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Proteomic toolbox to standardize the separation of extracellular vesicles and lipoprotein particles

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Abstract

Circulating in blood, extracellular vesicles (EVs) and lipoprotein particles (LPs) have diagnostic and prognostic value. To unambiguously define their functions, separation protocols need to be developed. However, because of their similar size and density, traditional approaches to separate EVs and LPs often fail to provide the required resolution. Further development and standardization of affinity-based protocols is necessary and a quantitative method is needed to assess the efficiency of LPs depletion from EVs samples. In the present study, we propose the simultaneous quantification of three groups of proteins by mass spectrometry as a toolbox to evaluate prospective separation protocols. We generated ¹⁵N-labeled internal standards for quantification of (i) EVs-specific proteins, (ii) all classes and subclasses of apolipoproteins constituting LPs, and (iii) several major serum proteins. These standards were then used in multiple reaction monitoring assay to evaluate the performance of size exclusion chromatography, heparin-Sepharose, lipopolysaccharide-Sepharose, (2-hydroxypropyl)- β -cyclodextrin-Sepharose, and concanavalin A-Sepharose in separating serum EVs and LPs. The efficiency of a resin to separate EVs from non-EVs substances could be jeopardized by simultaneous EVs aggregation. Therefore, dynamic light scattering analysis was used in this study in addition to the proteomic toolbox when making a recommendation to use particular resin for EVs isolation. Based on our measurements, we concluded that none of the individual separation protocols used in this study resulted in LPs-free EVs and combination of two protocols may be complex due to low EVs yield. Overall, this further points to the importance of proposed proteomic toolbox for the future evaluation of EVs separation protocols.

Graphical Abstract

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Author Contributions

T.W. and I.V.T. designed the research, performed experiments, analyzed results, and wrote the paper.

SUPPORTING INFORMATION

The following supporting information is available free of charge at ACS website <http://pubs.acs.org>.

Keywords

extracellular vesicles; lipoprotein particles; separation; standardization; targeted proteomics; QconCATs; multiple reaction monitoring; size exclusion chromatography; heparin-Sepharose; chylomicrons

INTRODUCTION

Extracellular vesicles (EVs) are stable membrane structures that can deliver their cargo remotely and regulate fundamental cellular responses. Over the last two decades, a number of studies have demonstrated the pivotal role of EVs in intercellular communication of all multicellular organisms. One of the most exciting fields of EVs research is associated with their ability to harbor various RNA species, which have the potential to become diagnostic and prognostic biomarkers of different health conditions.^{1–4} However, lipoprotein particles (LPs) that are abundant in human circulation have a similar size and density range to EVs and are typically co-isolated with EVs.^{5–8} Given that LPs have been shown to carry extracellular microRNAs as well,^{9–11} isolation protocols which provide separation of EVs and LPs will help discriminate between EVs and non-EVs RNAs and unambiguously define the EVs functions.¹²

LPs are very heterogeneous in size and composition, predisposed to aggregation, and are present in circulation at concentrations several orders of magnitude higher than EVs. We propose that the development of a quantitative mass spectrometry method to simultaneously measure concentrations of three groups of proteins, (i) EVs-specific proteins, (ii) all classes and subclasses of apolipoproteins constituting LPs, and (iii) several major serum proteins, will provide a toolbox for evaluation of separation protocols and provide a better understanding of the prospects and limitations of their applications.

Based on density gradient ultracentrifugation, LPs can be separated into high-density, low-density, intermediate-density, very-low-density, and ultra-low-density lipoproteins (HDL, LDL, IDL, VLDL, and ULDL, respectively).¹³ ULDL are commonly called chylomicrons. Classes and sub-classes of apolipoproteins include 15 proteins, namely apo A-I, apo A-II, apo A-IV, apo A-V, apo B-48, apo B-100, apo C-I, apo C-II, apo C-III, apo C-IV, apo D, apo E, apo H, apo J, and apo M.

Although apo A-I is the major apolipoprotein in HDL and apo B-100 is the major apolipoprotein in LDL and VLDL, the overall protein composition of LPs is complex and variable. This is due to the apolipoprotein exchange between different particles.^{13, 14} For example, many major apolipoproteins (such as apo A-I, apo A-IV, apo A-V, apo C-III, and

apo E) are also called exchangeable apolipoproteins, because they are able to dissociate from one lipoprotein and reassociate with another lipoproteins in the circulation.¹⁴ Even more, some apolipoproteins can appear in a lipid-free form.¹⁵ In addition, fragments of apolipoproteins can also be found associated with LPs.¹⁶ Taking all of these levels of complexity of LPs composition, we have to acknowledge that the measurement of a specific apolipoprotein in the sample is not necessarily attributed to the presence of specific LPs in the same sample.

We first developed ¹⁵N-labeled quantification concatamers (QconCATs), which can be used as internal standards,^{17, 18} to quantify all of the 15 human apolipoproteins using a multiple reaction monitoring (MRM) assay.^{19, 20} The generation of QconCATs for quantification of common EVs proteins and major serum proteins has been recently reported.²¹ Taken together, this pattern of internal standards permitted us to evaluate selected chromatography methods in terms of their efficiency in LPs depletion from EVs preparations. In the present study, the selected separation methods include size exclusion chromatography (SEC) and affinity chromatography on heparin-Sepharose, lipopolysaccharide (LPS)-Sepharose, (2-hydroxypropyl)- β -cyclodextrin (HCD)-Sepharose, and concanavalin A (ConA)-Sepharose. The rationale for their use is summarized below.

SEC is broadly used to separate particles based on their size and has been reported to isolate EVs since the time of their discovery.²² Gradually, it has become a popular technique for EVs isolation since it is less prone to contamination from non-vesicular proteins and macromolecules than ultracentrifugation.^{23–25} However, separation of EVs and LPs based on particle size has a conceptual limitation. EVs are a heterogeneous group of particles and currently there is no consensus on the exact size range of EVs.⁵ The size of LPs could also cover a broad range from approximately 10 nm to hundreds of nm and overlap with the general particle size range of EVs.

Affinity chromatography assumes the existence of affinity binding sites on the target for isolation. For example, it was previously reported that the addition of heparin to EVs almost completely blocked their uptake by 293T cells and a follow up study showed that heparin can directly interact with EVs.²⁶ This provides us with the rationale to use immobilized heparin to separate EVs and LPs. Additionally, it is well documented that LPS circulating in blood binds to LPS-binding protein, which can be associated with LPs.^{27, 28} This suggests the possibility of using immobilized LPS for depletion of LPs from EVs samples. It is also well established that β -cyclodextrins interact with cholesterol and can shuttle cholesterol between cell membranes and serum LPs.^{29, 30} Therefore, immobilized HCD has the potential to be used as an affinity matrix to separate EVs and LPs. Lastly, both EVs and LPs carry glycosylated proteins with a different pattern of glycosylation.^{31, 32} Lectins are proteins that can reversibly and highly specifically bind glycoproteins, giving credence to use immobilized lectin, ConA, for potential EVs and LPs separation.

EXPERIMENTAL SECTION

¹⁵N-Labeled QconCAT expression, purification, and characterization

The amino acid sequences of LP1 and LP2 QconCATs designed for quantification of 15 apolipoproteins are shown in Figure S1 (Supporting Information). The synthetic genes encoding these sequences were synthesized and incorporated into the pET21a expression vector with codon optimization for *E. coli* (Biomatik, Cambridge, Ontario). The plasmid was transformed into One Shot BL21 (DE3) competent *E. coli* cells (Invitrogen, Grand Island, NY) and grown in M9 minimal media with 1 g/L ¹⁵NH₄Cl (Cambridge Isotope Laboratories, Andover, MA) as the sole nitrogen source at 37 °C until the optical density reached 0.6 to 0.8 at 600 nm. Protein expression was induced by 0.5 mmol/L isopropyl β-D-1-thiogalactopyranoside. After 3 h of growth, the cells were harvested by centrifugation at 5000 g for 10 min. QconCATs were expressed as insoluble inclusion bodies. Collected cells were first washed with water and then disrupted by sonication in water. QconCATs were collected by centrifugation at 5000 g for 10 min and dissolved with 7 mol/L urea in water with sonication. After removal of insoluble material by centrifugation at 5000 g for 10 min, the samples were supplemented with 2 % SDS and separated using SDS polyacrylamide gel electrophoresis. The proteins were eluted from the gel at 100 mA for 40 min into 14 fractions using a Mini Whole Gel Eluter (Bio-Rad Laboratories, Hercules, CA). The fractions containing QconCATs as compared to molecular mass standards were collected and concentrated using Amicon Ultra-4 centrifugal filters with 10K molecular weight cutoff. The QconCAT concentration was measured in the presence of 2 % SDS using Nanodrop 2000 (Thermo Fisher Scientific, Wilmington, DE). Sample (2 μL) was applied for each measurement and the Protein A280 module was selected to determine the concentration of the QconCAT. The purified QconCATs were aliquoted and kept frozen at –20 °C. Expression, purification, and characterization of ¹⁵N-labeled QconCATs to quantify major serum proteins (albumin, transferrin, and α-2-macroglobulin (A2M)) and EVs proteins (moesin, flotillin-1, TSG101, L1CAM, HSPA8, HSP90AA1 and HSP90AB1) was previously described.^{21, 33}

Optimal MRM transitions for Q-peptides were experimentally selected after tryptic digestion of QconCATs (Table S1, Supporting Information). These transitions were then used to determine the level of stable isotope incorporation into the QconCATs. The isotope incorporation was calculated as the percentile of the area of the labeled peak to the sum of the unlabeled and labeled peaks. Calculation based on three representative Q-peptides resulted in 98.5 ± 0.6 % and 98.6 ± 0.6 % isotope incorporation for LP1 and LP2 QconCATs, respectively (Table S2, Supporting Information). These values were accepted as a complete labeling and no correction for labeling efficiency was applied during data analysis.

Separation of EVs and LPs

Human serum was pooled from 10 individual male samples (BioreclamationIVT, Westbury, NY). The processing scheme is summarized in Figure S2 (Supporting Information). All centrifugations were performed at 4 °C. For initial sample processing, serum (1.5 mL/tube) was centrifuged at 2000 g for 15 min to collect the supernatant. This step was repeated once

and the supernatant was subjected to 12000 g centrifugation for 30 min. This step was repeated once and the supernatant was further centrifuged at 50000 rpm ($r_{\max}$ 154000 g) for 60 min using a Beckman TLA-55 rotor and TL-100 ultracentrifuge. The pellet was dissolved in PBS and centrifuged again at 50000 rpm for 60 min. The PBS washed pellet was denoted as EVs(154K).

For SEC, an AKTA FPLC (Amersham Biosciences, Piscataway, NJ, USA) was used to separate the EVs(154K) on a Superdex 200 Increase 10/300 GL column in PBS. The flow rate was 0.4 mL/min. The void volume fraction was collected and called EVs(SEC). AKTA FPLC was also used to separate EVs(154K) on HiTrap Heparin HP (1 mL) column (GE Healthcare). The column was equilibrated in PBS. EVs(154K) in PBS was loaded and the column was washed with 10 column volumes of PBS. The elution was performed with 20 mmol/L phosphate buffer (pH 7.3)/1.0 mol/L NaCl and called Heparin_{bound}.

Three more resins were evaluated for LPs separation from EVs(154K), including in-house synthesized LPS-Sepharose and HCD-Sepharose and commercially available ConA-Sepharose 4B (GE Healthcare). To prepare LPS-Sepharose, LPS from *Klebsiella pneumoniae* (Sigma-Aldrich, St. Louis, MO) was immobilized on CNBr-activated Sepharose 4 Fast Flow (Amersham Biosciences) following the manufacturer's protocol. The density of immobilization was calculated, based on the absorbance at 259 nm before and after immobilization, to be approximately 4 mg of LPS/mL. To prepare HCD-Sepharose, (2-hydroxypropyl)- β -cyclodextrin (Sigma-Aldrich, St. Louis, MO) was immobilized on epoxy-activated Sepharose 6B (Sigma-Aldrich, St. Louis, MO) in 0.1 mol/L NaOH at 37 °C for 17 hours.³⁴ The density of immobilization was calculated, based on the absorbance at 205 nm before and after immobilization, to be approximately 2.5 mg of HCD/mL.

Separation of EVs(154K) on these resins was performed in a batch mode. For this purpose, the EVs(154K) obtained from 30 mL of serum were dissolved in 800 μ L of 20 mmol/L phosphate buffer (pH 7.3) and aliquoted into 4 tubes (200 μ L per tube). One tube was used as a control. Three other tubes were supplemented with 0.4 mL of either LPS-, HCD-, or ConA-Sepharose, and gently agitated at room temperature every 5 min for 45 min. For the ConA-Sepharose sample, the binding buffer was supplemented with 0.1 mmol/L CaCl_2 and 0.1 mmol/L MnCl_2 . After agitation, the mixture was centrifuged at 10000 g for 5 min. The pellets were then washed with 1 mL of 20 mmol/L phosphate buffer (pH 7.3), centrifuged again, and supernatants were discarded. For ConA-Sepharose sample, the washing buffer was also supplemented with 0.1 mmol/L CaCl_2 and 0.1 mmol/L MnCl_2 . The washed pellets were eluted to obtain LPS_{bound}, HCD_{bound}, and ConA_{bound} samples, respectively. For elution from LPS- and HCD-Sepharoses, 200 μ L of 20 mmol/L phosphate buffer (pH 7.3)/0.5 mol/L NaCl was used. For elution from ConA-Sepharose, 200 μ L of 0.5 mol/L methyl- α -D-glucopyranoside was used.

Dynamic light scattering

A DynaPro NanoStar (556-DPN, WYATT Technology, USA) was used for the dynamic light scattering (DLS) measurements. The laser was set at $\lambda = 661$ nm with the detector angle at 90° for measurements. The samples were adjusted with PBS to give concentration ranges from 0.2 mg/mL to 2.0 mg/mL. PBS buffer was filtered through a Millipore Millex-GV 0.22

µm PVDF filter. DLS spectra were acquired in a disposable cyclic olefin copolymer cuvette at 25 °C. Each spectrum was collected over 5 runs consisting of 10 ten-second scans. The 5 runs were then averaged. Dynamics software (7.5.0.17, WYATT Technology, USA) was used to acquire and analyze the spectra. The regularization method was used to fit the autocorrelation functions.

Sample processing for mass spectrometry

The protein samples were processed in 50 mmol/L NH_4HCO_3 /2 % SDS or in PBS/2 % SDS. 2 µL was used for total protein concentration determination by Nanodrop 2000. Samples were supplemented with various amounts of specific ^{15}N -labeled QconCATs and 10 mmol/L dithiothreitol. After incubation for 60 min at room temperature, samples were treated with 30 mmol/L iodoacetamide for another 60 min in the dark and precipitated with chloroform/methanol.³⁵ The pellets obtained from precipitation were sonicated in 100 µL of 50 mmol/L NH_4HCO_3 /0.1 % RapiGest and treated with trypsin (1:2.5 w/w) for 15 h at 37 °C. After trypsinolysis, the samples were acidified with 0.5 % trifluoroacetic acid for 30 min at 37 °C and centrifuged at 16000 g for 30 min to collect the supernatant. Finally, samples were dried in a vacuum centrifuge (Eppendorf AG, Hamburg, Germany), redissolved in water, and dried again.

LC-MS/MS Analysis

For LC-MS/MS analysis, dried peptides were reconstituted in 3 % acetonitrile/ 0.1 % formic acid (volume fraction) in water. Separation was performed on an Agilent Zorbax Eclipse Plus C18 RRHD column (2.1 mm × 50 mm, 1.8 µm particle) and MRM analysis was performed on an Agilent 6490 iFunnel Triple Quadrupole LC/MS system (Santa Clara, CA). Peptides were eluted at a flow rate of 200 µL/min using the following gradient of solvent B in solvent A: 3 % B for 3 min, 3 % to 30 % B in 30 min, 30 % to 50 % B in 5 min, and 50 % to 3 % B in 5 min. Solvent A was water containing 0.1 % formic acid (volume fraction) and solvent B was acetonitrile containing 0.1 % formic acid (volume fraction). The acquisition method used the following parameters in positive mode: fragmentor 380 V, collision energy 20 V, dwell time 100 ms, cell accelerator 4 V, electron multiplier 500 V, and capillary voltage 3500 V. MRM transitions for 2+ charge precursor ions and 1+ charge product ions were predicted using PinPoint software (Thermo Fisher Scientific, Waltham, MA).

Data analysis

MRM peak area integration was performed using Skyline (4.1.0.11714) (University of Washington). Excel was used to calculate peak area ratios. Peak integration was manually inspected and adjusted, if necessary. The peak ratios from transitions were averaged to yield the peptide ratios. All experiments were performed in duplicate with three replicate injections. Data are represented as the mean ± SD.

RESULTS AND DISCUSSION

Design of QconCATs for MRM Assay

There are eight classes of apolipoproteins and several sub-classes.¹³ We have selected tryptic peptides from all of the human apolipoproteins and assembled them into two QconCATs,

LP1 and LP2. Peptides were selected so that they did not include cysteine and methionine residues and were 8–16 amino acid residues in length. In addition, these peptides were supplemented with six amino acid long extensions from their natural sequences on both sides of the peptides.^{18, 36} To prevent oxidation and disulfide bond formation, the methionine and cysteine residues in the extension sequences were replaced with isoleucine and alanine residues, respectively. The amino acid sequences of LP1 and LP2 QconCATs are shown in Figure S1 Supporting Information. LP1 includes tryptic peptides from apo A-I, apo A-II, apo A-IV, apo A-V, apo C-I, apo C-II, apo C-III, apo C-IV, and apo J. LP2 includes tryptic peptides from apo B-100/B-48, apo B-100, apo D, apo E, apo H, and apo M.

We note here that despite our efforts, we were not able to quantify apo H and apo M. While they are not major apolipoproteins, both proteins are broadly reported as components of various LPs.³⁷ The signal for apo H in serum was low and non-reproducible; therefore, data for apo H were omitted. In addition, the signal from apo M was not detected at all, in either serum or from LP2 QconCAT, presumably because of the poor MRM performance of Q-peptides selected from apo M.

Removal of Chylomicrons

Human apo B-100 is a 4535 amino acid residues protein, which can be translated as a truncated protein, apo B-48. Apo B-48 represents the 2151 N-terminal amino acid residues of apo B-100. Therefore, quantification based on peptides from the N-terminal portion of apo B-100 actually results in their combined concentration (apo B-100/B-48) while quantification based on peptides from the C-terminal portion of apo B-100 represents the concentration of apo B-100 only. Taken together, these measurements can determine whether apo B-48 is present in a particular biological sample. This is an important analytical measurement because apo B-100 is a major protein constituent of LDL and VLDL, while apo B-48 is a major protein constituent of chylomicrons.¹³ Chylomicrons are large lipoprotein particles (200–600 nm in diameter), which consist of 98–99% of lipids and only 1–2% of proteins.¹³ Due to their buoyancy, they create a creamy top layer after low-speed centrifugation. In this study, chylomicrons-free serum (Figure 1) was carefully collected from the bottom of the tube after 12000 g centrifugation using long gel-loading tips. Figure 2 shows the quantification of apo B-100 and apo B-100/B-48 in EVs(154K). Measurements were performed based on three C-terminal peptides (given values for apo B-100 only) and based on three N-terminal peptides (given values for the sum of apo B-100 and apo B-48). The amount of apo B-100 and apo B-100/B-48 in the EVs(154K) sample was not statistically different. This is an important observation, which proves that a careful collection of the supernatant after low-speed centrifugation is sufficient for removal of chylomicrons from EVs preparations.

SEC

EVs(154K) were separated on the Superdex 200 Increase 10/300 GL column in PBS (Figure 1) and the void volume was collected as EVs(SEC). We first measured concentrations of specific proteins in both EVs(154K) and EVs(SEC), and then made an adjustment to the volume of both samples and calculated the total amount of specific protein in EVs(154K) and EVs(SEC). This allowed us to present the data as a percentile of recovery, which is the

amount of a specific protein in EVs(SEC) divided by the amount of this protein in EVs(154K) and multiplied by 100. This value reflects both the efficiency of EVs separation and the EVs yield after chromatography.

The first observation from this analysis is related to EVs proteins themselves (Figure 3A). If membrane proteins (moesin, flotillin-1, and EHD4) can be safely called EVs proteins, the classification of molecular chaperons (HSPA8, HSP90AA1, and HSP90AB1) as EVs proteins is less obvious. Lower recovery for HSP90AA1 and HSP90AB1 than membrane EVs proteins raises a question of whether these molecular chaperons are EVs proteins or proteins commonly co-isolated with EVs. Furthermore, higher recovery for HSPA8 than membrane EVs proteins likely results from the aggregation of HSPA8 and subsequent co-isolation with EVs on SEC.

Another observation from this analysis is related to the major serum proteins (Figure 3A). The insert shows that only a small amount of the major serum proteins was recovered in EVs(SEC): 0.6 % of albumin, 0.3 % of A2M, and 1.4 % of transferrin. This makes SEC a high priority separation technique for depletion of serum proteins from EVs samples.

Finally, low recovery for several apolipoproteins makes SEC a good choice for the separation of EVs and LPs: apo B-100 (3 %), apo C-I (1.7 %), apo C-II (1.8 %), apo D (3.6 %), and apo E (3.6 %) were well depleted.

Apo B-100 is a non-exchangeable apolipoprotein and is a major protein constituent of LDL and VLDL. Based on the 3 % recovery of apo B-100 versus 41 % for moesin and 53 % for flotillin-1, we can conclude that SEC efficiently depletes LDL and VLDL from EVs(SEC). At the same time, the 10% recovery for apo A-I and apo A-V was unexpected. Apo A-I and apo A-V are a major component of HDL, but they are also exchangeable apolipoproteins and can be found in various lipid-bound and lipid-free forms. Why SEC has a moderate ability in depleting apo A-I and apo A-V from EVs is not clear since the diameter of HDL (7–13 nm) is smaller than LDL (21–27 nm). Presumably, either aggregation of HDL or presence of other forms of apo A-I and apo A-V contributes to their moderate separation from EVs on SEC.

While interpreting the SEC results, we have to keep in mind that the original concentration of major serum proteins, molecular chaperons, and apolipoproteins is several orders of magnitude greater than the concentration of EVs. Even 1 % recovery can keep these contaminating proteins at concentrations far greater than EVs; therefore, additional steps of isolation are required. This statement concurs well with DLS data for EVs(SEC) shown in Figures 3B and 3C. When presented by *number*, the graph shows a single peak at around 7 nm, which is too small for EVs and better fits to the expected size of HDL (Figure 3B). Consistent with MRM quantification, apo A-I and apo A-V remains a major contamination of the EVs(SEC). When presented by *intensity*, the graph shows a broad peak at 170 nm (Figure 3C). This broad peak has high polydispersity, suggesting the presence of multiple species, namely EVs and probably some aggregates of LPs.

Heparin-Sepharose

Heparin-Sepharose has been used for EVs isolation (Figure 1) because heparin directly interacts with EVs.²⁶ However, it is also well established that apo B-100 and apo E of LDL bind heparin.³⁸ Apo B-100 actually has 7 heparin-binding sites.³⁹ Figure 4A shows that the chromatography on heparin-Sepharose is good for removal of apo A-I, apo A-II, and apo A-IV, which were recovered on the level of 1.5 % only, and apo A-V was not detected at all. As expected, the recovery of LDL in Heparin_{bound} was high. Apo B-100 and apo E were recovered at 30 % and 27 %, respectively.

Very low recovery of albumin, A2M, and transferrin in Heparin_{bound} can be seen as obviously positive. However, this conclusion can be somewhat misleading for EVs isolation from plasma, which has a large group of heparin-binding proteins (such as thrombin and antithrombin III), which were not measured in the present study, but presumably can be enriched in EVs samples after heparin-Sepharose chromatography.

DLS data for Heparin_{bound} look similar to those of EVs(SEC). The broad peak in distribution by intensity probably represents EVs (Figure 4C). However, distribution by number still points to particles much smaller than EVs as a major component of the sample (Figure 4B).

LPS-Sepharose

LPS is detoxified in the circulation by incorporation into the LPs and all LPs were reported to bind LPS.⁴⁰ However, depending on the physiological conditions, the preference of LPS binding to HDL, LDL, and VLDL varies broadly.²⁸ Our expectation was that the separation on LPS-Sepharose (Figure 1) will result in the selective depletion of certain classes of apolipoproteins. Figure 5A shows that this was not the case. With a few small exceptions, all measured apolipoproteins in LPS_{bound} were detected at the similar level, from 5 % to 7 % recovery. Basically, the affinity of LPS binding in vivo is pre-determined by the presence of LPS-binding protein and by properties of two major apolipoproteins, apo A-I and apo B-100. Our in vitro measurements show that the LPS-Sepharose does not discriminate between apo A-I and apo B-100 and in general shows relatively low-affinity to all apolipoproteins. This can be seen as a positive result because the recovery for EVs protein moesin was 23%. There are no reports of direct LPS binding to EVs, although LPS interacts with cell surface receptors and therefore potentially could interact with any particle derived from the biological membrane, including EVs.

The enthusiasm for using LPS-Sepharose for EVs isolation was, however, decreased by the DLS data. Although not being a direct proof, the broad peak at 255 nm in Figure 5C could be interpreted as the EVs aggregation. This is an important observation because there is a concern that certain column separations may cause EVs aggregation. Good efficiency of a resin to separate EVs from non-EVs substances could be jeopardized by simultaneous EVs aggregation. We show here the importance in using size-assessing methods while making a recommendation to use particular resin for EVs isolation.

HCD-Sepharose

The only reason to use the HCD-Sepharose to separate EVs and LPs (Figure 1) was the ability of HCD to chelate cholesterol.^{29, 30} It was expected that this ability of HCD could lead to the selective separation of the different classes of apolipoproteins and EVs on HCD-Sepharose. Indeed, recovery of apolipoproteins in HCD_{bound} was as low as 5 % to 7 % while moesin shows 27 % recovery (Figure 6A), and this is a positive sign for the HCD-Sepharose application. Recovery of major serum proteins in a range from 2 % to 3.5 % only is also a positive sign. Initial serum protein concentrations are so much higher than EVs that every resin recommended for EVs isolation needs to demonstrate as low as possible recovery of major serum proteins.

However, as with LPS-Sepharose, DLS data for HCD-Sepharose once again raised a concern of EVs aggregation (Figure 6C). In addition to chelating cholesterol, HCD has an ability to extract cholesterol from biological membranes.²⁹ This may be relevant to DLS data in Figure 6C and require a separate consideration before any recommendation is made.

ConA-Sepharose

All apolipoproteins are glycoproteins.^{32, 41–43} There is evidence that EVs carry oligo- and polysaccharides on their surface and can interact with ConA.^{31, 44} The rationale to use ConA-Sepharose for EVs and LPs separation (Figure 1) is based on the different affinity of different glycoproteins to ConA and the probability that this would allow the selective depletion of some apolipoproteins from EVs sample. Figure 7A shows that the recovery of EVs proteins in ConA_{bound} was slightly higher than the recovery of apolipoproteins. However, even with the highest value for moesin of 13 %, the yield is still too low to be recommended for EVs isolation. Recovery of albumin (4.5 %) and transferrin (6.5 %) was also too high to seriously consider ConA-Sepharose for EVs isolation. DLS data are positively assuming no EVs aggregation in ConA_{bound} (Figure 7C), but as with all previous separations, EVs remain a minor component of the sample (Figure 7B).

SEC and Heparin-Sepharose

Up to this point, our study suggests that the combination of SEC and heparin-Sepharose could be an efficient method for basic separation of EVs and LPs. SEC is efficient in depleting apo B-100 while less efficient in depleting apo As. Heparin-Sepharose has an opposite action, it efficiently depletes several apo As while keeping apo B-100. Figure 8 shows that the addition of heparin-Sepharose after SEC further decreases the level of apo A-I (0.6 % recovery), apo A-II (0.2 % recovery), and apo A-IV (not detected at all). It also decreased the recovery of apo C-II, apo C-III, and apo C-IV to as low as 0.05 %. However, the major concern is associated with the overall low yield of EVs after two-step isolation. Therefore, the combination of SEC and heparin-Sepharose results in a better purity of EVs sample, but will require the upscale of the original serum sample taken for isolation in order to obtain a practical amount of EVs.

CONCLUSIONS

We have demonstrated that the mass spectrometry-based simultaneous quantification of (i) EVs-specific proteins, (ii) all classes and subclasses of apolipoproteins, and (iii) several major serum proteins provides a robust approach to evaluate the efficiency of EVs and LPs separation protocols. As a proof of principle, six separation protocols were evaluated to reveal their advantages and disadvantages.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

A2M	α -2-macroglobulin
ConA	concanavalin A
EVs	extracellular vesicles
HCD	(2-hydroxypropyl)- β -cyclodextrin
LPS	lipopolysaccharide
MRM	multiple reaction monitoring
QconCATs	quantification concatamer
SEC	size exclusion chromatography

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Figure 1. Scheme depicting separation protocols used. Extended scheme summarizing conditions of separation is shown in Figure S2, Supporting Information.

Figure 2. Removal of chylomicrons. Apo B-100 alone and combined amount of apo B-100 and apo B-48, apo B-100/B-48, were measured in EVs(154K) sample. All experiments were performed in duplicate with three replicate injections. Data is represented as the mean $\pm$ SD.

Figure 3.

Representative SEC separation of EVs(154K) sample. (A) MRM analysis shows a percent recovery of apolipoproteins, major serum proteins, and EVs proteins in void volume fraction called EVs(SEC). (B and C) DLS analysis of EVs(SEC). The size distribution of particles is presented by *number* (B) and by *intensity* (C).

Figure 4.

Representative separation of EVs(154K) sample on heparin-Sepharose. (A) MRM analysis shows a percent recovery of apolipoproteins, major serum proteins, and EVs proteins in 20 mmol/L phosphate buffer/1.0 mol/L NaCl elution fraction called Heparin_{bound}. (B and C) DLS analysis of Heparin_{bound}. The size distribution of particles is presented by *number* (B) and by *intensity* (C).

Figure 5.

Representative separation of EVs(154K) sample on LPS-Sepharose. (A) MRM analysis shows a percent recovery of apolipoproteins, major serum proteins, and EVs proteins in 20 mmol/L phosphate buffer/0.5 mol/L NaCl elution fraction called LPS_{bound}. (B and C) DLS analysis of LPS_{bound}. The size distribution of particles is presented by *number* (B) and by *intensity* (C).

Figure 6.

Representative separation of EVs(154K) sample on HCD-Sepharose. (A) MRM analysis shows a percent recovery of apolipoproteins, major serum proteins, and EVs proteins in 20 mmol/L phosphate buffer/0.5 mol/L NaCl elution fraction called HCD_{bound}. (B and C) DLS analysis of HCD_{bound}. The size distribution of particles is presented by *number* (B) and by *intensity* (C).

Figure 7.

Representative separation of EVs(154K) sample on ConA-Sepharose. (A) MRM analysis shows a percent recovery of apolipoproteins, major serum proteins, and EVs proteins in 0.5 mol/L α -D-methylglucoside elution fraction called ConA_{bound}. (B and C) DLS analysis of ConA_{bound}. The size distribution of particles is presented by *number* (B) and by *intensity* (C).

Figure 8. Representative two-step separation of EVs(154K) sample on SEC and heparin-Sepharose. The individual separation protocols are identical to those described for SEC and heparin-Sepharose in the Experimental Section. MRM analysis shows a percent recovery of apolipoproteins, major serum proteins, and EVs proteins.