubation at 70°C for 10 min. Electrophoresis was then performed on a mini-protean TGX precast gel (Bio-Rad) at 90 V for 10 min, followed by 200 V for 25 min. The gel was stained with the Sigma silver staining kit following the manufacturer's instruction.

2.6 TEM analysis

Carbon film (CFTH200-Cu) was obtained from Electron Microscopy Sciences (Hatfield, PA, USA). Glow discharge on the carbon film was performed to make the surface of the carbon film hydrophilic. The sample (3 μL) was then loaded on the carbon film and incubated for 2 min. After removing the supernatant liquid by absorbing it using filter paper, 5 μL of 2.5% w/v glutaldehyde in PBS was loaded for the fixation of the exosomes. After 5-min incubation, the supernatant liquid was removed and the carbon film was washed with water three times. After removing the last water, the film was stained with 5 μL of 1% uranyl acetate for 1 min. The TEM image was obtained using a CM-100 TEM instrument from Philips.

2.7 Lysis and Western blotting

The lysis of the enriched exosomes involved incubation of the exosomes at 4°C for 30 min in a 1:1 ratio with a 2× RIPA buffer. The 2× RIPA buffer solution was composed of 100 mM Tris-HCl, 300 mM NaCl, 2.0% NP-40 (US Biological), 1.0% sodium deoxychlorate, 0.2% SDS, 1 mM EDTA, and protease inhibitors (cOmplete, EDTA-free Protease Inhibitor Cocktail Tablets, Roche).

For Western blot analysis, the lysed exosome proteins (20 μL each) were separated on a gel as described above and transferred onto a PVDF membrane (catalog number: 162–0177, Bio-Rad). The membrane was then first incubated in PBS blocking buffer containing 5% milk for 1 h at room temperature and then with primary mouse anti-CD63 (catalog number: ab59479, Abcam) diluted in a 1:500 ratio in PBST (0.1% Tween 20 in PBS buffer solution) overnight at 4°C. The membrane was then washed three times with PBST and incubated with secondary goat anti-mouse IgG H&L (horseradish peroxidase) preadsorbed (ab97040, Abcam) in PBST (1:1000 dilution) and visualized by incubating sections with 3,3-diaminobenzidine tetrahydrochloride (ImmPACT DAB peroxidase substrate; Vector Laboratories, Burlingame, CA, USA)

2.8 Tryptic digestion

Following the lysis of the exosome samples, the filter-aided sample preparation (FASP) method was used to perform tryptic digestion. The lysed sample was reduced with 100 mM DTT for 10 min at 70°C. The solution was allowed to cool down and then was mixed with 200 μL of 8 M urea in 100 mM Tris-HCl (pH 8.5), transferred to a centrifugal spin filter with a MW cutoff of 30 kDa (YM-30, Millipore), and centrifuged for 15 min at 14 000 × *g*. The same centrifugation conditions were used for the following steps: The sample was washed again with 200 μL of the urea buffer. Alkylation was performed by adding 100 μL of 50 mM iodoacetamide in the urea buffer, followed by vortexing for 1 min and incubation for 20 min in the dark at room temperature. To remove the remaining iodoacetamide, the protein mixture was centrifuged and washed twice with the urea buffer. The sample was washed three times with 100 μL of 50 mM ammonium bicarbonate. Then, tryptic digestion was performed overnight at 37°C by adding trypsin (Sequencing grade modified, Promega) in a 1:20 ratio w/w. Digested peptides were collected by centrifugation with 40 μL of 50 mM ammonium bicarbonate three times. After tryptic digestion, the samples were desalted using Thermo Scientific Pierce C18 Spin Columns before LC-MS/MS analysis.

2.9 LC-MS/MS analysis

The samples were analyzed in duplicate. For each LC-MS/MS analysis, ~0.5 μg exosome proteins were used. Peptide

mixtures dissolved in 0.5% formic acid (FA) were loaded onto a Proxeon Easy-nLC II system (Thermo) with a flow rate of 400 nL/min. The samples were first desalted on an RP trap column (100 μm × 20 mm, C18AQ particles, 5 μm, 200 Å, Michrom Bioresources, Auburn, CA, USA) and separated on a C18 analytical column (75 μm × 250 mm, C18AQ particles, 5 μm, 200 Å) coupled to an Orbitrap Elite mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). Peptides were separated with 0.1% FA in water (solvent A) and 0.1% FA in ACN (solvent B) using a 70-min linear gradient from 5 to 35% solvent B at a flow rate of 400 nL/min.

The mass spectrometer was operated in positive ion mode with an electrospray voltage of +2.5 kV and a capillary temperature of 300°C. Full scan mass spectra were acquired from m/z 400.0–1800.0 in the Orbitrap analyzer with a resolution $R = 120\,000$, followed by HCD MS/MS scans with resolution $R = 15\,000$ on the top 15 most intense ions. The isolation width was set to 1.5 and the normalized collision energy was 35.0%. Dynamic exclusion was enabled with a ±10 ppm exclusion window with a repeat count of 1 using an exclusion duration of 30 s.

All MS/MS spectra were searched against the human UniProt database (downloaded June, 2014) containing 26 152 entries using SEQUEST (Proteome Discoverer 1.4, Thermo Fisher Scientific). The search parameters were as follows: (1) static carbamidomethylation of cysteine residues (+57.021 on Cys); (2) dynamic oxidation of methionine residues (+15.995 on Met); (3) allowing two missed cleavages; (4) peptide ion mass tolerance 10 ppm (Isotopic MW); (5) fragment ion mass tolerance 0.6 Da (Isotopic MW). Identified peptides were filtered using a 1% false discovery rate.

2.10 Ingenuity Pathway Analysis (IPA)

IPA (Ingenuity Systems) was performed to obtain the detailed molecular information. The identified protein lists were uploaded into the IPA tool and analyzed. The result files contained gene symbols, descriptions, locations, and types of the proteins. The location has four different categories, such as extracellular, cytoplasm, plasma membrane, nucleus, and other.

2.11 CD antigen list and comparison

The common cluster of differentiation (CD) antigen list was obtained from the cdlist on UniProt (http://www.uniprot.org/docs/cdlist) released on July 9, 2014. The CD antigen list from CD1 through CD363 was used for comparison. A total of 445 entries from the common CD antigen list were used for comparison where some CD antigens have more than one entry; for example CD235a and CD235b. The Swiss-Prot entry names from the common CD antigen list and from the currently identified protein list were compared to obtain the CD antigen name for each identified protein in the currently identified protein list.

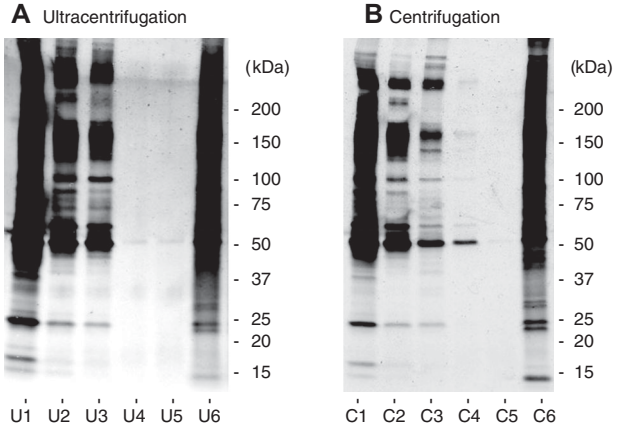


Figure 1. 1D gel images for the samples from (A) ultracentrifugation and (B) centrifugation processes of 2.0 mL human serum. The samples from 1 through 5 (U1–U5 and C1–C5) are the supernatants from the corresponding enrichment processes. The samples of U6 and C6 are from the enriched exosomes. The samples of 1 and 2 were diluted 500-fold and 20-fold with a PBS buffer solution prior to loading to reduce their concentrations and provide weaker bands.

3 Results

3.1 Enrichment of exosomes

Currently, the most common enrichment method of exosomes is using UC with a speed of 110 000 × *g*. In this study, we have explored whether a reduced speed (e.g. 40 000 × *g*) would provide similar performance for the enrichment of exosomes. Figure 1 shows the 1D gel images of the samples obtained from the UC and centrifugation procedures for the enrichment of exosomes from 2.0 mL human serum. Samples 1 through 5 are from the supernatants from the first through the fifth enrichment steps while sample 6 is from the enriched exosome pellet. The enriched exosome proteins were visualized using silver-staining. As shown in Fig. 1, the protein separation patterns for the corresponding samples of supernatants and enriched exosomes between UC and centrifugation were very similar, showing that these two enrichment methods provided similar efficiencies for exosome enrichment.

The concentrations of the first, second, and third supernatants for both UC and centrifugation were ~50, ~1, and ~0.01 mg/mL, respectively, based on the BCA assay. In the fourth and fifth supernatant samples, no protein was detected using BCA assay. Most proteins are believed to be eliminated after four UC steps or centrifugation steps. Few bands were still visualized on gel using sliver-staining (Fig. 1), which illustrates 3× UC enrichment or 3× centrifugation enrichment is not sufficient to remove nonexosomal proteins.

3.2 Exosome protein yield

The amount of exosome proteins obtained from 1, 2, or 4 mL human serum was around 2.2, 14.3, or 28.5 μg, respectively,

Table 1. Quantities of exosomes obtained from 2 mL serum using 5× ultracentrifugations and 5× centrifugations

Sample number	Ultracentrifugation (μg)	Centrifugation (μg)
1	3.4	4.3
2	4.0	5.6
3	7.5	7.8
4	10.9	9.7
5	11.3	11.5
6	12.7	14.7
Average ± SD	8.3 ± 4.0	8.9 ± 3.9

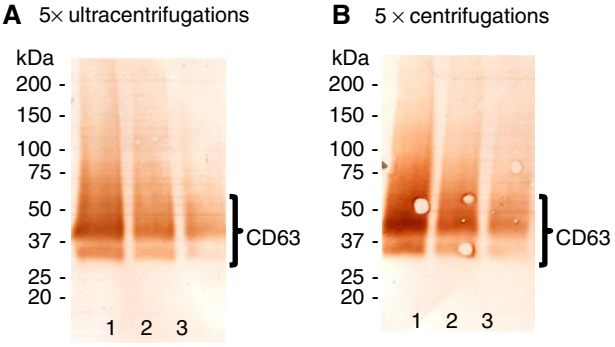


Figure 2. Western blot analyses detecting CD63 in exosomes purified from human serum using (A) the ultracentrifugation enrichment for five times and (B) the centrifugation enrichment for five times. The columns of "1," "2," and "3" for each image are from the exosome proteins obtained from 4, 2, and 1 mL human serum, respectively.

from the 3× UC enrichment and 2.1, 8.3, or 20.7 μg from the 5× UC enrichment while 3.8, 8.6, or 21.1 μg from the 3× centrifugation enrichment, and 2.9, 8.5, or 16.3 μg from the 5× centrifugation enrichment was obtained using the BCA assay. Around 20% less exosome proteins were observed from the five-cycle enrichment process compared to the corresponding three-cycle enrichment process. Based on the amount of exosome proteins obtained from 2 mL serum where both enrichment methods provided a similar yield of ~8.5 μg proteins (Table 1), the current yield is around 0.005%, assuming the protein concentration in human plasma is 60 ~ 80 mg/mL. The yields from all the enrichment conditions in this study were within the reasonable range between 0.001 and 0.01% [1, 37].

3.3 Western blot analysis

Figure 2 shows the Western blot analyses detecting CD63 in the exosome proteins isolated from human serum using UC or centrifugation, where the intensities of the bands become weaker as the starting amount decreases. Similar intensities in Western blot analysis were observed from the UC and centrifugation enrichment procedures for the same starting amounts of exosome proteins, confirming the similar performances between UC and centrifugation. The broad band ~50 kDa is characteristic of CD63 [19, 26]. In the current investigation, CD63 was only detected when the exosome proteins were not reduced. In additional Western blot analyses, CD9 and CD81 were also detected from reduced exosomes proteins and nonreduced proteins, respectively (data not shown).

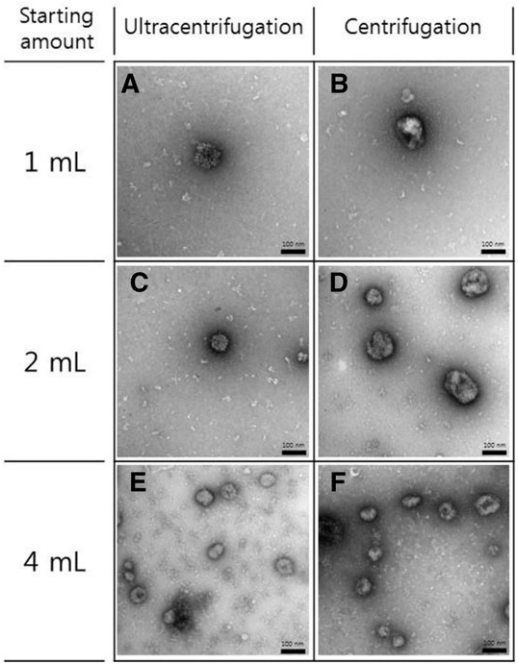


Figure 3. TEM images of exosome samples enriched from human serum using 5× ultracentrifugations and 5× centrifugations. The first, second, and third rows show the images for the exosomes enriched from starting amounts of 1, 2, and 4 mL, respectively. Scale bars: 100 nm.

3.4 Size distribution of exosomes

Figure 3 shows the TEM images of the enriched exosome samples from different starting amounts using either 5× UCs or 5× centrifugations. Exosomes were observed in a size range of around 100 nm. The number of exosomes increased with all experimental conditions as the amount of starting serum increased.

Figure 4 shows the histograms of size distribution of exosomes enriched from human serum using 5× UCs and 5× centrifugations. The size distributions from the 5× UCs and 5× centrifugations were found to be very similar where the average diameters of the purified exosomes were 72 (±21) nm and 73 (±20) nm, respectively.

3.5 Application of FASP

The FASP method has recently been published for the successful digestion of the sample containing SDS, where SDS is exchanged to urea on a standard filtration device [38, 39]. In the digestion of exosome proteins, in-gel digestion is frequently used since in-gel digestion can circumvent problems associated with SDS [30, 40].

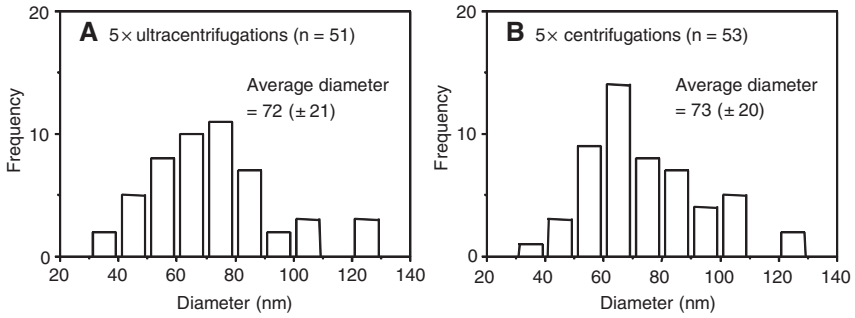


Figure 4. Histograms showing the diameter distribution of exosomes enriched from 4 mL human serum using (A) 5× ultracentrifugation enrichment and (B) 5× centrifugation enrichment. The total number of exosome particles used for each histogram is shown as "n".

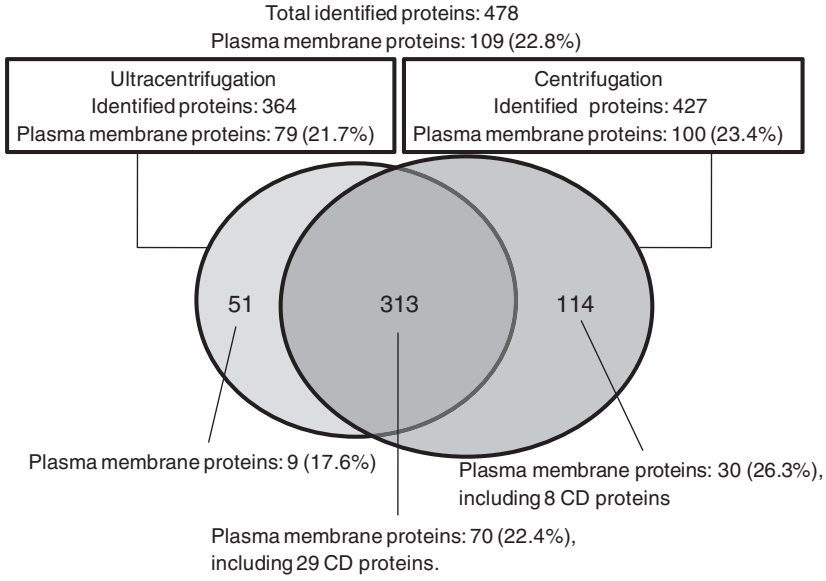


Figure 5. Venn diagram showing the overlap of exosome proteins enriched from 5× ultracentrifugations and 5× centrifugations. A total of 37 CD proteins were identified.

In the current investigation, protease inhibitors were added during the lysis step to avoid any protease activity during lysis. The protease inhibitors in the sample also inhibit trypsin activity. With the application of the FASP method, we successfully digested the exosome proteins, while without the use of the FASP method, the digestion was not successful. The successful digestion using the FASP method appears to be due to the protease inhibitors along with detergents such as SDS and NP-40 being removed during the filtration processes.

3.6 Proteins identified from LC-MS/MS analysis

A total of 478 proteins were identified from the LC-MS/MS analyses of exosome proteins enriched from 4 mL serum using 5× UCs and 5× centrifugations. Figure 5 shows the Venn diagram showing the overlap of exosome proteins enriched using 5× UCs or 5× centrifugations, where 313 proteins (65.5%) were commonly observed in both enrichment processes. The complete list of the 478 proteins can be found in Supporting Information Table 1. The total number of plasma membrane proteins is 109, where 9 plasma membrane proteins were only observed from the exosomes enriched using 5× UCs and 30 plasma membrane proteins were only observed from the exosomes enriched using 5× centrifugations.

Among the 478 proteins, 196 proteins (41.0%) were identified with a single unique peptide of each protein. The high mass accuracy of an Orbitrap Elite mass spectrometer (peptide ion mass tolerance < 10 ppm) and high peptide confidence level (false discovery rate $< 1\%$) are believed to be sufficient to provide a confident peptide list and corresponding protein list even with a single unique peptide for the identification of proteins. The proteins identified with single unique peptides are assumed to be low-abundance proteins [41].

CD antigens are cell surface molecules recognized by specific monoclonal antibodies [42]. CD antigens are defined when surface molecules on human cells interact with at least one new monoclonal antibody [43]. CD antigens perform a variety of roles in immune reactions of organisms [44]. A total of 37 CD proteins were identified from 5× centrifugations, while 29 CD proteins were identified from 5× UCs (Table 2). Additional identification of CD proteins from the centrifugation purification process showed that the centrifugation is more efficient in isolating exosomes than the UC. The detailed information of the 37 CD proteins and their related peptides are shown in Table 2 and Supporting Information Table 2, respectively. The MS/MS spectra of the identified peptides from the 37 CD proteins are included in Supporting Information Fig. 1.

Table 2. List of 37 CD proteins identified in the current investigation

Number	CD number	Swiss-Prot name	Accession number	Gene name	Description	*n*[a)]	U[b)]	C[c)]
1	CD9	CD9_HUMAN	P21926	CD9	CD9 antigen	5	V	V
2	CD10	NEP_HUMAN	P08473	MME	Neprilysin	1	-[d)]	V
3	CD11b	ITAM_HUMAN	P11215	ITGAM	Integrin alpha-M	1	-[d)]	V
4	CD13	AMPN_HUMAN	P15144	ANPEP	Aminopeptidase N	7	V	V
5	CD18	ITB2_HUMAN	P05107	ITGB2	Integrin beta-2	4	-[d)]	V
6	CD29	ITB1_HUMAN	P05556	ITGB1	Integrin beta-1	12	V	V
7	CD31	PECA1_HUMAN	P16284	PECAM1	Platelet endothelial cell adhesion molecule	4	V	V
8	CD36	CD36_HUMAN	P16671	CD36	Platelet glycoprotein 4	6	V	V
9	CD41	ITA2B_HUMAN	P08514	ITGA2B	Integrin alpha-IIb	26	V	V
10	CD42a	GPIX_HUMAN	P14770	GP9	Platelet glycoprotein IX	4	V	V
11	CD42b	GP1BA_HUMAN	P07359	GP1BA	Platelet glycoprotein Ib alpha chain	2	V	V
12	CD42c	GP1BB_HUMAN	P13224	GP1BB	Platelet glycoprotein Ib beta chain	4	V	V
13	CD43	LEUK_HUMAN	P16150	SPN	Leukosialin	1	V	V
14	CD45	PTPRC_HUMAN	P08575	PTPRC	Receptor-type tyrosine-protein phosphatase C	3	V	V
15	CD47	CD47_HUMAN	Q08722	CD47	Leukocyte surface antigen CD47	3	V	V
16	CD49b	ITA2_HUMAN	P17301	ITGA2	Integrin alpha-2	4	-[d)]	V
17	CD49f	ITA6_HUMAN	P23229	ITGA6	Integrin alpha-6	19	V	V
18	CD53	CD53_HUMAN	P19397	CD53	Leukocyte surface antigen CD53	1	V	V
19	CD59	CD59_HUMAN	P13987	CD59	CD59 glycoprotein	3	V	V
20	CD61	ITB3_HUMAN	P05106	ITGB3	Integrin beta-3	27	V	V
21	CD63	CD63_HUMAN	P08962	CD63	CD63 antigen	2	V	V
22	CD66b	CEAM8_HUMAN	P31997	CEACAM8	Carcinoembryonic antigen-related cell adhesion molecule 8	1	-[d)]	V
23	CD71	TFR1_HUMAN	P02786	TFRC	Transferrin receptor protein 1	23	V	V
24	CD82	CD82_HUMAN	P27701	CD82	CD82 antigen	2	-[d)]	V
25	CD91	LRP1_HUMAN	Q07954	LRP1	Prolow-density lipoprotein receptor-related protein 1	11	V	V
26	CD92	CTL1_HUMAN	Q8WWI5	SLC44A1	Choline transporter-like protein 1	5	V	V
27	CD98	4F2_HUMAN	P08195	SLC3A2	4F2 cell-surface antigen heavy chain	1	V	V
28	CD107a	LAMP1_HUMAN	P11279	LAMP1	Lysosome-associated membrane glycoprotein 1	2	-[d)]	V
29	CD107b	LAMP2_HUMAN	P13473	LAMP2	Lysosome-associated membrane glycoprotein 2	1	V	V
30	CD148	PTPRJ_HUMAN	Q12913	PTPRJ	Receptor-type tyrosine-protein phosphatase eta	7	V	V
31	CD151	CD151_HUMAN	P48509	CD151	CD151 antigen	2	V	V
32	CD156c	ADA10_HUMAN	O14672	ADAM10	Disintegrin and metalloproteinase domain-containing protein 10	13	V	V
33	CD225	IFM1_HUMAN	P13164	IFITM1	Interferon-induced transmembrane protein 1	1	V	V
34	CD233	B3AT_HUMAN	P02730	SLC4A1	Band 3 anion transport protein	17	V	V
35	CD240CE	RHCE_HUMAN	P18577	RHCE	Blood group Rh(CE) polypeptide	1	-[d)]	V
36	CD241	RHAG_HUMAN	Q02094	RHAG	Ammonium transporter Rh type A	1	V	V
37	CD321	JAM1_HUMAN	Q9Y624	F11R	Junctional adhesion molecule A	3	V	V

a) Number of identified unique peptides.
b) Detection in the samples from 5× ultracentrifugations.
c) Detection in the samples from 5× centrifugations.
d) Not detected.

3.7 Comparison with other identified proteins

Comparison of the 213 human plasma exosome proteins identified from three different isolation methods (DG, UC, and EI) [2] with the currently identified 479 exosome proteins revealed 108 common exosome proteins as shown in Supporting Information Table 3. Among the 108 common exosome proteins, 7 proteins were found to be CD proteins (CD31, CD41, CD42c, CD61, CD71, CD233, and CD321). Eleven proteins were found in all of the five enrichment methods as shown in Table 3. Among the 11 commonly observed proteins, four proteins (alpha-2-macroglobulin, albumin,

Table 3. List of 11 proteins identified from all of the five different exosome enrichment methods (two methods in the current investigation and three methods in the previous investigation from human plasma [2])

Number	Swiss-Prot name	Gene name	Description
1	A2MG_HUMAN	A2M	Alpha-2-macroglobulin
2	ALBU_HUMAN	ALB	Albumin
3	FIBA_HUMAN	FGA	Fibrinogen alpha chain
4	HPT_HUMAN	HP	Haptoglobin
5	IGJ_HUMAN	IGJ	Immunoglobulin J polypeptide
6	K2C6A_HUMAN	KRT6A	Keratin 6A
7	K1C14_HUMAN	KRT14	Keratin 14
8	K1C16_HUMAN	KRT16	Keratin 16
9	K1C17_HUMAN	KRT17	Keratin 17
10	DCD_HUMAN	DCD	Dermcidin
11	IGLL5_HUMAN	IGLL5	Immunoglobulin lambda-like polypeptide 5

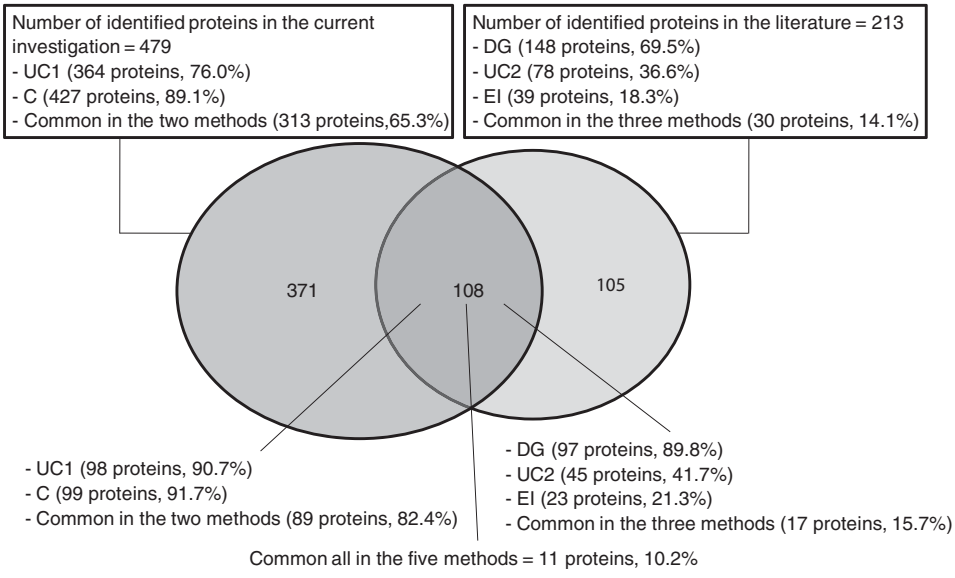


Figure 6. Venn diagram showing the overlap of exosome proteins enriched from the current investigation and from the three different enrichment methods [2]. UC, ultracentrifugation; C, centrifugation; DG, density gradient; EI, EpCAM-based immunoaffinity pull-down.

fibrinogen alpha chain, and haptoglobin) are well-known abundant plasma proteins [45], which might have been enriched as impurities. Five proteins (immunoglobulin J polypeptide, keratin 6A, keratin 14, keratin 16, and keratin 17) were already identified in the exosome fraction of human parotid saliva [2]. Ten proteins (all proteins except immunoglobulin lambda-like polypeptide 5 in Table 3) were also previously identified in the exosome fraction of normal human urine [7]. In Fig. 6 is shown the Venn diagram comparing the overlap of exosome proteins from the three different isolation methods and the current investigation, where 97 (89.8%), 45 (41.7%), and 23 (21.3%) exosome proteins were from proteins from DG, UC, and EI, respectively.

Among the 24 common exosomal proteins reported by Simpson and his co-workers [12], 18 proteins were identified in the current investigation, where 14 proteins were identified in both the 5× UC and 5× centrifugation procedures (Table 4). Two proteins (HSP90AB1 and YWHAG) were only identified from 5× UC and the other two proteins (HSP90AA1 and PGK1) were only identified from 5× centrifugations. The other six unidentified proteins were identified in similar forms as shown in Table 4.

4 Discussion

A recent study showed that a single cycle by UC or the ExoQuick kit to purify exosomes from human serum was not sufficient to remove high amounts of albumin and immunoglobulin G, where it was suggested that two or more cycles were required to increase exosome purity [24]. Another study showed that two cycles of UC are not sufficient to increase the purity of exosomes from nonexosomal protein contamination in the enrichment of exosomes from human serum [25]. The current methodology using UC twice for enriching exosomes may be effective for enriching exosomes from cells, while for serum or plasma samples, which contain several high abundant proteins, multiple cycles (more than 4) of UC or centrifugation are necessary.

Table 4. Comparison between the 24 commonly identified exosomal proteins [12] and proteins identified in the current analysis

Gene name	Description	Current analysis[a)]
ACTB	Actin, cytoplasmic 1	U, C
ACTC1	Actin, alpha cardiac muscle 1	U, C
ACTG1	Actin, cytoplasmic 2	ACTN1 (U, C), ACTR2 (C), ACTR3 (C)
ANXA11	Annexin A11	U, C
ANXA6	Annexin A6	U, C
ARF1	ADP-ribosylation factor GTPase-activating protein 1	ARF3 (U, C)
CFL1	Cofilin-1	U, C
ENO1	Alpha-enolase	U, C
GNAI3	Guanine nucleotide-binding protein G(k) subunit alpha	GNAI2 (U, C), GNAQ (C), GNAZ (C)
GNB1	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	U, C
HSP90AA1	Heat shock protein HSP 90-alpha	C
HSP90AB1	Heat shock protein HSP 90-beta	U
HSPA8	Heat shock cognate 71 kDa protein	U, C
PDCD6IP	Programmed cell death 6-interacting protein	U, C
PGK1	Phosphoglycerate kinase 1	C
PKM2	Pyruvate kinase PKM	PKM[b)] (U, C)
RAB5A	Ras-related protein Rab-5A	RAB5C (U, C)
RAB5B	Ras-related protein Rab-5B	RAB5C (U, C)
RAB5C	Ras-related protein Rab-5C	U, C
RAP1B	Ras-related protein Rap-1b	U, C
YWHAB	14-3-3 Protein beta/alpha	U, C
YWHAE	14-3-3 Protein epsilon	U, C
YWHAG	14-3-3 Protein gamma	U
YWHAZ	14-3-3 Protein zeta/delta	U, C

a) U and C mean detection from 5× ultracentrifugation and 5× centrifugation purifications, respectively. For the proteins that were not identified in the current analysis, the gene names of the identified similar proteins were provided.
b) The alternative name is PKM2.

The TEM image showed that the exosome enrichment using 5× centrifugations is similar to 5× UCs in removing proteins and protein aggregates, providing a similar average diameter of exosomes. There are several advantages to the use of centrifugation in that a centrifuge instrument is relatively inexpensive and widely disseminated compared to a UC instrument. In addition, it is easy to handle the samples without contamination since most tubes for centrifugation have lids, while extra care is required for sample handling using UC since most tubes for UC do not have lids.

In conclusion, in the current investigation we have shown that five-cycle repetition with the use of UC or centrifugation is necessary for a successful enrichment of exosomes from human serum based on 1D gel analysis and the comparison of protein yield between three cycles and five cycles. In addition, we have shown that 5× centrifugations provided comparable results to those obtained using 5× UCs. Both enrichment procedures provided similar performances in terms of exosome protein amounts and Western blot analyses detecting CD-63 antigen, while significantly higher numbers of identified exosome proteins and CD proteins were obtained from 5× centrifugations. A comparison between the exosome protein list from the current investigation with the previously reported exosome protein list shows that the current method is successful in isolating exosomes from human serum. Additionally, a total of 37 CD proteins were identified, which will be important in future exosome research for providing a means for rapid detection of exosomes using targeted antibodies or mass spec assays. This will be especially important in biomarker studies of disease states and therapeutic response based on monitoring of proteins from exosomes in serum.

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