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Profiling of phospholipids in lipoproteins by multiplexed hollow fiber flow field-flow fractionation and nanoflow liquid chromatography–tandem mass spectrometry

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ABSTRACT

This study describes a coupled analytical method to carry out the systematic profiling of phospholipids (PLs) in high-density lipoproteins (HDL) and low-density lipoproteins (LDL) from human blood plasma. HDL and LDL of healthy human plasma samples were separated by size and collected on a semi-preparative scale using multiplexed hollow fiber flow field-flow fractionation (MxHF5). Phospholipid mixtures contained in the resulting HDL and LDL fractions were analyzed by shotgun nanoflow liquid chromatography–tandem mass spectrometry (nLC–ESI-MS–MS). We utilized a dual scan method for the separation and simultaneous characterization of complicated PL mixtures by nLC–ESI-MS–MS, such that phosphatidylcholine (PC) and phosphatidylethanolamine (PE) molecules were detected in positive ion mode in a first LC run. In a second LC run, phosphatidylinositol (PI), phosphatidylglycerol (PG), and phosphatidic acid (PA) were detected in negative ion mode. In this study, a total of 56 PLs from HDL and 52 PLs from LDL particles were characterized by their molecular structures from data dependent collision-induced dissociation (CID) experiments, and their relative abundances were compared.

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1. Introduction

Lipoproteins are macromolecular globules that contain both proteins and lipids, with hydrophilic groups consisting of phospholipids, cholesterol, and apolipoproteins oriented toward the surface of the structure while more hydrophobic triglyceride and cholesteryl ester components are located in the lipoprotein interior. The function of lipoprotein particles is to transport fats and cholesterol around the body in human plasma [1]. Lipoprotein particles are classified as high-density lipoproteins (HDL), low-density lipoproteins (LDL), and very low-density lipoproteins (VLDL) depending on the relative amounts of fats and proteins that they contain. Among subfractions of lipoproteins, abnormalities of LDL (smaller and denser than normal LDL) are associated with coronary artery disease (CAD) risk in clinical diagnosis [2–4]. High levels of HDL are associated with a lower incidence of atherosclerosis due to the protective effect of HDL facilitating the removal of cellular free cholesterol from peripheral tissues [5].

Phospholipids (PLs) are major components of all lipoproteins [6]. PLs are classified into molecular species based on differences in the lengths and degrees of unsaturation of their acyl chains and in the polar head group [7–9]. In the area of lipidomics research, the study of changes in the distribution and concentration of PLs is of particular interest to understand lipid transport and synthesis. Most studies of lipoprotein PLs have been carried out by analysis of fatty acid composition using gas–liquid chromatography (GLC) after transmethylation of the PLs [6,10,11]. However, this method is laborious and time consuming since it requires pre-fractionation using column chromatography for the selective isolation of different PL polar head groups and then derivatization of the polar head groups. On the other hand, the analysis of PLs in cells or tissue samples is facilitated by sophisticated mass spectrometry (MS) techniques and, when coupled with liquid chromatography (LC), allows intact PL separation without derivatization and simultaneous structural identification of PL molecules. Recently, capillary LC with electrospray ionization-tandem mass spectrometry (LC–ESI-MS–MS) has been employed to analyze PL mixtures from tissues and to provide structural identification while simultaneously lowering the detection limit [12–15]. It has been demonstrated that nLC–ESI-MS–MS can be powerfully utilized for the characterization of urinary PLs from only a 1 mL urine sample [16,17]. However, due to the difficulties in fractionating lipoprotein subfractions by current techniques, a comprehensive analysis of PLs in different lipoproteins has not yet been carried out.

Flow field-flow fractionation (F4) is an elution-based separation technique that results in size separation of macromolecules,

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proteins, protein aggregates or complexes, and nanometer sized materials [18–20]. F4 utilizes an unobstructed open channel having a rectangular or cylindrical cross-section as a separation chamber, and size separation in the F4 channel can be carried out by applying two different flow streams: an elution flow acting along the channel axis and a crossflow that is driven perpendicular to the elution flow. The role of the crossflow is to drive sample components toward one wall of the channel while sample components protrude from the wall, such that sample components distribute themselves against the channel wall according to size. When a migration flow is applied to differentially distributed sample materials, they elute at different rates, which leads to size separation as smaller molecules elute before larger molecules. Since the F4 channel is empty and contains no packing media, it is suitable for the size separation of lipoprotein particles. Size differentiation of lipoprotein particles has previously been carried out by a few analytical methods: ultracentrifugation [21], gel electrophoresis [3,22], and chromatography [23]. These methods utilize a strong field that induces shear, or packing media that may cause blockage of separation paths. In earlier studies, it was demonstrated that HDL and LDL in human plasma from CAD patients can be separated by F4 [24] using Sudan Black B for selective detection, and also by hollow fiber flow field-flow fractionation (HF5) [25]. HF5 utilizes a hollow fiber membrane as a separation channel. The membrane is disposable, and therefore this method reduces carryover problems [26–28]. Separation in an HF5 follows the simple F4 principle of size separation except that a radial flow, acting toward the inner wall of the fiber, replaces the crossflow of a rectangular F4 channel due to the cylindrical geometry of the hollow fibers. This technique has been applied with on-line multi-angle light scattering (MALS) for the size and shape characterization of blood lipoproteins [29]. Recently, asymmetrical F4 (or AF4) has been utilized for the separation and on-line enzymatic determination of cholesterol and triglycerides in serum lipoproteins [30].

In this study, we introduce a comprehensive analytical method to characterize five different classes of intact PLs in different lipoproteins. HDL and LDL particles isolated from human plasma samples were directly separated by a multiplexed HF5 (MxHF5) system, which was recently developed for semi-preparative scale separation using a parallel connection of six HF5 modules [31], and the collected fractions of HDL and LDL particles were analyzed by nanoflow LC–ESI-MS–MS for the separation and structural determination of intact PL species. By employing MxHF5 for the separation of lipoproteins, blood plasma sample injections of 50 μL can accumulate a sufficient amount of material for the analysis of PL mixtures after two runs. PL mixtures extracted from lipoproteins were separated by capillary LC column followed by data dependent collision-induced dissociation (CID) for structural determination of PL molecules in both positive ion and negative ion MS modes in two consecutive LC runs. The PL types examined in this study were phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylglycerol (PG), and phosphatidic acid (PA). The quantitative profiling of PLs in different lipoproteins from healthy donors is made in a semi-preparative way by comparing the MS peak area of each PL species.

2. Experimental procedures

2.1. Materials and reagents

HF5 channel fibers were manufactured of polyacrylonitrile (PAN) (Chemicore, Inc., Daejeon, Korea) with dimensions of 1.0 mm × 1.4 mm × 25 cm (I.D. × O.D. × length) and a molecular weight cut-off of 30 kDa. Silica capillaries (75 μm—I.D., 360 μm—O.D.) were used for preparing capillary LC columns (Polymicro Technology, LLC, Phoenix, AZ, USA). The reverse-phase resin Magic C18 (5 μm—100 Å) was used for column packing

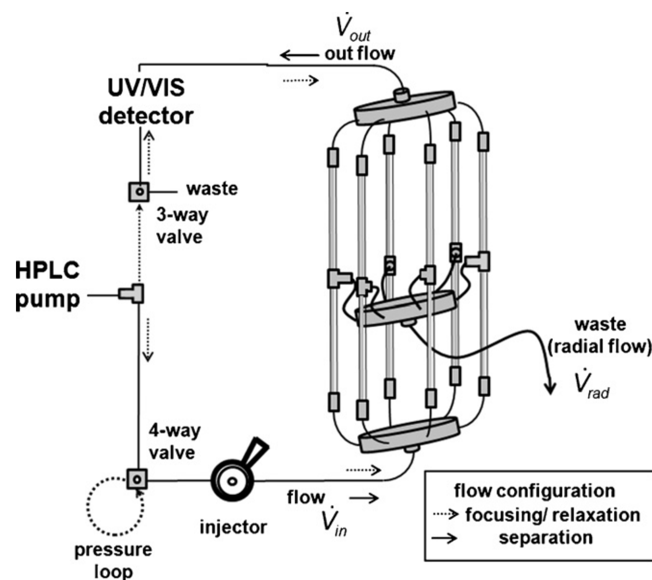

Fig. 1. System configuration of MxHF5 for the semi-preparative separation and collection of lipoproteins. Flow directions are expressed by solid lines for separation and dotted lines for focusing/relaxation.

(Michrom Bioresources, Inc., Auburn, CA, USA). HPLC grade solvents (CH_3CN, CH_3OH, $CHCl_3$, isopropanol, and dH_2O) were used.

Protein standards used for the optimization of MxHF5 separation conditions were bovine serum albumin (BSA, 66 kDa), apoferritin (444 kDa), and thyroglobulin (670 kDa) (Sigma, St. Louis, MO, USA). The lipoprotein sample used in this study was human blood plasma from a healthy donor, which was stained with Sudan Black B (SBB) only for confirmation of retention time of HDL and LDL particles before injection into the MxHF5. Plasma samples were injected for the collection of fractionated HDL and LDL without staining.

2.2. Multiplexed hollow fiber flow field-flow fractionation (MxHF5)

The MxHF5 module utilized in this study is identical to the one previously described [31] and was assembled by connecting six individual HF5 channels in parallel using a seven-port manifold as shown in Fig. 1. Each individual HF5 module was assembled by inserting a PAN HF into two pieces of glass tubing (1.8 mm-I.D., 3.5 mm-O.D.) which were connected via a Teflon tee (Upchurch Scientific, Oak Harbor, WA, USA). The tee connects only the two glass tubes using 1/8 in. hand-tight ferrules and nuts without holding the hollow fiber so that radial flow can exit. At both ends of the hollow fiber, each glass tube was connected to a Teflon union using a 1/8 in. hand-tight ferrule and the other side of the union was connected to PEEK tubing (1/32 in.-O.D. and 0.175 mm-I.D.) using 1/16 in. hand-tight ferrules and nuts. For the connection with PEEK tubing, the hollow fiber was extended about 1/2 cm so that the PEEK tubing could be inserted into the extra portion of the hollow fiber and 1/16 in. hand-tight ferrules hold the fiber and PEEK tubing together.

For the delivery of carrier solution to the MxHF5, a Model SP930D solvent delivery pump (Young-Lin Instruments, Seoul, Korea) was used. Carrier solution was 10 mM NH_4HCO_3 solution prepared with ultrapure water (>18 MΩ), which was filtered before use with a 0.22 μm membrane filter (Millipore Corp., Bedford, MA, USA). During sample loading and focusing/relaxation, the pump flow was divided into two portions using a metering valve: one (1/10) for the channel inlet via a model 7125 loop injector (Rheodyne, Cotati, CA, USA) with a 90 μL loop and the other (9/10) for

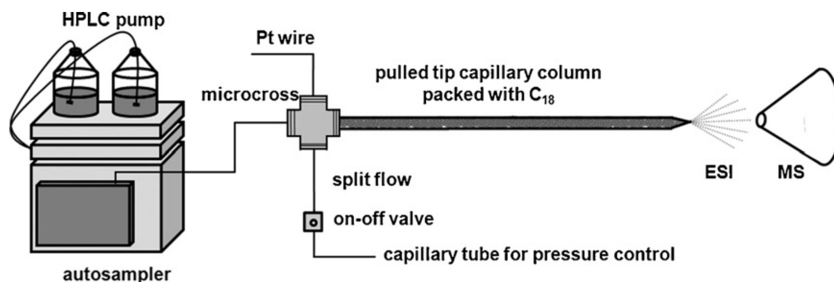

Fig. 2. Diagram of the nanoflow LC setup for the connection of pulled tip capillary column, Pt wire, and on–off valve with a microcross connector.

the channel outlet. In this mode, sample components are expected to accumulate at a position from the fiber inlet corresponding to 1/10 of the total fiber length from the injector and all flows exit through the fiber wall as radial flow. After a certain period of time had elapsed to ensure sample relaxation, an equilibrium distribution of sample components from the two counter-directing forces (hydrodynamic forces of radial flow and diffusion of sample materials), the flow converting valve was switched to the direction at which pump flow was directed to the channel inlet so that separation begins. We applied 5 min of relaxation time for lipoprotein separation.

Eluted sample components were monitored with an UV730D UV–VIS detector (Young-Lin Instruments) at wavelengths of 280 nm for protein standards and 600 nm for SBB stained lipoproteins. The detector signals were recorded using Autochro-2000 software (Young-Lin). Lipoproteins in the plasma sample were stained by mixing 100 μL of raw plasma with 10 μL of 1% SBB in dimethylsulfoxide. The mixture was vortexed for 20 min and then stored overnight at 4 °C. The mixture was injected directly into the MxHF5 without further treatment. For the collection of fractionated lipoproteins, unstained plasma samples were injected, 50 μL for each injection, and two sample fractionations were sufficient to accumulate enough lipoprotein for nLC–ESI-MS–MS analysis.

2.3. Extraction of lipids from HDL and LDL fractions

A total of 100 μL of plasma sample was fractionated by MxHF5 (50 μL for each injection) and HDL and LDL particles were collected using a fraction collector. HDL and LDL fractions contained in FFF carrier solution (20–40 mL volume) collected from MxHF5 runs were concentrated to ~500 μL by centrifuge at 4000 × *g* and then the concentrated fractions were evaporated with a SC110A SpeedVac® Plus (ThermoSavant, Waltham, MA, USA). The lyophilized lipid powder was dissolved in 0.9 mL of 2:1 (v/v) $CHCl_3$:CH_3OH, and the solution was sonicated to break up lipoprotein particles using a tip sonicator at 1 W for 10 min with a pulse interval of 1 s. Then the solution was mixed with 0.5 mL of water, the mixture was centrifuged at 15,000 × *g* at room temperature, and the bottom layer containing lipid mixtures was isolated. The organic layer was evaporated with a SpeedVac and the final lipid extracts were re-dissolved in 1:1 (v/v) CH_3CN:CH_3OH at a concentration of 1 μg/μL and were stored in a refrigerator.

2.4. nLC–ESI-MS–MS of phospholipids

Phospholipid mixtures of HDL and LDL fractions were characterized by nLC–ESI-MS–MS. Separation of PL mixtures of each lipoprotein fraction was accomplished with a homemade pulled tip capillary column (100 μm-I.D., 360 μm-O.D., and 17 cm) prepared by pulling one end of a tip softened in a flame to make a sharp needle, which served as a self-emitter for ESI. The pulled tip capillary was packed with 5 μm C18 resins at 1000 psi of He. The top of the capillary column was connected with a PEEK microcross (Upchurch Scientific) with one capillary tube from a microflow HPLC pump, a model 1200 capillary pump system (Agilent Technologies, Palo Alto, CA, USA) equipped with an autosampler. At the other two arms of microcross, another capillary tube was connected to vent the split flow which leads to an on-off valve at the end, and a Pt wire was connected for electrical contact to provide electrospray ionization for MS as shown in Fig. 2. The capillary column was directly interfaced with a LCQ Deca XP MAX ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) via ESI without a separate emitter.

Separation of PL mixtures was carried out for each set of PL mixture samples in two consecutive nLC–ESI-MS–MS runs in sequentially positive and negative MS ion modes. In the positive ion mode, PCs and PEs were characterized, while PIs, PGs, and PAs were identified in the negative ion mode. nLC separation of PL mixtures was carried out with a binary gradient elution. Mobile phase compositions for the binary gradient LC separation are the same for both ion modes except for the ESI modifiers: 50/50 (v/v) CH_3CN/dH_2O for mobile phase A and 90/10 isopropanol/CH_3CN for mobile phase B. The modifier for the positive ion MS mode analysis was 0.1% formic acid, which was added to both mobile phases, and 0.05% NH_4OH was added for the negative ion MS mode analysis. Samples were loaded directly onto the analytical column with mobile phase A at a flow rate of 300 nL/min for 10 min. About 5 μg of PL mixtures for each lipoprotein fraction were injected. During sample loading, the on–off vent valve was closed. When loading was finished, binary gradient elution began. During separation, pump flow was delivered at a rate of 7 μL/min with the on–off vent valve open so that only a small portion of flow (300 nL/min) reached the analytical column with the rest exiting through the vent tubing. A relatively high speed flow (7 μL/min) from the pump to the microcross was utilized to minimize dwell time. Gradient elution began at 0% B, ramped up to 55% B over 1 min, and then linearly increased to 90% B over 90 min (for positive ion mode) or to 90% B over 60 min (for negative ion mode). ESI voltages were 2.5 kV for the positive ion mode and 3.0 kV for the negative ion mode. For CID experiments, data dependent analysis was carried out for the three most intense ions at each precursor scan under 40% (positive ion mode) or 45% (negative ion mode) of normalized collision energies. Mass ranges of MS detection were 500–1000 amu in the negative ion mode and 700–1000 amu in the positive ion mode for precursor scans and 200–900 amu for MS–MS runs. Identification of PL molecules was accomplished manually.

3. Results and discussion

Fig. 3 illustrates the performance of the MxHF5 channel for the separation of three protein standards and for lipoproteins from human plasma samples obtained at an outflow rate of 1.7 mL/min and a radial flow rate of 1.3 mL/min. Each protein standard shown in Fig. 3a was a 15 μg injection. In Fig. 3b, MxHF5 separation of lipoproteins in human plasma samples yielded two distinctive HDL and LDL peaks which were selectively detected with the help of

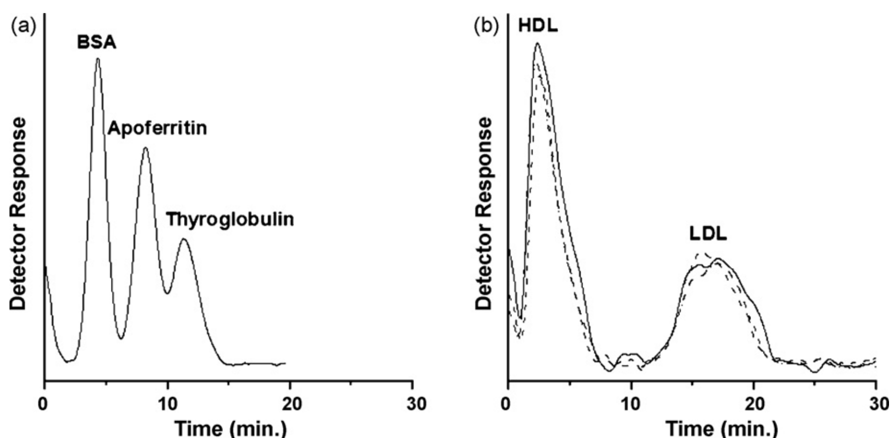

Fig. 3. MxHF5 fractograms of (a) the separation of protein standards (10 μg of each standard) at 280 nm and (b) the separation of HDL and LDL particles from a human plasma sample (50 μL stained with Sudan Black B) at 600 nm. The outflow rate was 1.7 mL/min and the radial flow rate was 1.3 mL/min for both runs.

SBB staining prior to injection. However, to collect HDL and LDL particles for PL analysis, approximately 50 μL of unstained plasma sample was injected at each injection. For the profiling of phospholipids, fractions of the two consecutive runs were accumulated. Fig. 3b shows the superimposed fractograms obtained from three consecutive runs after a thorough cleaning (3.0 mL/min without applying radial flow rate for 30 min).

HDL and LDL fractions collected during the MxHF5 run were processed to remove plasma proteins and to extract lipids. The resulting lipid extracts of both HDL and LDL fractions were analyzed by nLC–ESI-MS–MS. Fig. 4 shows the base peak chromatograms (BPCs) of lipid extracts of HDL and LDL fractions which were examined by nLC–ESI-MS–MS in both positive and negative ion modes. Since the lipid extract samples from the HDL and LDL fractions contain many different non-polar molecules including complex phospholipid mixtures, cholesterols, and other forms of lipids, the BPCs shown in Fig. 4 do not represent unique profiles of PL molecules in HDL and LDL fractions, however they do exhibit a clear difference in total lipid distribution.

Structural identification of intact PL molecules during nLC separation was accomplished by a precursor MS scan followed by data dependent CID experiments for the three prominent ions of each precursor scan. Fig. 5 shows how PC and PE molecules from the HDL fraction were identified in the positive ion mode with the precursor MS spectra at 40.97 min (marked in Fig. 4) and the two CID spectra obtained for the two ions (m/z 787.3 and 794.7). The

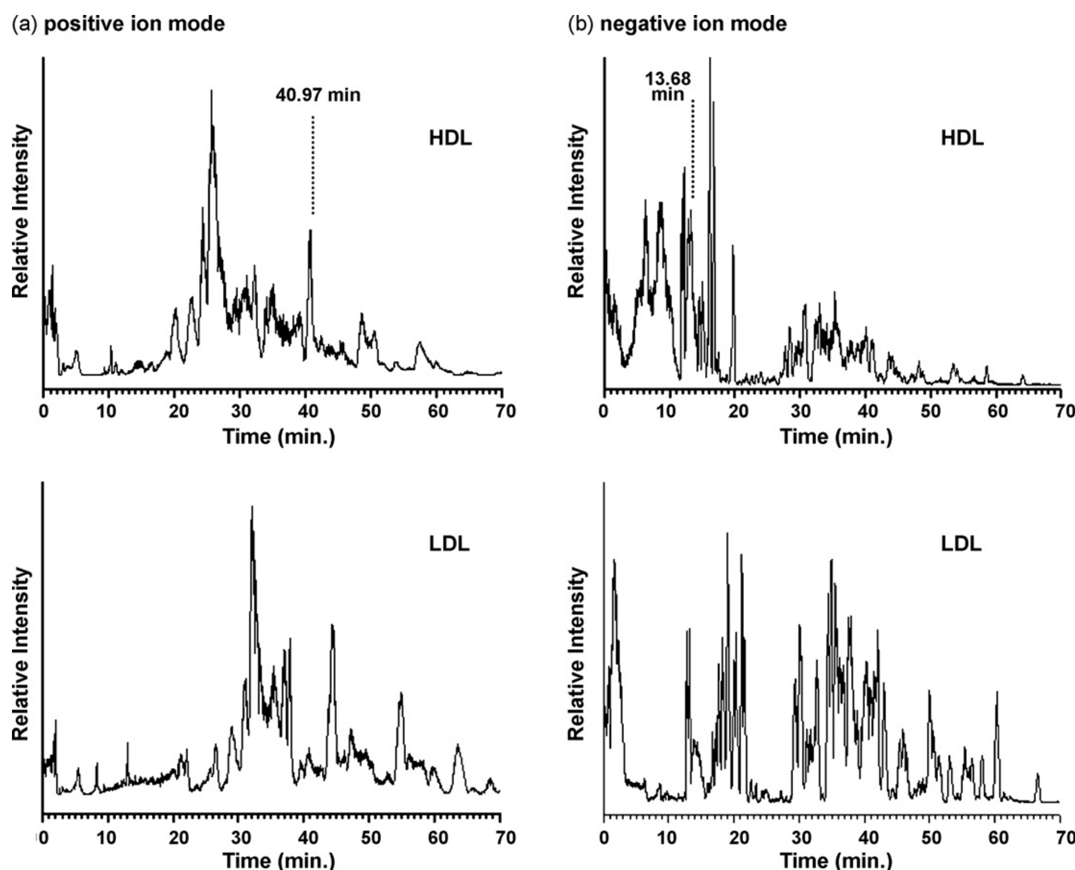

Fig. 4. Base peak chromatograms (BPCs) of lipid extracts from HDL and LDL fractions obtained from nLC–ESI-MS–MS in (a) positive ion mode and (b) negative ion mode.

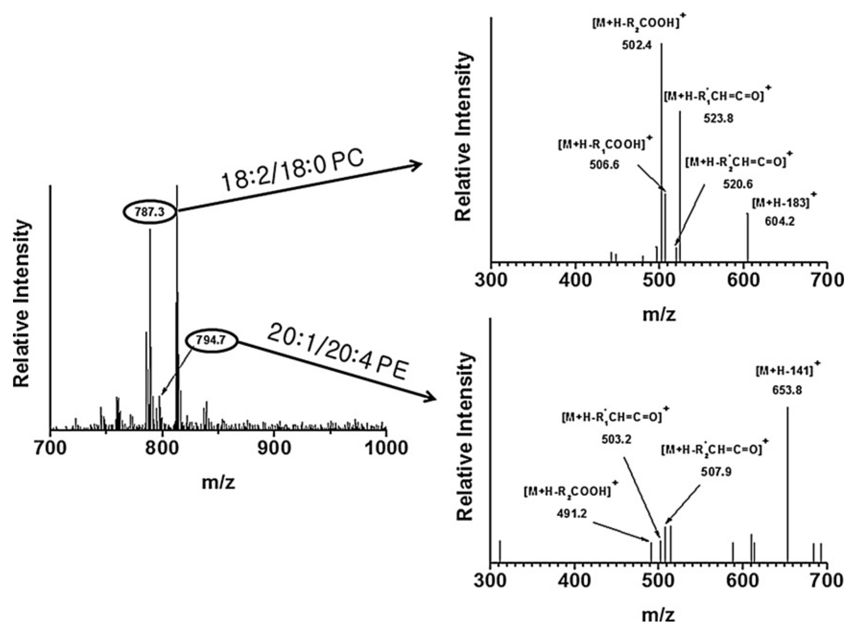

Fig. 5. Precursor scan MS spectra (left side) at nLC time of 40.97 min for the HDL fraction in positive ion mode, and the corresponding fragmentation spectra of *m*/*z* 787.3 and 794.7 obtained by data dependent CID experiments leading to the identification of 18:2/18:0-PC and 20:1/20:4-PE, respectively.

CID spectrum of the ion *m*/*z* 787.3 resulted in the identification of 18:2/18:0-PC based on characteristic fragmentation patterns. The fragment ions can be assigned as *m*/*z* 604.2 for $[M+H-183]^+$ which is the loss of choline ($HPO_4(CH_2)_2N(CH_3)_3$, 183 amu), *m*/*z* 523.8 and 520.6 for the loss of a fatty acid in the form of a ketene ($[M+H-R_1'CH{=}C{=}O]^+$ and $[M+H-R_2'CH{=}C{=}O]^+$), and *m*/*z* 506.6 and 502.4 for the loss of fatty acid (sn-1 and sn-2) in the carboxylic acid form, respectively. The positive ion *m*/*z* 794.7 of the precursor scan was identified as 20:1/20:4-PE based on characteristic fragment ions shown in the lower right side of Fig. 5: *m*/*z* 653.8 $[M+H-141]^+$ for the loss of ethanolamine ($HPO_4(CH_2)_2NH_3$, 141 amu), *m*/*z* 503.2 and 507.9 for the loss of a fatty acid in the form of ketene ($[M+H-R_1'CH{=}C{=}O]^+$ and $[M+H-R_2'CH{=}C{=}O]^+$, respectively), and *m*/*z* 491.2 for the loss of a fatty acid in the carboxylic acid form ($[M+H-R_2COOH]^+$). In the positive ion mode, PC and PE molecules were identified exclusively in this study and are listed in Table 1. In Table 1, 16 PCs were identified from both HDL and LDL fractions but among these only 11 were commonly found in both fractions. It is interesting to compare the relative peak areas of components in the HDL and LDL fractions. For instance, the two PC species such as 18:1/18:3-PC and 18:3/16:1-PC occupy almost 49% of the total peak area for the HDL fraction, but the same species were not detected in the LDL fraction. On the other hand, 16:1/22:5-PC, 18:2/16:1-PC, and 16:1/20:3-PC appear to be around 20% of the relative abundance of identified PCs in the LDL fraction, but the same species are negligible (not detectable for 16:1/22:5-PC or 0.9% for 18:2/16:1-PC) or have a very low occurrence (3.1% for 16:1/20:3-PC) in the HDL fraction. In the case of PE, five species (22:2/14:1, 20:1/18:2, 22:0/16:4, 18:1/18:0, and 20:1/20:4) were

Table 1
PCs and PEs contained in HDL and LDL from positive ion mode.

Class	Molecular species	*m*/*z*	Relative peak area (%)		Class	Molecular species	*m*/*z*	Relative peak area (%)	
			HDL	LDL				HDL	LDL
PC	16:1/20:5	779.5	0.6	0.4	PE	18:2/20:4	764.9	3.6	2.0
	18:1/18:3	782.6	22.0	ND		18:1/20:4	767.4	12.3	8.7
	18:3/16:1	759.1	26.9	ND		22:2/14:1	743.2	7.2	ND
	16:0/18:2	758.5	2.6	ND		20:0/20:4	794.7	15.8	12.7
	16:1/22:5	807.0	ND	17.9		20:1/18:2	776.8	9.2	ND
	22:4/16:1	808.6	8.9	2.2		18:1/20:3	768.8	7.8	12.5
	20:2/18:3	809.0	ND	0.1		22:0/18:5	794.6	28.3	16.4
	18:2/16:1	758.9	0.9	23.6		18:1/18:1	744.8	6.6	6.2
	16:1/20:3	782.8	3.1	21.6		22:0/16:4	768.9	1.7	ND
	20:4/16:1	780.7	1.9	1.5		18:1/18:0	746.5	5.1	ND
	16:2/16:4	723.4	ND	2.1		20:1/20:4	794.7	2.4	ND
	18:0/16:1	760.9	7.1	7.2		20:2/18:0	772.8	ND	5.0
	16:3/20:0	784.7	4.4	17.1		18:2/22:1	798.8	ND	34.3
	18:0/18:1	789.9	1.3	ND		20:0/16:0	748.8	ND	2.2
	22:4/18:1	835.9	2.7	2.5					
	16:1/16:1	731.0	ND	0.4					
	18:2/18:0	787.8	8.9	ND					
	20:2/18:1	813.4	6.6	0.4					
	18:1/18:2	785.5	2.0	2.1					
	20:2/20:0	842.8	ND	0.8					
	18:3/16:0	757.0	0.1	0.1					

ND: not detected.

Table 2
PGs, PAs and PI contained in HDL and LDL from Negative ion mode.

Class	Molecular species	*m/z*	Relative peak area (%)		Class	Molecular species	*m/z*	Relative peak area (%)	
			HDL	LDL				HDL	LDL
PG	16:0/18:6	737.4	1.3	ND	PA	20:6/18:1	716.6	ND	7.8
	18:2/18:4	766.4	0.8	ND		20:5/20:2	746.3	6.0	ND
	22:6/18:0	821.5	8.2	ND		22:6/18:1	746.4	ND	16.4
	18:2/16:0	746.5	4.8	1.7		20:4/20:2	749.6	24.5	42.3
	18:1/18:1	774.4	3.4	10.9		20:4/18:0	724.4	10.0	14.1
	16:2/22:1	798.6	16.4	9.1		20:4/20:1	750.3	5.3	5.0
	18:2/20:3	795.6	1.2	9.6		20:4/20:0	751.2	30.9	ND
	18:2/18:1	770.6	2.3	14.5		18:2/20:1	726.4	19.1	ND
	18:2/18:0	774.6	1.9	10.8		20:4/22:1	776.9	4.2	14.4
	20:4/18:0	798.6	3.8	3.1	PI	16:0/22:6	881.5	2.9	1.4
	18:1/20:2	801.6	52.0	ND		16:0/20:3	859.6	4.9	6.4
	18:1/20:1	802.6	3.9	13.9		16:0/18:2	833.5	8.2	4.2
	18:3/20:3	794.2	ND	18.4		16:0/22:4	857.6	8.5	4.8
	16:0/22:3	800.5	ND	8.0		18:0/22:6	909.5	8.0	2.9
						18:0/20:3	887.4	43.0	8.0
						18:0/20:4	885.5	11.9	54.9
						18:1/18:1	861.4	3.3	13.5
						18:1/20:2	887.7	ND	1.0
						18:0/22:4	913.6	5.3	1.6
						18:0/18:1	864.6	4.0	1.3

ND: not detected.

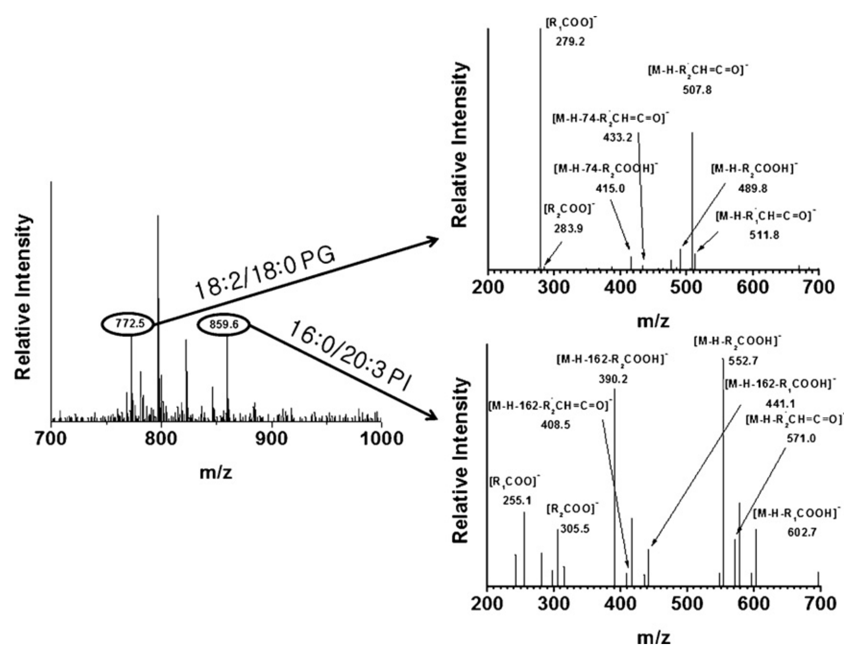

Fig. 6. Precursor scan MS spectra (left side) at a nLC time of 13.68 min for the HDL fraction in negative ion mode, and the data dependent CID spectra of negative ions *m/z* 772.5 and 859.6 leading to the identification of 18:2/18:0-PG and 16:0/20:3-PI, respectively.

not detected in the LDL fraction while they were identified in the HDL fraction.

PL molecules identified in the negative ion mode were PGs, PIs, and PAs. The precursor MS scan shown in the left side of Fig. 6 was observed at a time of 13.68 min which was marked in the upper right BPC in Fig. 4. In the negative ion mode, the precursor ions observed were mostly $[M-H]^-$, and the two ions (*m/z* 772.3 and 859.6) marked in Fig. 6 were identified as 18:2/18:0-PG and 16:0/20:3-PI, respectively, based on the corresponding CID spectra shown in the right side of Fig. 6. Fragment ions shown in the CID spectra of PG and PI molecules in Fig. 6 appeared with similar fragmentation patterns, such as the loss of fatty acid in the form of carboxylic acid or ketene, as was observed with PC and PE molecules. In the cases of PG and PI molecules, fragment ions representing the loss of fatty acid were obtained from two sources of ions: *m/z* 489.8 for $[M-H-R_2COOH]^-$ from direct cleavage of the precursor ion and *m/z* 415.0 for $[M-H-74-R_2COOH]^-$ from cleavage of the precursor ion without the glycerol attached to the head group ($CH_2CH(OH)CH_2OH$, 74 amu). Similar observations were made from CID spectra of PI molecules in Fig. 6 with

Table 3
Numbers of PL species from HDL and LDL fractions identified by nLC–ESI-MS–MS.

	HDL	LDL	Common from both fractions
PC	16	16	11
PE	11	8	6
PG	12	10	8
PI	10	11	10
PA	7	6	4
Total	56	52	39

the loss of fatty acid as $[M-H-162-R_1COOH]^-$ for m/z 441.1 and $[M-H-162-R'_2CH{=}C{=}O]^-$ for m/z 408.5 which results from loss of an inositol head group (162 Da). For both PG and PI molecules detected in negative ion mode, two cleaved carboxylate anions, $[R_1COO]^-$ and $[R_2COO]^-$, were clearly distinguishable. In Table 2, identified PGs, PAs, and PIs are listed along with their relative peak areas for each HDL and LDL fraction. Significant variation in the relative abundance of each of these molecules is observed. For instance, 18:1/20:2-PG was found to be more than 50% in relative peak area among identified PGs in the HDL fraction; however, it was not detected in the LDL fraction. In the case of PAs, 20:4/20:0 and 18:2/20:1 occupied ∼50% of the PAs in the HDL fraction but were not found in the LDL fraction, and the PAs 20:6/18:1 and 22:6/18:1 which were not detected in the HDL fraction appeared as 7.8% and 16.4% respectively of the LDL fraction. Though the relative peak area values expressed as percentages did not directly correspond to the relative concentrations due to the influences on MS peak intensity of acyl chain length and the number of PL double bonds, the data did show the relative variation of each component between HDL and LDL fractions. In Table 3, the total number of PL molecules from both HDL and LDL fractions are listed. In this study, we identified a total of 56 and 52 PL molecules from the HDL and LDL fractions, respectively, among which 39 PLs (70–75%) were found to be common to both fractions.

4. Conclusions

The present study demonstrates that size fractionation of lipoproteins followed by profiling of intact phospholipids from different lipoproteins can be accomplished through an off-line combination of MxHF5 and nLC–ESI-MS–MS. This combined analytical method provides first qualitative information on individual phospholipids from the HDL and LDL of human plasma samples by intact PL analysis. And it may be applied to investigate the relative variation of specific PL molecules according to disease status in relation to diagnosis or therapy in the future. For instance, the LDL fractions of plasma from coronary artery disease (CAD) patients are denser and smaller in their sizes than fractions from normal controls. Based on what is currently known about PL compositions, high speed scanning of PL molecules could be accomplished for a quantitative analysis of CAD patients with multiple plasma samples to develop disease markers. The method we present in this paper requires only 100 μL of plasma sample for comprehensive analysis of PLs. The proposed method could be further expanded to examine other components of lipoproteins such as lipoprotein associated proteins, lysophospholipids, and cholesterol in relation to artery diseases. Since the F4 technique utilizes unobstructed open channel space for its separation chamber, it offers a high compatibility in handling biological nano-globules without the risk of sample loss or deformation during passage through packing media. In addition, the current MxHF5 channel can be manufactured in disposable form, which reduces the risk of cross-contamination for clinical samples.

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