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Technical Note

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A Comparison of an Optimized Ultracentrifugation Method versus Size-Exclusion Chromatography for Isolation of Exosomes from Human Serum

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Abstract:

Exosomes are nanosized vesicles which are abundant in biological fluids. In recent years, exosomes have attracted increasing attention where their cargo may provide promising biomarkers for the early diagnosis and therapy for many diseases, such as cancer. In addition to ultracentrifugation (UC), many alternative methods including size-exclusion chromatography (SEC) have been developed for isolating exosomes. It has been reported that the SEC method provided improved performance relative to the UC method in isolating exosomes from plasma, where the former contained less residual blood protein contamination. Herein, we have compared the SEC method with an optimized UC method in isolating exosomes from human serum. This was based on dilution of the serum to reduce the viscosity, and a prolonged cycle of UC followed by another 4 cycles. We found that more than 95% of serum proteins were removed without significant loss of exosome proteins relative to SEC. We also combined 1 cycle of UC with SEC and found this method provided improved results relative to the SEC method, although the serum protein contamination was several folds higher than our optimized UC method. The TEM showed that the size distribution of exosomes isolated from each of the three methods was similar.

Keywords: exosomes, SEC, serum, UC

Introduction

Exosomes are 30-120 nm sized membrane-bound vesicles[1,2] originating from the luminal membrane of multivesicular bodies (MVB) in all types of normal and tumor cells.[3-6] They are believed to participate in cell-cell communication by loading cargo proteins, metabolites and nucleic acids (mRNA, miRNA) from their original cells,[2,5,7,8] which can be released into the extracellular space upon fusion with the plasma membrane.[9-11] There are many cell surface membrane proteins that can be detected in exosomes, some of which may be used for early detection, diagnosis and prognosis of cancer.[12-18]

The commonly used method for isolation of exosomes is ultracentrifugation (UC). This method can be time consuming and requires access to an ultracentrifuge. Several other methods, such as immunoaffinity,[19] ultrafiltration,[20] polymeric precipitation[21] and size-exclusion chromatography (SEC), have been recently developed as alternatives.[22,23] However, the polymeric precipitation method has little specificity and usually captures unwanted extracellular vesicles other than exosomes. The immunoaffinity methods require large amounts of antibodies, which are costly and may extract larger vesicles other than exosomes. Ultrafiltration generally has low exosome yield because of the adhesion of exosomes to filters. Several types of microfluidic chip separations based on immunoaffinity or SEC have also been developed to isolate exosomes.[24,25] The throughput of these methods is generally low and not suitable for pre-analytical processes, such as discovery.[24]

SEC has the ideal performance in separating extracellular vesicles from blood proteins.[26,27] Although it was reported to have the limitation of low exosome yield, the SEC method has been applied for the analysis of clinical samples.[28,29] A recent study used a commercial qEV SEC column (Izon Science Ltd, Christchurch, New Zealand) and compared it with the exoEasy kit.[30] The authors found that the SEC method resulted in better protein yield and size distributions for isolation of exosomes from human plasma compared to the exoEasy kit. Another study compared the SEC method

with the UC method in isolating exosomes from plasma, and found the former provided improved performance where there was less serum protein contamination.[31]

In the current study, we compare the commercial qEV SEC column with our optimized UC method for exosome isolation from human serum. We studied human serum since this will be essential for clinical applications. Briefly, our optimized UC method was based on dilution of the serum with PBS to reduce the viscosity, and a prolonged first cycle of UC followed by another 4 cycles of UC. We have also studied the use of one cycle of UC coupled with the qEV SEC column for isolating exosomes for further analysis. NanoSight and transmission electron microscopy (TEM) were used to compare the yield and size distribution of exosomes. We have performed LC−MS/MS based proteome analysis to compare the protein categories, exosome protein markers and degree of exosome purification from blood proteins for the three methods.

Experimental Section

Human Serum Sample

The commercially available serum (Innovative Research, Novi, MI) was pooled from a cohort of 1500 to 3000 healthy normal humans. Each donor unit was tested and found negative for HIV 1&2, HCV, HBsAg, and PRP by FDA approved methods. The serum was stored at −80 ^{o}C until use. The initial volume of serum was 18 mL. It was diluted with an equal volume of PBS (AppliChem, St. Louis, MO) to decrease the viscosity. The diluted serum was centrifuged at 2000g for 10 min and 10000g for 30 min at 4 °C to remove cell debris and large extracellular vesicles. The supernatant of serum was divided into 3 equal aliquots, which were designated as Serum sample 1 (SS1), Serum sample 2 (SS2) and Serum sample 3 (SS3). Each sample contained 12 mL of diluted serum.

Five Cycles of Ultracentrifugation

The SS1 was further divided into 6 equal aliquots (SS1-1, SS1-2, SS1-3, SS1-4, SS1-5, SS1-6), where each aliquot contained 2 mL of diluted serum. Each aliquot of the SS1 was transferred to Ultra-Clear^{TM} tubes (Beckman Coulter, Indianapolis, IN), diluted by PBS into 4 mL and centrifuged at 100000g using a Beckman Optima XL-70 ultracentrifuge for 120 min at 4 °C. The supernatant was removed by pipette carefully. To avoid the loss of exosome pellets, 2 mm of supernatant was left in the tubes. Compared with exosome protein, the presence of serum protein was dominant. Thus, a couple of cycles of ultracentrifugation were not sufficient to remove the serum protein for analysis of the exosome proteome. In our previous work, we showed that five cycles of ultracentrifugation were necessary to efficiently remove the serum protein contamination.[32] The sedimentary pellets in each tube were suspended in 4 mL of PBS to dilute the supernatant and centrifuged at 100000g for 70 min at 4 °C followed by removal of the supernatant. In total, five cycles of ultracentrifugation were

performed to purify exosomes in order to eliminate serum protein contamination. Samples SS1-1 and SS1-2 were analyzed by NanoSight and TEM. Samples SS1-3, SS1-4, SS1-5, SS1-6 were each analyzed by mass spectrometry separately to provide replicate runs.

SEC columns

The SS2 was further divided into 6 equal aliquots (SS2-1, SS2-2, SS2-3, SS2-4, SS2-5, SS2-6), where each aliquot contained 2 mL of diluted serum. The qEV SEC 10 mL columns (iZON Science Ltd, Christchurch, New Zealand) were used here to isolate exosomes. After rinsing the columns with 15 mL of PBS for equilibration, each aliquot of SS2 was transferred on the SEC columns. It should be noted that the loading volume of sample was 0.5 mL each time. PBS was continually added to the columns so that they did not run dry. The first 3.5 mL of elute was discarded since it was the void volume. The next 0.5 mL of elute was collected for the exosome analysis. The columns were then flushed using 30 mL of PBS and cleaned using 10 mL of 0.5 M NaOH. The columns were rinsed by 50 mL of PBS for equilibration, which were then ready for the next loading. To prepare each sample, two columns were used where each column was used twice. Samples SS2-1 and SS2-2 were analyzed by NanoSight and TEM. Samples SS2-3, SS2-4, SS2-5, SS2-6 were each analyzed by mass spectrometry separately.

One Cycle of Ultracentrifugation & SEC columns

The SS3 was further divided into 6 equal aliquots (SS3-1, SS3-2, SS3-3, SS3-4, SS3-5, SS3-6), where each aliquot contained 2 mL of diluted serum. Each aliquot of SS3 was transferred to Ultra-Clear^{TM} tubes (Beckman Coulter, Indianapolis, IN), diluted by PBS into 4 mL and centrifuged at 100000g using a Beckman Optima XL-70 ultracentrifuge for 120 min at 4 °C. After removing the supernatant,

the pellets were suspended and diluted by PBS to a final volume of 0.5 mL. Then the sample was transferred to a pre-equilibrated qEV SEC 10 mL column. PBS was continually added to the columns. The first 3.5 mL of eluent was discarded and the next 0.5 mL of eluent was collected for the exosome analysis. To prepare each sample, only one column was used. Samples SS3-1 and SS3-2 were analyzed by NanoSight and TEM. Samples SS3-3, SS3-4, SS3-5, SS3-6 were each analyzed by mass spectrometry separately.

NanoSight Analysis

The recoveries of exosomes by the above three methods were evaluated using the NanoSight NS300 (Malvern, Worcestershire, UK). Each exosome sample was diluted by PBS to 1 mL and continuously infused into the NanoSight by an automatic syringe pump at a flow rate of 30 μL/min. The focus was adjusted and the temperature was set as 25 °C. The exosome movement was captured for 5 times, where each time was 60 seconds. The concentration of exosomes was then calculated by a built-in application.

Transmission Electron Microscopy (TEM)

TEM was used to measure the size of the exosomes. First, glow discharge was performed to make the surface of the carbon film (Hatfield, PA, USA) hydrophilic. Each exosome sample was diluted by PBS to 1 mL, where 5 μL was dropped on the carbon film and incubated for 2 min. Next, 5 μL of 2.5% w/v glutaldehyde was used to fix the exosomes for 5 min. The film was negatively stained using 5 μL of 1% uranyl acetate for 1 min. After each of the above steps, the liquid was removed by a small piece of filter paper. Exosomes on carbon films were then imaged in a Philips CM-100 TEM instrument.

NanoLC−MS/MS

The tryptic digests of exosome samples were desalted by homemade C_{18} tips[33] and then separated on an EASY−nLC 1000 liquid chromatograph system (Thermo Fisher Scientific, San Jose, CA) with a 250 mm reverse−phase (RP) C_{18} column. The samples were resolved under a 120 min linear gradient from 2% to 35% acetonitrile in 0.1% formic acid at a constant flow rate of 300 nL/min.

The samples were analyzed on an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific, San Jose, CA) operated in positive ion mode. The capillary temperature and the spray voltage were set as 200 °C and 2.5 kV. The data was acquired in a data-dependent mode, where up to 20 strongest MS1 peaks were selected for subsequent MS2 analysis. For every selected peak, collision induced dissociation (CID) was performed. The MS1 spectra (m/z 350-1650) and the MS2 spectra were acquired in the Orbitrap and LTQ, respectively.

Data Analysis

All raw data files were processed by the MaxQuant computational proteomics platform (version 1.6.1.0).[34] The parameters were set as follow: database, human UniProt; enzyme, trypsin; fixed modifications, carbamidomethyl (C); variable modification, oxidation (M) and protein N-terminal acetylation; up to 2 missed cleavages allowed. The MS1 mass tolerance was set as 20 ppm and 6 ppm for the first search and main search, respectively; the MS2 mass tolerance was set as 0.5 Da. The false discovery rates (FDR) for peptides and proteins were both set as 1%.

Western blot

Exosomes proteins from each method were separated on a 4-15% SDS-PAGE gradient gel (Bio-Rad, Berkeley, CA) and transferred to a PVDF membrane (Bio-Rad, Berkeley, CA). After blocking, the

membrane was incubated overnight with anti-CD63, anti-ALBU or anti-Calnexin antibody (Sigma-Aldrich, St. Louis, MO), followed by incubation with HRP-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA) and was visualized using a chemiluminescent method kit (Merck Millipore, Billerica, MA).

Results and Discussion

Comparison of Exosome Recoveries

In this work we have compared three methods for isolating exosomes. These include using multiple cycles of ultracentrifugation (UC method), a commercial qEV SEC column (SEC method) and a combination of these two methods which first uses one cycle of UC followed by further purification with the qEV SEC column (UC&SEC method). The workflow of the three methods for isolating exosomes from human serum is shown in Figure 1. It has been reported that the efficiency of the UC method for isolating exosomes from highly viscous biofluid is very low.[35] Reducing the viscosity of the serum and increasing the time of UC were found to significantly increase the exosome recovery.[36] Herein we have mixed the serum with an equal volume of PBS to reduce the viscosity of serum before using each of the isolation methods. We set 120 min for the first cycle of UC and 70 min for each of the next 4 cycles since the viscosity of PBS was much lower.

We used the NanoSight method to measure the number of exosomes isolated. Before the use of UC or SEC column isolation, the concentration of serum protein is too high for this light scattering method so that we could not read any information from NanoSight. After the first and the fifth cycle of UC, we found the number of exosomes based on 1 mL of starting material for serum was around 1.5×10^9 and 1.0×10^9, respectively. The results showed that the loss of exosomes in PBS by UC was not significant even after multiple cycles of UC. To evaluate the recovery of exosomes in serum from the first cycle of UC, we collected the supernatant and performed UC again to isolate exosomes. The NanoSight result showed that only around 2.0×10^8 exosomes were acquired from the collected supernatant, which was much less than the number of exosomes from the serum by the first cycle of UC. It seems that the prolonged UC time and reduced serum viscosity by PBS efficiently isolated

exosomes. Also the precipitated exosomes might not stick to the tube bottom firmly. In order to make sure these exosomes were not lost, around 2 mm of supernatant above the bottom was left when we slowly removed the supernatant after each cycle of UC.

In comparison, we investigated the use of the qEV SEC 10 mL column for isolating exosomes from serum. The instructions of the commercial SEC column show that the eighth, ninth and tenth fractions (each fraction = 0.5 mL) of the eluent contain exosomes; while the eighth fraction of eluent contains very little serum protein. We also measured the protein amount of each of the three fractions using the BCA assay and found that the ninth and tenth fractions had a much higher protein amount compared to the eighth (Figure 2). To eliminate the serum protein contamination, we only collected the eighth 0.5 mL of eluent. After the isolation of exosomes by the SEC column, the number of exosomes from 1 mL of serum was around 1.3×10^9. Compared with 1 cycle and 5 cycles of UC, the SEC column method resulted in the relative isolation of 85% and 130% of exosomes, respectively. We also investigated the combined use of 1 cycle of UC followed by the qEV SEC column. The number of exosomes isolated by the UC&SEC method from 1 mL of serum was around 1.2×10^9. Although its recovery was lower than that by the SEC column method, it was still 20% higher than that by 5 cycles of UC. It should be noted that potential protein aggregates in the SEC samples could possibly add concentration to the signal, although the serum underwent centrifugation at 10000g for 30 min beforehand.

Before the isolation by the three methods, low speed centrifugations were performed to remove cell debris and large extracellular vesicles. The transmission electron microscopy (TEM) images showed that the exosomes isolated from the human serum by the three different methods had similar morphology and size distribution (72 ± 5 nm), where the mean diameters of exosomes from the optimized UC, UC&SEC and SEC methods were 67, 75 and 77 nm, respectively (Figure 3). In addition, these exosomes had intact membrane structures.

Recovery and purity comparison of exosome protein by LC-MS/MS

We processed and digested exosome proteins using the filter-aided sample preparation (FASP) method,[37,38] followed by desalting and LC−MS/MS analysis. Herein, we measured the protein concentration of the sample from each method by BCA assay and found that the sample from the SEC method had a much higher protein amount. We loaded each sample prepared from the same volume (1 mL) of serum for LC−MS/MS analysis. However, the protein amount in the sample prepared by the SEC method was too high to load on the mass spectrometer. We finally loaded 10% of sample from the SEC method for LC−MS/MS analysis. Usually, the loading protein amounts are statistically normalized among different samples using the intensities of total proteins or sometimes the highest abundance protein. However, such normalization is not suitable in this case. The highest abundance proteins in SEC samples were the serum proteins which were efficiently removed in the UC and UC&SEC methods. Meanwhile, the protein number and the abundance of each protein in samples from the 3 methods were significantly different.

The experiment was performed 4 times for each sample of the 3 different methods. Thus, a total of 12 samples were analyzed by mass spectrometry. We identified totally 578 proteins (Supplemental Table S-1) using the MaxQuant 1.6.1.0 computational proteomics platform, where 495, 474 and 347 proteins were from UC, UC&SEC and the SEC method, respectively. The number of common proteins identified in all the four runs for each of the methods was 242 for UC, 216 for UC&SEC and 162 for SEC, respectively. The Venn diagram shows the overlap of the identified proteins from the three isolation methods (Figure 4a). The samples from the SEC method had the highest protein amount but identified the fewest proteins, while the samples from the optimized UC method identified the most proteins. We believe that the optimized UC method is the optimal method for the identification of the exosome proteome. The SEC method alone cannot avoid excessive contamination

of serum proteins. Although it retains more exosome proteins and should be ideal for RNA analysis, the SEC has obvious limitations in the discovery of the exosome proteome. It should be noted that the SEC would be improved using well-known modifications in the liquid chromatography (LC) method to reduce shoulder effects whereby the exosomes could be better enriched with less serum protein contamination. The heat map of Pearson correlation coefficient (PCC) of the mass spectrometry results was used to reflect the reproducibility of each method and the differences among the methods (Figure 4b). The PCC of four replicates in each method was around 0.95; while the PCC of two runs in different methods was generally between 0.67 and 0.90. Since a smaller PCC meant a larger difference between two samples, the heat map showed that the proteomes of different methods had a larger variation than that of four replicates of the same method. The reproducibility of each method appeared overall to be very good. The proteomes observed using the SEC method were much more different than those of the other two methods. We speculate that it might be caused by the larger amount of blood proteins in the SEC samples.

We also studied various exosome markers that were detected by mass spectrometry analysis. Among these proteins, we did not detect the negative exosome marker Calnexin. We performed Western blot assay and verified the absence of Calnexin. We compared the recovery of exosome protein by the label free method for quantifying the exosome markers CD9, CD63 and CD81 among the three methods since these marker proteins were identified in samples from all three methods. We also detected another positive exosome marker TSG101 in samples from UC and UC&SEC methods but not in samples from the SEC method possibly due to the low ratio of markers to serum proteins.

Since the loading amount of the SEC sample was one tenth of that prepared by the other two methods, we amplified by 10 folds of the peak intensity of the SEC data when compared with the peak intensities of the other two methods. The protein amounts of three exosome markers CD9, CD63 and

CD81 in samples from the UC&SEC column method are 1.3, 1.4 and 2.7 folds of that from optimized UC method, respectively (Figure 5a). NanoSight results showed that the SEC method had more exosomes than the UC&SEC method, but the mass spectrometry data suggested the amount of CD63 prepared by the UC&SEC method was larger. We speculate that there may be a large amount of serum protein contamination that suppresses the signal from exosome proteins in the SEC sample in the mass spectrometer. We then performed Western blot assay of CD63 and found its abundance prepared by the optimized US was 80% of that from the other two methods (Supplemental Figure S1).

In the samples from the SEC method, the amount of exosome protein and serum protein were the highest relative to those from the other two methods, but the amount of serum protein had a much larger ratio. According to the intensities of proteins in the mass spectrometer, APOB was the most abundant serum protein in samples from the SEC method. The protein amounts of ALBU, APOA1 and APOB in samples from the SEC column method are 149.2, 38.6 and 127.5 folds of that from the optimized UC method, respectively (Figure 5b). Western blot results verified that the abundances of ALBU from samples prepared by the optimized UC and SEC methods were the least and the most abundant, respectively (Supplemental Figure S2). Although it had a slightly lower exosome recovery than that from other two methods, the sample from our optimized UC method had the highest purity of exosome protein which is critical for further proteomic analysis. Although the amount of various IgG in samples from the SEC method was slightly less than that from other two methods, the SEC method alone was limited for proteome analysis since serum proteins and various lipoproteins were dominant.

Theoretically, multiple cycles of the SEC column method could also probably remove serum protein more efficiently than one cycle with the accompanying loss. The speed of the flow through a used column becomes slower than a new one even after thorough washing suggesting the content of fractions might have a tiny shift, although the protein amounts in various fractions were not

significantly changed between new columns and those used once according to our experiment. The use of UC before the SEC column was found to remove the serum protein contamination relative to the SEC method. As a result, the speed of the flow through a used column did not become significantly slower. Thus the UC&SEC method saves the lifetime of the qEV SEC column and increases the purity of exosomes for proteomics although the qEV SEC columns were only used once in the UC&SEC method.

Conclusions

In this study, we optimized the UC method by diluting the serum with PBS to reduce the viscosity, prolonging the first cycle of UC and using another 4 cycles of UC. Our results suggest that serum proteins were efficiently removed without significant loss of exosomes by our optimized UC method relative to the SEC method. We also combined 1 cycle of UC with SEC and found this method provided improved results relative to the SEC method, although the blood protein contamination was slightly higher than our optimized UC method. The use of each these methods will depend on the application where the UC method would be best for proteomic discovery, while the UC&SEC method would be better for targeted proteomics. The SEC method has obvious limitations in the discovery of the exosome proteome due to large amounts of serum protein contamination, but it retains more exosome proteins and should be ideal for RNA analysis.

Supporting Information:

The following files are available free of charge at ACS website http://pubs.acs.org:

Supplemental Table S1: Total proteins identified and quantified in the three methods. (XLSX)

Figure S1: Western blot results of CD63 from the samples prepared by the optimized UC, UC&SEC and SEC methods. (PDF)

Figure S2: Western blot results of ALBU from the samples prepared by the optimized UC, UC&SEC and SEC methods. (PDF)

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Figure Legends

Figure 1: The workflow of exosomes isolated by three different methods from human serum.

The human serum was diluted with an equal volume of PBS to reduce the viscosity followed by the centrifugation to remove cell debris and large extracellular vesicles. Next, exosomes were isolated using multiple cycles of ultracentrifugation (UC method), a commercial qEV SEC 10 mL column (SEC method) and a combination of these two methods which first uses once cycle of UC followed by further purification with the qEV SEC column (UC&SEC method).

Figure 2: Protein concentration of fraction 8, 9 and 10 eluted from SEC column.

In the SEC method, 0.5 mL of diluted serum was loaded onto the SEC column. The eluent was collected by fractions, where each fraction was 0.5 mL. The protein amount of each fraction was then measured using the BCA assay 3 times and it was found that the ninth and tenth fractions had a much higher protein amount compared to the eighth.

Figure 3: TEM images and the size distribution of the exosomes.

The TEM images showed that the exosomes isolated from the human serum by the three different methods had similar morphology and size distribution. The mean diameters of exosomes from optimized UC, UC&SEC and SEC method were 67, 75 and 77 nm, respectively.

Figure 4: Proteomics analysis of exosomal proteins isolated from the three isolation methods.

(a) The Venn diagram showed the overlap of the identified proteins from the three isolation methods. (b)The heat map of Pearson correlation coefficient showed that the proteomes of different methods had a larger variation than that of four replicates of the same method.

Figure 5: The relative abundance of exosome markers and serum contamination protein in the samples from the three methods.

(a) The relative abundance of three exosome markers (CD9, CD63 and CD81) identified in the samples from the three methods was similar. The protein amounts of CD9, CD63 and CD81 in samples from the UC&SEC column method are 1.3, 1.4 and 2.7 folds of that from the optimized UC method, respectively. (b) The protein amounts of ALBU, APOA1 and APOB in samples from the SEC column method are 149.2, 38.6 and 127.5 folds of that from the optimized UC method, respectively.

Figure 1.

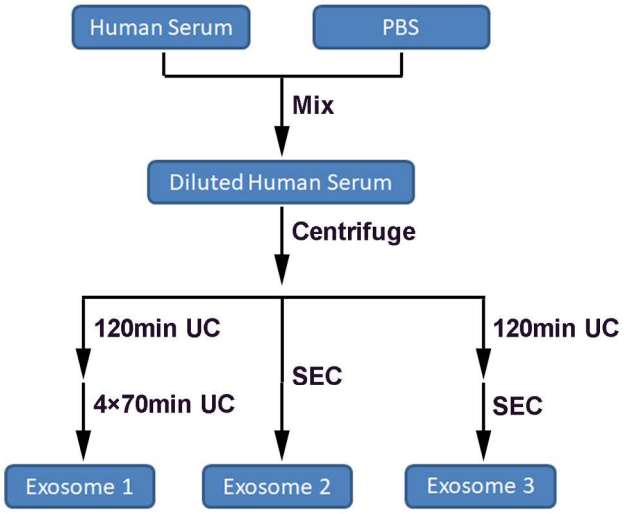

Human Serum
PBS
Mix
Diluted Human Serum
Centrifuge
120min UC
4×70min UC
SEC
120min UC
SEC
Exosome 1
Exosome 2
Exosome 3

Figure 2.

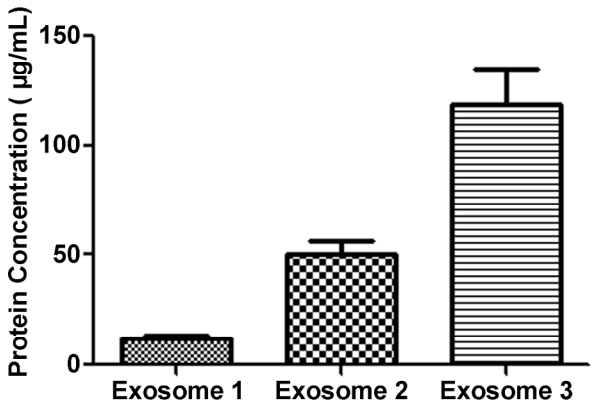

Protein Concentration (µg/mL)
150
100
50
0
Exosome 1
Exosome 2
Exosome 3

Figure 3.

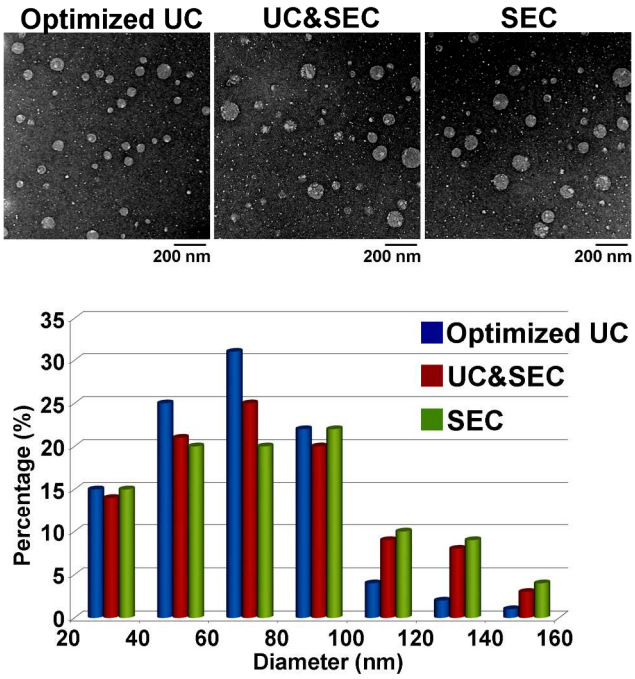

Optimized UC
UC&SEC
SEC
200 nm
200 nm
200 nm
Optimized UC
UC&SEC
SEC
Percentage (%)
Diameter (nm)
0
5
10
15
20
25
30
35
20
40
60
80
100
120
140
160

Figure 4.

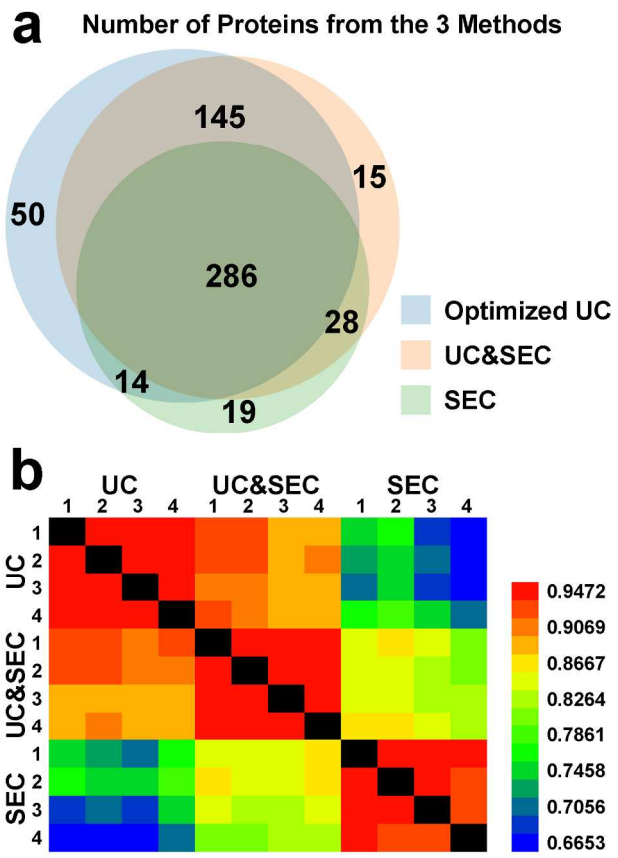
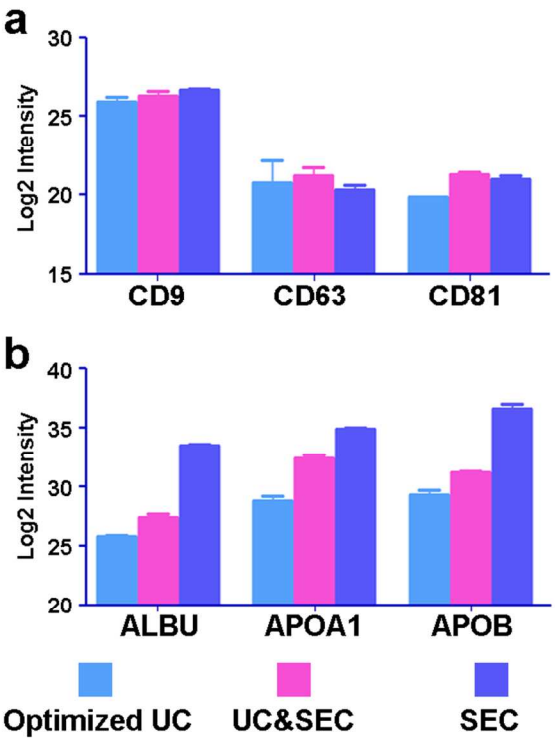

a
Number of Proteins from the 3 Methods
145
15
50
286
28
14
19
Optimized UC
UC&SEC
SEC
b
UC
UC&SEC
SEC
1 2 3 4 1 2 3 4 1 2 3 4
UC
UC&SEC
SEC
0.9472
0.9069
0.8667
0.8264
0.7861
0.7458
0.7056
0.6653

Figure 5.


a
Log2 Intensity
30
25
20
15
CD9
CD63
CD81
b
Log2 Intensity
40
35
30
25
20
ALBU
APOA1
APOB
Optimized UC
UC&SEC
SEC

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