A Comparative Proteomics Study of Six Serum Exosome Isolation Procedures

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ABSTRACT

Purpose: Comprehensive evaluation of six exosome isolation strategies using mass spectrometry-based proteomics and western blotting.

Methods: Exosomes were isolated from a pool of normal human serum using ultracentrifugation and five commercial exosome isolation kits: Exosomes were isolated, lysed using RIPA buffer or SDS, and the total protein was quantified using the BCA protein assay. A novel and rapid proteomic approach was performed for the profiling and quantification of exosome proteomes and biomarkers using a Thermo Scientific™ Q Exactive™ Plus platform. Biomarkers (CD9, CD81 CD63) and the contamination proteins were further validated by western blot. The proteome data were analyzed by Thermo Scientific™ Proteome Discoverer™ and the identified proteins were compared with the exosome database (ExoCarta) to evaluate the purity of the exosomes isolated using different strategies.

Results: We evaluated exosome isolation by ultracentrifugation and five commercial kits (Total Exosome Isolation Reagent for serum (Thermo Fisher Scientific), qEV iZON original (iZON Science), MagCapture Exosome Isolation PS (Wako Chemical), ExoEasy Maxi Kit (Qiagen), and Exo-spin™ Kits (Cell Guidance Systems)) that use different isolation methods. The initial evaluation comparing particle size and concentration showed that all methods yielded particles in the appropriate size range for exosomes (30-150nm). For proteomic profiling of different methods for exosome purification, the distribution of identified proteins was also compared. To assess the quality of the exosomes isolated from different strategies, we quantitatively monitored the enrichment of the identified exosome protein markers. In addition, western blotting was performed on each sample in parallel using known exosome surface markers. HSA was used as a negative control to assess purity. More proteins (620-700 protein groups) were identified from qEV iZon, MagCapture, UC_S (Ultracentrifugation with sucrose cushion purification) and ExoEasyMaxi than ExoSpin and Total Exosome Isolation Reagent (TEI). The results indicate that exosome purity and protein patterns are method dependent. MagCapture gives the best purity of exosomes for proteomics study among the six exosome isolation technologies. Higher purity of the isolated exosomes allows the identification of a higher number of proteins/exosome proteins. MagCapture gives the best exosome purity but the least protein yield (too low). Differential ultracentrifugation coupled with sucrose cushion does not provide the best exosome purity for proteomics study.

INTRODUCTION

Exosomes are small extracellular vesicles (30-150nm) that play a key role in cell-to-cell communication. They are a rich source for biomarkers, including proteins, RNA and DNA. Isolation of exosomes from biological fluids has become an area of focus for liquid biopsy development and disease diagnostics. Exosome proteins are a larger pool of biomarkers, but are underutilized due to detection difficulties and isolation protocols that result in low purity. As an original and widely used exosome isolation method, ultracentrifugation can be tedious and time consuming. With the help of exosome isolation reagents/kits, intact exosomes can be easily enriched. However, the downstream compatibility of the isolation technology and high purity of exosomes is required for proteomic analysis. In this study we compared six exosome isolation methods with subsequent analysis of protein content by proteomics and western blotting.

MATERIALS AND METHODS

Sample Preparation

Exosomes were isolated from a pool of normal human serum using ultracentrifugation and five commercial exosome isolation kits.

Exosomes Isolation Using Differential Ultracentrifugation

The serum samples were centrifuged at 300 × g, 2,000 × g and 10,000 × g to remove dead cells and cell debris. The 1st ultracentrifugation at 100,000 × g was applied to spin down the raw exosomes. The raw exosomes were either washed with PBS and centrifuged at 100,000 × g or further purified using sucrose cushion ultracentrifugation. The 2nd Ultracentrifugation with sucrose cushion: raw exosomes were cleaned using a 30% sucrose cushion in conjunction with ultracentrifugation at 100,000 × g. The 3rd Ultracentrifugation: the exosomes in the sucrose cushion were further cleaned by dilution with PBS and centrifuged at 100,000 × g.

Exosome Isolation Using five commercial exosome isolation kits

Serum exosomes were also isolated using these five commercial kits according to the vendor's protocol. The five exosome isolation kits include the following: Total Exosome Isolation Reagent for serum (Thermo Fisher Scientific), qEV iZON original (iZON Science), MagCapture Exosome Isolation PS (Wako Chemical), ExoEasy Maxi Kit (Qiagen), and Exo-spin™ Kits (Cell Guidance Systems). The isolated exosomes were either lysed with RIPA (Thermo Fisher Scientific, P/N 8990) for BCA quantitation and Western blotting, or with 5% SDS for downstream in-solution proteomics sample preparation.

Lysis of exosomes and tryptic digestion

The exosome samples were lysed by 5% sodium dodecyl sulfate (SDS) for downstream proteomics study. Buffer exchange was performed using Pierce™ Protein Concentrators PES (10K MWCO, Thermo Fisher Scientific, P/N 88513) and the exosomes proteins were finally buffered in 0.1% SDS, 50mM TEAB (freshly prepared using 1M TEAB, Thermo Fisher Scientific, P/N 90114). The total protein was measured using BCA assay again (Thermo Fisher Scientific, P/N 23225) after buffer exchange. Twenty micrograms of exosome proteins were reduced by tris(2-carboxyethyl)phosphine (TCEP, Thermo Fisher Scientific, P/N 77720), alkylated by chloroacetamide and digested by MS-grade Trypsin (Thermo Fisher Scientific, P/N 90058) following an in-house developed in-solution fast protein digestion protocol. The SDS in the tryptic peptides were removed using an in-house developed SCX cleanup protocol. Duplicates of the exosomes isolated from each technology were analyzed.

Liquid Chromatography and Mass Spectrometry

Tryptic digest samples were enriched and desalted on-line using the Thermo Scientific™ Acclaim™ PepMap 100 C18 Trap Column. The samples were analyzed by nanoLC-MS/MS using a Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLCnano System and Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap Mass Spectrometer.

Data Analysis

Raw data were analyzed with Thermo Scientific™ Proteome Discoverer™ 2.2 and Thermo Scientific™ Proteome Discoverer™ 1.4. The identified protein groups (with at least one unique high confidence peptide) were compared with the top103 and top 25 exosome markers that are often identified in exosomes (ExoCarta Exosome Database).

RESULTS

Figure 1. Total protein yield by the six exosome isolation procedures.

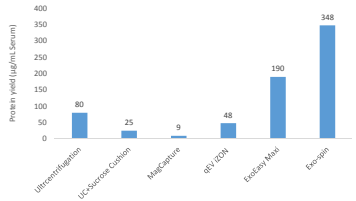
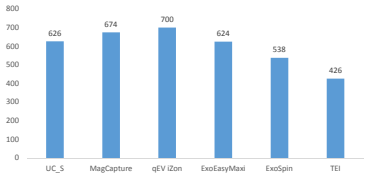


Figure 2. Protein Groups Identification.



The sample elution buffer is different from kit to kit, to minimize the matrix effect, buffer exchange was performed for the all of the exosome samples before BCA quantification. The protein yield was calculated per 1mL serum, TEI (Total Exosome Isolation Reagent) gave the highest protein yield (~ 5.2 – 8.3 mg per 1 mL serum, not shown in Figure 1). Using proteome analyses, the number of identified protein groups was compared across the six technologies. More proteins (620-700 proteins) were identified from qEV iZon, MagCapture, UC_S and ExoEasyMaxi than ExoSpin and TEI (Figure 2). The protein yield was very high using ExoSpin and TEI exosome isolation products, which indicate that more serum high abundant proteins were co-isolated with exosomes. The presence of these high abundant proteins will decrease the identification of exosome proteins due to the ion suppression of lower abundant proteins. This issue is especially problematic due to the use of a short LC gradient (100 min) in our study. This was further proved by the western blot results of HSA (Serum Albumin, Figure 7), where albumin was shown to be more abundant in ExoSpin and TEI than other samples.

Figure 3. Exosome Protein Coverage Compared to ExoCarta Exosomes Markers.

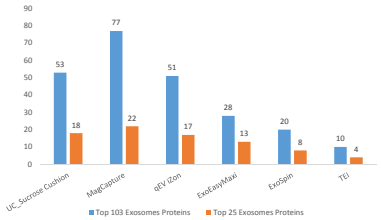
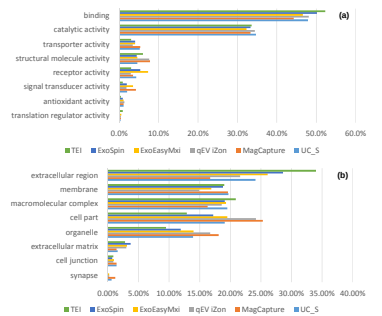


Figure 4. Gene Ontology (a) molecular function / (b) cellular component annotations of the exosome proteins (http://geneontology.org/).



To assess the performance of the exosome isolation technologies, Top103 and Top25 ExoCarta Exosomes Markers were selected for a comparative evaluation of the purity of the isolated exosomes. As shown in Figure 3, our data suggests that the exosomes prepared using the MagCapture technology are the most pure compared to other technologies used in this work. About 75% of the Top 103 most often identified exosome proteins were found in the exosome sample prepared using MagCapture (Figure 5a). Exosome markers CD9, CD81, CD63, HSPA8, PDCD6IP (Alix), ANXA5, ICAM1, EPCAM, FLOT1 and HSPA1A were observed among these identified proteins.

In comparison, traditional differential ultracentrifugation (UC) was used for isolating exosomes. The raw exosomes were further purified using a sucrose cushion ultracentrifugation method (UC_S). Compared to MagCapture, exosomes isolated by UC_S show less exosome protein coverage, which indicates that ultracentrifugation coupled with a sucrose cushion does not provide the best exosome purity for proteomics study. A density gradient ultracentrifugation method may be preferred for proteomic exosome preparation if commercial products are not desired.

Gene Ontology annotations of proteins (Figure 4) show that the highest portion of proteins from MagCapture exosomes (in orange) are located in cell part and organelle, which is different from other lower purity exosomes. The proteins identified from the top three methods indicate that different isolation technologies give different protein/exosome patterns (Figure 5).

To further compare the purity of exosomes from different procedures, nine commonly used exosome protein markers were quantitatively analyzed using the MS data. The results clearly indicate that MagCapture, UC_S and qEV iZon produced exosomes in higher purity than other kits. Among the six exosome isolation technologies tested, the MagCapture kit provided the best purity. These quantitative results were comparable with the western blotting (CD63, CD9 and CD81, Figure7).

Figure 5. (a) MagCapture Protein compared with ExoCarta Exosomes Markers; (b)Proteins identified in UC_S, MagCapture and qEV iZon.

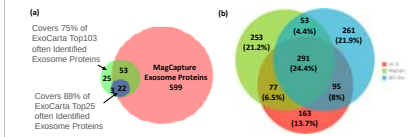


Figure 6. Quantitative analysis of nine exosome markers using protein intensities from MS data.

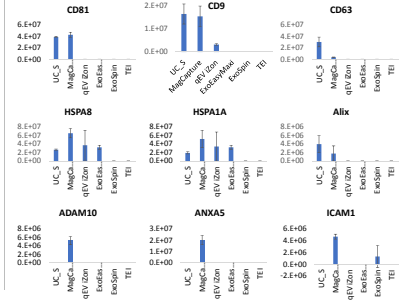
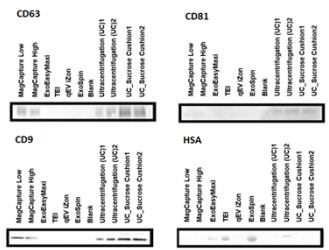


Figure 7. Western blotting of three exosome markers (CD63, CD81, CD9) and a serum high abundant protein (Serum Albumin, HSA).



CONCLUSIONS

- Highest exosome purity for proteomic analysis was obtained using the MagCapture kit among the six exosome isolation technologies tested. Higher purity of the isolated exosomes allows the identification of higher numbers of proteins/exosome proteins. However, MagCapture provides the best exosome purity but the lowest protein yield.
- Differential ultracentrifugation coupled with sucrose cushion does not provide the best exosome purity for proteomics study. A density gradient ultracentrifugation method is recommended for proteomic exosome preparation if no commercial products are desired.
- Gene Ontology cellular component analysis of the high purity serum exosome proteins shows that the highest portion of proteins are cell part proteins, then organelle and extracellular region proteins.
- This work evaluated exosome isolation products/procedures based on MS-based proteomic analysis and western blot. The results do not reveal the performance of these products/procedures with other exosome cargo, including RNA and lipids, where high purity of exosomes is not strictly required.

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