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A Comparison of Methods To Enhance Protein Detection of Lipoproteins by Mass Spectrometry

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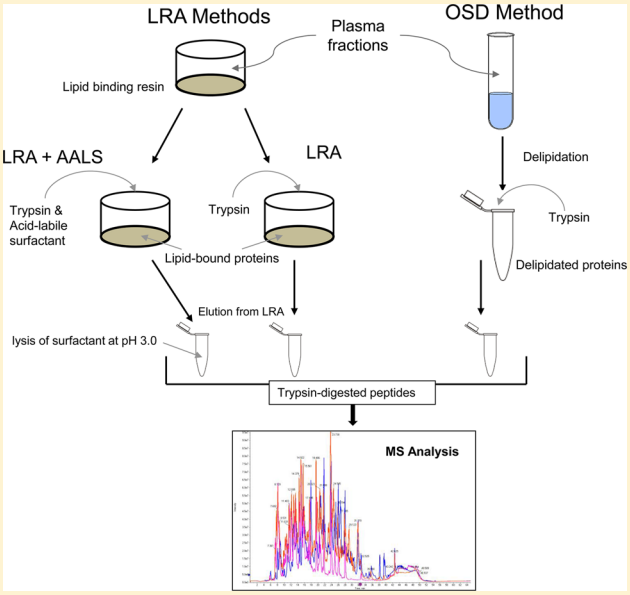
Supporting Information

ABSTRACT: We sought to develop a new method to more efficiently analyze lipid-bound proteins by mass spectrometry using a combination of a lipid removal agent (LRA) that selectively targets lipid-bound proteins and a mass spectrometry compatible detergent, anionic acid labile surfactant (AALS), that is capable of eluting proteins off the LRA. This method was compared to established methods that use the lipid removal agent alone and straight proteomic analysis of human plasma after organic solvent delipidation (OSD). Plasma from healthy individuals was separated by gel filtration chromatography and prepared for mass spectrometry analysis by each of the described methods. The addition of AALS to LRA increased the overall number of proteins detected in both the high and low density lipoprotein size range, the number of peptide counts for each protein, and the overall sequence coverage. Organic solvent delipidation detected the most proteins, though with some decrease in overall protein detection and sequence coverage due to the presence of nonlipid-bound proteins. The use of LRA allows for selection and analysis of lipid-bound proteins. The addition of a mass spectrometry compatible detergent improved detection of lipid-bound proteins from human plasma using LRA.



KEYWORDS: *lipoproteins, mass spectrometry, detergents, lipids, surfactant*

INTRODUCTION

Plasma lipoproteins, such as low- and high-density lipoproteins (LDL and HDL), play important roles in lipid transport and metabolism. The roles of these lipoproteins are largely mediated by the sequence and structure of their associated apolipoproteins (apo), apoB for LDL and apoA-I for HDL. Prior to 2000, both LDL and HDL were thought to contain <15 different proteins.[1] However, with the development and application of modern mass spectrometry (MS) techniques, our understanding of the proteomic diversity of both LDL and HDL has dramatically increased. Recent MS studies have identified proteins that point to novel roles for HDL including complement activation, anti-inflammation,[2] hemostasis, vitamin transport, and even glucose metabolism.[3,4] Similarly, LDL may also have a role in complement activation and hemostasis.[5,6]

While modern MS technology has increased the ability to identify proteins, there is concern that some of the proteins may not be truly lipoprotein associated. For example, upward of 200 proteins have been described to associate with HDL, but given HDL size, it is not possible for all of these proteins to be associated with HDL. In fact, only about 90 or so have been described in ≥ 3 or more studies by independent laboratories, probably representing the best estimate of the HDL proteome.[4] The other 115+ proteins include intracellular and cell surface proteins, antibodies, immunoglobulins, and even skin keratin. These findings suggest that improved MS methods are needed to detect proteins truly associated with lipoproteins.

Historically defined by density, lipoproteins are most commonly isolated by density-gradient ultracentrifugation into three major classes, VLDL, LDL, and HDL.[7] Our laboratory has elected to analyze lipoproteins by size through gel-filtration

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chromatography because we and others have noted fewer alterations in the particle proteome compared with ultracentrifugation.[8,9] This gel filtration chromatography method in combination with a calcium silicate hydrate resin (trade name lipid removal agent (LRA)) was originally developed to selectively target lipid associated proteins for proteomic analysis directly in plasma. Previously, the most common way to analyze lipoprotein proteomes was to first isolate lipid containing particles by density ultracentrifugation separation prior to the MS analysis. The LRA method opened up the possibility of studying plasma lipoproteins separated by a host of non-centrifugal methods including gel filtration, anion exchange, and isoelectric focusing.[9,10] One potential drawback of the LRA method is the fact that phospholipids bind nearly irreversibly to the resin. Ideally, the resulting peptides are released from the lipoproteins using trypsin, eluted from the resin, and analyzed. However, there are several issues that could result in overall poor peptide yield by this method. First, only the most polar and exposed regions of lipid-bound proteins are likely accessed by trypsin. As a result, some peptides may be either buried in lipid or remain associated with it even when proteolyzed. Thus, there may be a selection bias in the analysis. Second, steric issues caused by the interaction between trypsin and the resin may limit the extent of proteolysis. Third, even if liberated, some peptides may nonspecifically associate with the resin and therefore escape detection. Thus, we have expended considerable effort to develop ways to elute LRA bound lipoproteins in their native form by altering pH, ionic strength, competition, and mild organic solvents. Unfortunately, none of these approaches has been successful to date. In fact, the only way we have found that reliably removes LRA bound proteins is by sodium dodecyl sulfate (SDS) detergent solubilization, a method that is incompatible with MS.

For the current study, we had two goals in mind. First, we sought to develop ways to more efficiently (if possible) elute and analyze LRA retained peptides. Since SDS is not MS compatible, we focused on one of the several hydrolyzable detergents that have recently been developed for MS. Anionic acid labile surfactant (AALS) is an anionic detergent with similar solubility properties to SDS, but upon exposure to acidic conditions, hydrolyzes into small, noninterfering organic molecules that are compatible with subsequent reverse-phase separations and MS ionization. We reasoned that AALS would result in (i) a more complete solubilization and elution of LRA bound proteins, and (ii) subsequent denaturation of proteins by the AALS could result in significant improvements in the number of proteins detected and the overall sequence coverage of each protein by MS. Second, we sought to compare both LRA and LRA+AALS to a straight proteomic analysis of gel filtration fractions from human plasma prepared by organic solvent delipidation (OSD) that had not been exposed to LRA.

MATERIALS AND METHODS

Participants

Three healthy, nonsmoking, normolipidemic (plasma cholesterol <190 mg/dl and plasma triglycerides <150 mg/dl) males with a mean age of 26 years were recruited. Fasting blood lipids levels were confirmed. A second aliquot of blood was collected in BD Vacutainer (BD Biosciences) tubes using citrate as anticoagulant and spun at ∼1590*g* for 15 min at room temperature (25 °C) to isolate plasma. Plasma was stored at 4 °C and was never frozen. This study was reviewed and approved by the institutional review board at Cincinnati Children's Hospital Medical Center, and all participants provided informed consent.

Plasma Separation by Gel Filtration Chromatography

Within 4 h of collection, 370 μL of plasma was separated via three Superdex 200 columns arranged in series (GE Healthcare) as previously described.[10] Eluate was collected as 47 fractions in 1.5 mL volumes maintained at 4 °C. Fractions 13−30 contain detectable phospholipid that corresponds to plasma lipoproteins LDL/HDL. To relate gel filtration results to traditional density-centric definitions, we use the presence of apoB, the core constituent of LDL, as the key distinguisher. Therefore, the VLDL/LDL range is defined as fractions 13−19 due to the presence of apoB. We assigned the remaining fractions 20−30 as the HDL range because their diameters are consistent with measurements for density-isolated HDL and because of the abundance of the major HDL protein, apoA-I. Supplement Figure 1 of the Supporting Information shows the choline containing phospholipid (PL) content (Wako) of our gel filtration plasma fractions compared to LDL and HDL isolated by ultracentrifugation.

MS SAMPLE PREPARATION

Calcium Silicate Hydrate (LRA)

A commercially available synthetic calcium silicate hydrate, LRA (Supelco) was used to bind and isolate lipoprotein particles from each of the gel filtration fractions.[10] In a 96-well 0.45 μm filter plate (Millipore), 15 μL of LRA [100 mg/mL stock solution in 50 mM ammonium bicarbonate (AB buffer)] was added to 300 μL of each gel filtration fraction. The plate was mixed for 30 min at RT. The supernatant containing lipid-free plasma proteins was then removed from the plate using a vacuum manifold and later assayed for PL to ensure that the phospholipid-containing particles were fully bound to the LRA.[10] The LRA resin in the filter plate was washed three times with 150 μL of 50 mM AB buffer. One microgram of sequencing grade trypsin in a volume of 32.5 μL of AB buffer was then added to each well. The plate was sealed with parafilm to prevent evaporation and incubated at 37 °C overnight on a shaker plate. To ensure the complete digestion of proteins from the LRA resin, an additional 1 μg of trypsin was added to each sample in a volume of 16.3 μL of AB buffer, and the samples were incubated on a shaker plate for 2 h at 37 °C. Following digestion with trypsin, samples were eluted by vacuum manifold from the filter plate into a clean 96-well plate. The LRA resin was washed and eluted twice more with 50 μL of AB buffer. Peptides were reduced with dithiothreitol (10 mM concentration for 30 min at 37 °C) and then carbamidomethylated with iodoacetamide (40 mM concentration for 30 min at room temperature in the dark). Ninety-four microliters of each sample was transferred to a rinsed microfuge tube, lyophilized to dryness, and stored at −20 °C until analyzed by MS.

Calcium Silicate Hydrate + Anionic Acid Labile Surfactant I (LRA+AALS)

After the gel filtration fractions were incubated with LRA as above and the LRA resin was washed three times with 150 μL of AB buffer, each fraction was then resuspended in 1 μg of trypsin and 3.25 μL of 0.1% anionic acid labile surfactant I (AALS I, CMC 7.7 mM, Progenta) in a total volume of 32.5 μL of AB buffer. The sample plate was wrapped and incubated overnight at 37 °C. The trypsin digest and remainder of the protocol was continued the proceeding day in the same manner as above.

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Organic Solvent Delipidation (OSD, no LRA)

In this method, 300 μL of each gel filtration fraction was dialyzed into 50 mM AB buffer, transferred to glass tubes, and lyophilized. Powdered samples were resolubilized in 1 mL of ice-cold chloroform:methanol (2:1 v/v), vortexed, and incubated on ice for 30 min. One milliliter of ice-cold methanol was then added to each sample, and the tubes were centrifuged at 4000*g* for 30 min at 4 °C. The pellet was resuspended in 2 mL of ice-cold methanol and spun as before. The pellet was resuspended in 90 μL of 20% methanol/80% AB buffer. Samples were briefly sonicated to aid in solubilization. Samples were reduced by adding dithiothreitol to a final concentration of 10 mM and incubating for 30 min at 42 °C, and carbamidomethylated by adding iodoacetamide to a final concentration of 40 mM and incubating at room temperature in the dark for 30 min. Twenty microliters of each sample was then added to a rinsed microfuge tube and digested with 1 μg of sequencing grade trypsin at 37 °C overnight. The following day, an additional 1 μg of trypsin was added to each sample and incubated again at 37 °C for at least 1 h. Samples were then lyophilized to dryness and stored at −20 °C until MS analysis.

Nanoliquid Chromatography Coupled Electrospray Tandem Mass Spectrometry (nLC−ESI−MS/MS)

Prior to MS analysis, lyophilized fractions containing AALS were resuspended in 5 μL of a 1% trifluoroacetic acid (TFA) solution to cleave the AALS detergent into small, noninterfering organic molecules. After 20 min, 10 μL of high-performance liquid chromatography (HPLC)-grade water was added to adjust the sample to a 0.3% TFA concentration. LRA-prepared samples not containing AALS were resuspended in 15 μL of a 0.3% TFA solution. Samples that had been resuspended in equal volumes of TFA were then lyophilized to dryness once more.

All samples from each method were reconstituted in 50 μL of 0.1% formic acid and centrifuged at 10 000*g* for 10 min. Then 2 μL of the supernantant was diluted to a volume of 10 μL with 0.1% formic acid. nLC−ESI−MS/MS analyses were performed on a TripleTOF 5600+ (ABSciex, Toronto, On, Canada) attached to an Eksigent (Dublin, CA) nanoLC.ultra nanoflow system. Five microliters of the 0.1% formic acid solution was loaded (via an Eksigent nanoLC.as-2 autosampler) onto an IntegraFrit Trap Column (outer diameter of 360 μm, inner diameter of 100 μm, and 25 μm packed bed) from New Objective, Inc. (Woburn, MA) at 2 μL/min in formic acid/H_2O 0.1/99.9 (v/v) for 15 min to desalt and concentrate the samples. For the chromatographic separation of peptides, the trap-column was switched to align with the analytical column, Acclaim PepMap100 (inner diameter of 75 μm, length of 15 cm, C18 particle sizes of 3 μm, and pore sizes of 100 Å) from Dionex-Thermo Fisher Scientific (Sunnyvale, CA). The peptides were eluted using a variable mobile phase (MP) gradient from 95% phase A (formic acid/H_2O 0.1/99.9, v/v) to 40% phase B (formic acid/acetonitrile 0.1/99.9, v/v) for 35 min, from 40% phase B to 85% phase B for 5 min, and then keeping the same mobile phase composition for 5 more minutes at 300 nL/min. The nLC effluent was ionized and sprayed into the MS using NANOSpray III Source (AB Sciex, Toronto, On, Canada). Ion source gas 1 (GS1), ion source gas 2 (GS2), and curtain gas (CUR) were, respectively, kept at 15, 0, and 30 vendor specified arbitrary units. Interface heater temperature and ion spray voltage were kept at 150 C and at 2.5 kV, respectively. MS method was operated in positive ion mode set to go through 3993 cycles for 65 min, where each cycle performing one time-of-flight (TOF)-MS scan type (0.25 s accumulation time, in a 350−1800 m/z window) followed by 30 information-dependent acquisition (IDA)-mode MS/MS-scans on the most intense candidate ions having a minimum 250 counts. Each product ion scan was operated under vender specified high-sensitivity mode with an accumulation time of 0.075 s and a mass tolerance of 50 mDa. Former MS/MS-analyzed candidate ions were excluded for 15 s after its first occurrence, and data were recorded using Analyst-TF (v.1.6) software.

Mass Spectrometry Data Analysis

Acquired mass spectra were scanned against the UniProtKB/Swiss-Prot Protein Knowledgebase (release 2011, 533 657 sequences) for *Homo sapiens* (20 323 sequences) using Mascot (version 2.2.07) and X! Tandem (version 2010.12.01.1) search engines. Search criteria assumed digestion with the enzyme trypsin and included carbamidomethylation and Met oxidation as variable modifications, peptide tolerance set to ±35 ppm, MS/MS tolerance set to ±0.6 Da, and up to three maximum missed trypsin cleavage sites allowed. Validation of MS/MS-based peptide and protein identification using an X! Tandem subset search was performed within Scaffold (version 4.3.4, Proteome Software Inc., Portland, OR). Only peptides and proteins with >90% identification probability via both Mascot and X!Tandem, along with the Peptide Prophet algorithm[11] and Protein Prophet algorithm,[12] were considered in analysis. Protein identifications were accepted if they contained at least two identified peptides. Since equal starting volumes of sample were used in all three methods, the abundance of protein in a given fraction should be relative to the number of spectral counts (i.e., the number of MS/MS spectra assigned to a particular protein) in that fraction.[13] Raw spectral counts are presented, and no data normalization was performed.

RESULTS

Selection of the Anionic Acid Labile Surfactant (AALS)

Initial experiments were conducted to optimize the MS-compatible detergent needed to elute proteins from the LRA. Two MS-compatible detergents were chosen, each with a different critical micelle concentration (CMC), which influences solubility: AALS I (CMC 7.7 mM, Progenta) and AALS II (CMC 1.9 mM, Progenta). Comparisons were made to the non-MS compatible detergent SDS, which reliably elutes LRA bound proteins, and Triton X-100.

Compared to SDS, Triton X-100 (at 0.05% and 0.1%) and AALS I at 0.05% showed less protein recovery from the LRA by SDS-polyacrylamide gel electrophoresis (PAGE) gel. AALS I at 0.1% and AALS II at 0.1% and 0.05% were comparable to SDS (Supplement Figure 2, Supporting Information).

A determination of the PL loss from LRA in the presence of a detergent is important because lipid contamination in proteomic samples can damage the HPLC column and obscure MS results. We found that AALS I at both 0.1% and 0.05% eluted less PL from the LRA compared to both concentrations of AALS II. This was also less than with the use of SDS (Supplement Figure 3, Supporting Information). Increasing the amount of LRA resin used in this experiment showed no improvement in minimizing PL elution (data not shown).

To finalize the optimization of AALS, two samples were prepared for MS analysis using AALS I (0.1%) and AALS II (0.05%), which were conditions where we saw the greatest protein recovery and least PL coelution off the LRA.

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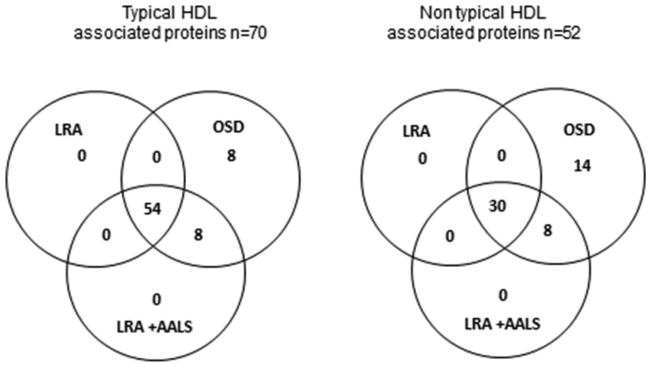

Figure 1. Venn diagram of the number of typical HDL associated and nontypical HDL associated proteins detected by MS. Number of proteins detected from HDL size range fractions (20−30) among three methods of preparation: lipid removal agent (LRA), lipid removal agent with anionic acid labile surfactant (LRA + AALS), and organic solvent delipidation (OSD).

Table 1. Presence or Absence of Lowest Abundance HDL Associated Proteins by Method[a]

	LRA	LRA+AALS	OSD
C4b-binding protein α-chain	−	−	+
zinc-α-2-glycoprotein	−	−	+
ApoM	−	+	+
ApoF	−	−	+
ApoL1	+	+	+
ApoC-II	+	+	+
afamin	−	+	+
glycoprotein phospholipase D	+	+	+
ApoD	−	+	+
comp. C2	+	+	+
sum	4	7	10

[a]Symbols indicate the presence (+) or absence (−) of the protein by each method.

Approximately 20% higher unweighted spectral counts were observed for apoA-I and apo A-II using AALS I (0.1%) versus AALS II (0.05%).

Results from the aforementioned experiments led us to use AALS I at a 0.1% concentration for subsequent MS preparations due to its compatibility with the MS, maximal protein recovery with minimimal PL contamination in the delipidated sample, and increased number of spectral counts detected by MS.

Comparisons of the Three MS Methods

Gel filtration fractions were prepared for MS analysis using each of the three different methods (LRA, LRA+AALS, OSD) in the HDL size range (fractions 20−30) and LDL size range (fraction 13−19).

First, we sought to determine which method detected the most proteins in the HDL size range. Proteins were classified as typical HDL associated versus nontypical HDL associated proteins, where typical HDL associated proteins were defined as those found to associate with HDL from three independent laboratories in ≥ three proteomic studies.[4] Nontypical HDL proteins are defined as those that have been identified as HDL associated, but only by a minority of laboratories ($n \leq 2$). Figure 1 shows that a total of 122 different proteins were identified across all three methods in the HDL range. Fifty-four typical HDL associated proteins were detected in all three methods. Eight additional proteins were detected with the addition of AALS to LRA. OSD identified these eight proteins and an additional eight. For a full list of HDL associated proteins identified by each method, see Supplement Table 1 of the Supporting Information. For nontypical HDL proteins, similar results were seen as above. LRA+AALS improved the number of proteins detected over LRA alone. OSD detected an additional 14 proteins (specific proteins not listed).

We next sought to determine if one method detected more low abundance HDL associated proteins. In this analysis, only typical HDL associated proteins were considered. Spectral counts were summed and ranked across fractions for known HDL associated proteins. The 10 proteins with the lowest total spectral counts in the HDL size range are listed in Table 1 and are marked as "+" if present by that method. OSD detected three additional low abundance HDL associated proteins compared to LRA+AALS. Both OSD and LRA+AALS detected three additional proteins over LRA alone.

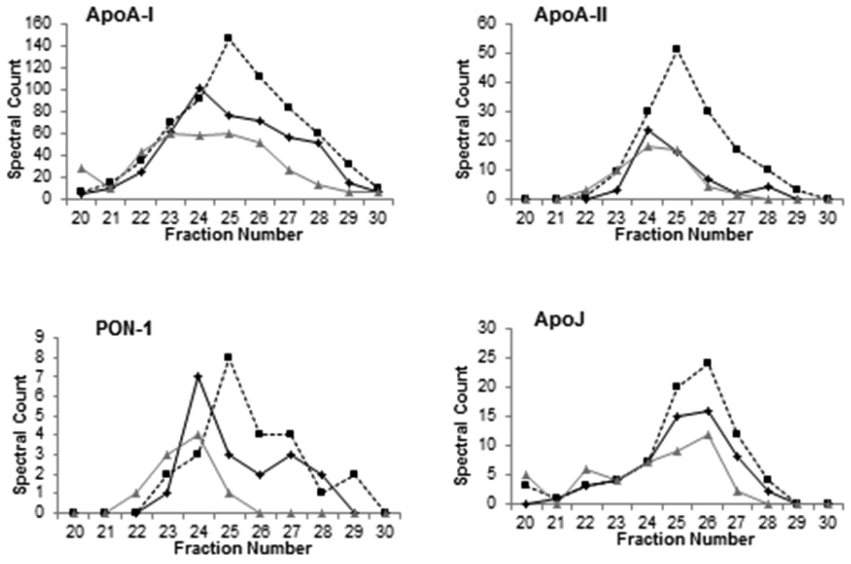

Figure 2. Unadjusted spectral counts for a given HDL associated proteins across gel-filtered plasma fractions in the HDL size range. LRA (black), LRA +AALS (dashed), and OSD (gray) methods.

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We next determined if one method improved spectral counts across the HDL lipoprotein range. All proteins were analyzed, and in general, all three methods located a given protein in similar regions of the elution profile as expected. However, we did note some key differences, with examples shown in Figure 2. For HDLs two most well recognized proteins, apoA-I and apoA-II, LRA+AALS improved spectral counts across the HDL size range compared to OSD and LRA alone. In other words, the addition of AALS to LRA produced taller peak profiles for these proteins. For PON-1 and apoJ (clusterin), less abundant but well-known HDL associated proteins, similar results were seen. The OSD method resulted in a blunted smaller peak with overall decreased spectral counts, whereas the LRA methods, particularly the LRA +AALS, resulted in a taller, more featured peak. In fact, the OSD method missed a peak shoulder (fractions 26–28) of PON-1 that is clearly apparent with both LRA methods. Table 2 shows the percent sequence coverage of some of HDL most recognized proteins. Addition of AALS to LRA improved the sequence coverage of almost all proteins.

Table 2. Percent (%) Sequence Coverage of Common HDL Associated Proteins[a]

	LRA	LRA+AALS	OSD
ApoA-I	55.8	62.6	44.9
ApoA-II	69.0	69.0	53.0
ApoA-IV	7.56	20.7	18.2
PON-1	8.5	22.0	3.9
ApoC-II		23.8	8.9
ApoC-III	16.2	27.3	27.3
ApoJ	19.6	23.8	19.4

[a]Percent sequence coverage of a given protein in fraction 25. These data were generated using Scaffold (version 4.3.4, Proteome Software Inc., Portland, OR).

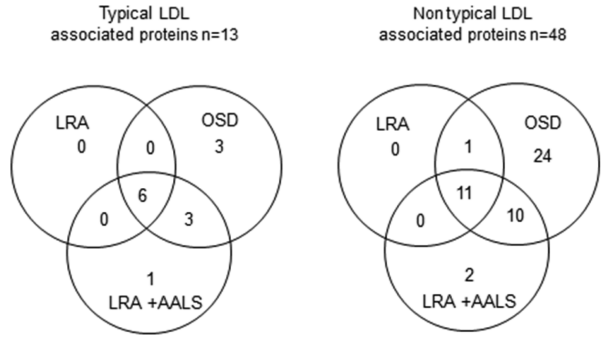

Figure 4. Venn diagram of the number of typical LDL associated and nontypical LDL associated proteins. Proteins detected by MS from LDL size range fractions (13–19) by LRA, LRA+AALS, and OSD.

Finally, we ranked the top 10 most abundant HDL proteins (as judged by overall spectral counts) detected by each method (Figure 3). ApoA-I was the top protein detected using the LRA +AALS method. Albumin was also the top protein for the LRA method, but with spectral counts comparable to ApoA-I. The top protein detected by OSD, with >1400 spectral counts, was albumin. ApoA-I was the fourth most abundant protein by OSD.

Similar analyses were conducted to evaluate proteins in the LDL size range. Proteins were classified as typical LDL associated versus nontypical LDL associated proteins, where typical LDL associated proteins were defined as those found to associate with

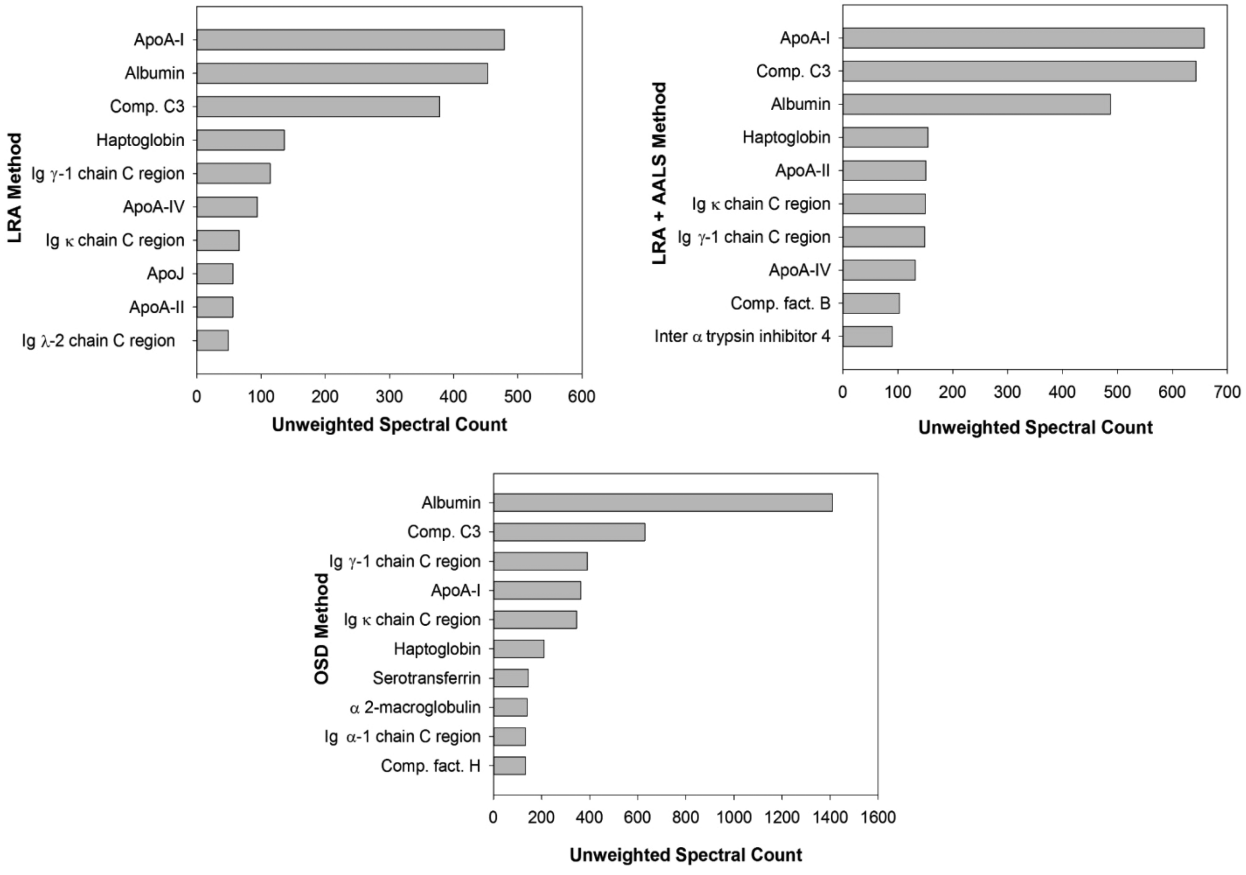

Figure 3. Top 10 HDL associated proteins by total spectral counts across the HDL size range detected by MS using LRA, LRA+AALS, and OSD.

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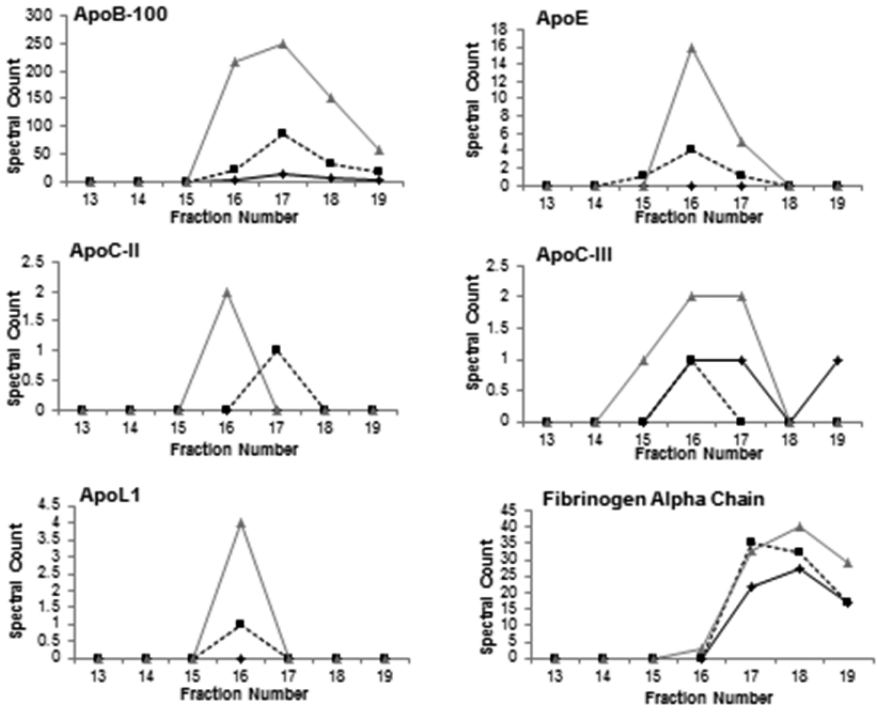

Figure 5. Unadjusted spectral counts for given LDL associated proteins across gel-filtered plasma fractions in the LDL size range. LRA (black), LRA+AALS (dashed), and OSD (gray).

LDL from independent laboratories in ≥ two proteomic studies (http://homepages.uc.edu/~davidswm/LDLproteome.html). Figure 4 shows that 61 different proteins were identified across all three methods in the LDL range. For typical LDL associated proteins, only six proteins including alpha-1-antitrypsin, apoA-I, apoB, apo C−III, fibrinogen alpha chain, and albumin were detected in all three methods. The addition of AALS increased the number of proteins detected for both typical and nontypical LDL associated proteins versus LRA alone. The addition of AALS identified the following LDL associated proteins: apoJ (clusterin), apoC-II, apoE, and apoL-I. Compared to both LRA methods, OSD detected the most proteins. Additional typical LDL associated proteins detected by OSD included apoA-IV, apoC, and apoM.

When spectral counts were evaluated across the LDL range, in contrast to what was seen in the HDL size range, there was improvement in resolution (taller peaks, more spectral counts) with OSD compared to both LRA methods (Figure 5).

We also ranked the top 10 most abundant proteins (as judged by overall spectral counts) detected by each method in the LDL size range. Figure 6 shows that apoB, LDL's most abundant protein, was the top protein by OSD and LRA+AALS. ApoB was the third most abundant protein by LRA alone.

DISCUSSION

The above study resulted in two overall conclusions with respect to the proteomic analysis of gel filtration fractions of human plasma. First, the addition of an MS-compatible detergent to our existing LRA method increased the overall number of proteins detected, the number of peptide counts for each protein, the resolution of each protein across the HDL size range, and the sequence coverage of each protein. Second, a simple organic solvent extraction of unmodified fractions, that is, without specifically isolating lipid-containing particles, identified the most proteins even those in low abundance compared to either LRA method, but with some important caveats. Each of these will be discussed in turn further.

We originally developed the LRA method in 2010 to rapidly select lipid-containing particles within plasma for proteomic analysis. This avoided the necessity of potentially disruptive ultracentrifugal separations in high salt concentrations and opened the possibility for a host of noncentrifugal separation methods to be applied to study proteins that associate with phospholipid. Using this technology allowed us to track proteins in the LDL and HDL size range across three orthogonal separation techniques (gel filtration, isoelectric focusing, and ion exchange chromatography) revealing important new information on the potential cohabitation of pairs of proteins on individual lipoprotein particles.[9] However, as described in the Introduction, the inability to determine the full extent to which we are eluting bound lipoproteins from the LRA prior to peptide generation and analysis offers several possible avenues for signal loss.

One way to remove lipid-bound proteins from the resin is to solubilize them in SDS. However, the use of SDS is limited in MS samples because of ion suppression effects and reduced sensitivity.[14−16] Anionic acid labile surfactant, on the other hand, is a long-chain derivative of 1,3-dioxolane sodium propyloxy sulfate with similar denaturing and electrophoretic properties to SDS, but has the advantage of being MS compatible.[14−16] Prior studies using AALS have shown increased protein detection by gel electrophoresis[15] and MS analysis.[17,18] The results here confirm increased protein detection and increased spectral counts with the addition of AALS to our LRA method. Further detailed analyses also revealed increased sequence coverage of prominent HDL-containing proteins over LRA and OSD. This suggests that addition of a detergent improves the ability of trypsin to access hydrophobic regions of lipoproteins while bound to the LRA, and results in liberation of additional peptides.

OSD was used as a comparison because it allows for a straight proteomic analysis of the gel filtration fractions without the use of LRA. While this method detects the most proteins, there are three important caveats to discuss. First, while OSD identified more

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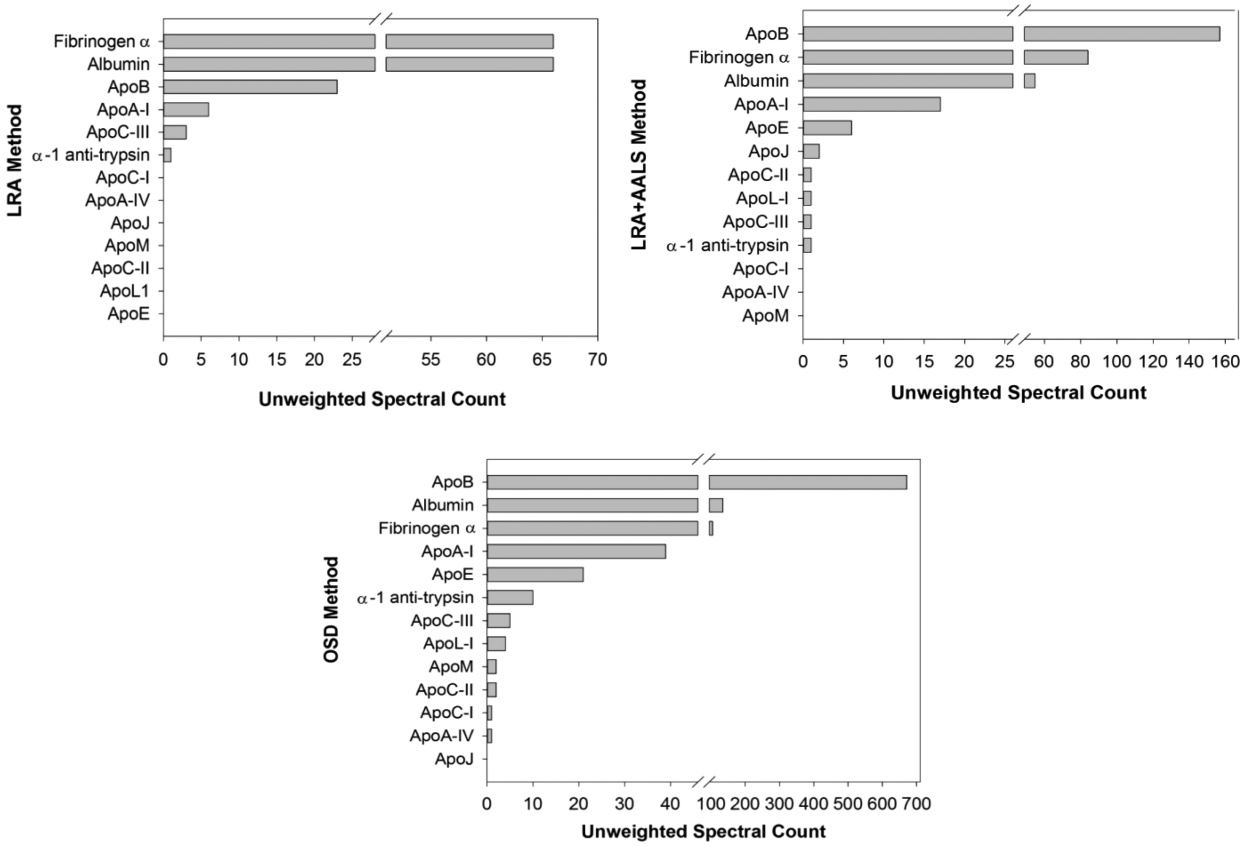

Figure 6. Top 10 LDL associated proteins by total spectral counts across the LDL size range detected by MS using LRA, LRA+AALS, and OSD.

proteins in general in both the HDL and LDL size range, some of these proteins may not necessarily be lipid bound and could represent free proteins found in plasma. This is reflected in the 14 additional nonconsensus HDL proteins and 24 nonconsensus LDL proteins detected by OSD, most of which were immunoglobulins. Thus, if one is focused on the proteome of lipoproteins, an additional method is required to establish whether identified proteins are bound to lipid. Second, for proteins in the HDL range, there was a decrease in detection and sequence coverage in the HDL size range by OSD. It is unclear why this occurred but may be due to the presence of high abundance nonlipid associated proteins (i.e., albumin >1400 counts) that monopolize the duty cycle of the mass spectrometer resulting in a lower mass spectrometer signal intensity and loss of protein detection. This idea is further supported by the improved protein detection in the LDL size range, where there were less proteins in general. Third, while not directly a MS data processing or analysis issue, it should be also noted from a work flow standpoint, OSD is a longer and more technically difficult process, which requires desalting by dialysis, solubilization of a protein pellet, and an organic layer extraction. This was one reason we developed the LRA method initially.

However, given the ability to detect more proteins in both the HDL and LDL size range, OSD does offer some advantages and may be ideal for identifying candidate proteins in biological samples. Additionally, OSD may offer improved detection in the LDL size range making it useful to study the LDL proteome; however, spectral counts were too low to definitively conclude this. The advantages and limitations of each method are outlined in Table 3.

It should be noted that although 70 typical HDL associated proteins were identified by at least one method, some of HDLs well-known proteins including cholesteryl ester transfer protein, phospholipid transfer protein, and lecithin:cholesterol acyltransferase were not detected by any method. Lack of detection is likely because these proteins are lower in abundance and fall below our limit of detection. Similar results have been reported by others.[19−23] Thus, the methods presented here are not ideal for quantitation of these low abundance proteins.

Table 3. Advantages and Limitations of Three Methods To Analyze Lipid Associated Proteins

method	advantages	limitations
LRA	can be prepared in a 96-well plate	fewer proteins compared to OSD
	no dialysis or desalting needed	
	selects for lipid-bound proteins	
LRA+AALS	can be prepared in a 96-well plate	fewer proteins compared to OSD
	no dialysis or desalting needed	added cost and steps of AALS
	selects for lipid-bound proteins	AALS requires complete lysis prior to MS.
	improved sequence coverage	
OSD	greater number of proteins detected	detects non lipid-bound proteins
		dialysis and solubilization required

CONCLUSIONS

In conclusion, we found that the addition of an MS-compatible detergent improves protein detection of lipid-bound proteins separated from human plasma by gel filtration when a lipid binding resin was used. While a straight analysis of the fractions without the

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J. Proteome Res. XXXX, XXX, XXX−XXX

use of a phospholipid binding agent detected more proteins, the inability to confidently determine the lipidation status of the identified proteins was viewed as a clear disadvantage.

■ ASSOCIATED CONTENT

Supporting Information

Phospholipid content in fractions separated by gel filtration of plasma versus HDL and LDL separated by ultracentrifugation from a single subject. Comparisons of protein elutions from the LRA with various detergents. Relative determination of phospholipid coeluting with proteins off of the LRA. HDL-associated proteins by the method identified. Data for all proteins identified and their peptide count distributions across each fraction. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.5b00270.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS:

LRA, lipid removal agent; AALS, anionic acid labile surfactant; OSD, organic solvent delipidation; RT, room temperature; AB, ammonium bicarbonate

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J. Proteome Res. XXXX, XXX, XXX−XXX