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# UC/MALDI-MS analysis of HDL; evidence for density-dependent post-translational modifications

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**Abstract**

The purpose of this study is to determine whether the nature of the post-translational modifications of the major apolipoproteins of HDL is different for density-distinct subclasses. These subclasses were separated by ultracentrifugation using a novel density-forming solute to yield a high-resolution separation. The serum of two subjects, a control with a normolipidemic profile and a subject with diagnosed cardiovascular disease, was studied. Aliquots of three HDL subclasses were analyzed by MALDI and considerable differences were seen when comparing density-distinct subclasses and also when comparing the two subjects. A detailed analysis of the post-translational modification pattern of apoA-1 shows evidence of considerable protease activity, particularly in the more dense fractions. We conclude that part of the heterogeneity of the population of HDL particles is due to density-dependent protease activity.


*Keywords:* HDL; Apolipoprotein; Post-translational modification; MALDI; Density profile

## 1. Some comments about Peter Roepstorff

In the early 1970s, the observation that intact molecular ions of large biomolecules could be desorbed intact from solids when excited with a short burst of energy led to a new direction in biological mass spectrometry. In the ensuing years, much effort was directed toward understanding the physics and chemistry underlying the process. Early on, a lone voice kept asking questions about the feasibility of applications to problems in the life sciences, live bugs, and bacteria: questions far removed from the focus of the time on desorption mechanisms. That lone voice came from the individual being honored in this special issue, the first life scientist to not only appreciate and predict the potential of desorption mass spectrometry when applied to life science problems, but to immediately apply the method to his field. For Peter, it did not matter whether the excitation source was a fission fragment, a laser pulse, or any other mode of ionic transportation; the problem and application were paramount. Now, three decades later, desorption mass spectrometry is an established analytical tool for the life sciences, continuing to grow, and expanding to adopt the latest developments emerging from fundamental mass spectrometry research. Peter was the first: a true explorer of the unknown, relishing the challenge of exploring new territories, a modern-day Viking. As we considered what topic our contribution should be, we decided to keep in the Roepstorff spirit, to share some of our recent experiences in applying MALDI mass spectrometry to learn more about the link between cardiovascular disease and the composition of HDL particles, not knowing what we might find but using the power of MALDI in revealing what is going on at the molecular level.

## 2. Introduction

High-density lipoprotein (HDL) plays a major role in lipid metabolism and cholesterol transport. It has a micelle-like structure with a diameter of ∼10 nm and a MW of 360,000. It consists of a lipid core made up of cholesterol esters and triglycerides

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surrounded by a surface structure consisting of phospholipids, cholesterol and apolipoproteins. Apolipoprotein, apoA-I (MW 28,078) is the major protein providing the backbone for the structure and has a serum concentration on the order of $5 \times 10^{-5}$ M. apoA-II, the second most abundant protein is a covalently linked dimer with a MW of 17,414 and a molar concentration of $2 \times 10^{-5}$ M. Other minor apolipoproteins include apoC-I, MW 6630, [$9 \times 10^{-6}$ M], apoC-II, MW 8914.9 [$1 \times 10^{-6}$ M] and apoC-III, MW 8764.7 [$8 \times 10^{-6}$ M] [1].

During the past decade, several studies have been carried out characterizing serum apolipoproteins by MALDI [2–4]. Numerous post-translational modifications of the apolipoproteins have been identified, indicating that during their lifetime in circulation, the lipoproteins are exposed to domains in the circulatory system that modify their structures [5,6]. Little is known about the origin or function of these modifications. While HDL plays a major role in maintaining a healthy lipid metabolism, recent evidence suggests that there are forms of HDL that are involved in the development of cardiovascular disease [7]. We recently found evidence that a form of HDL containing apoC-I is associated with newborns that are smaller than gestational age (SGA) [8]. These SGA infants tend to develop cardiovascular disease at an early age. Following up on this observation, we have initiated a clinical study involving adults with cardiovascular disease who have high-HDL cholesterol levels and normal LDL levels. Five HDL subclasses have been identified from previous studies covering a density range from 1.063 to 1.179 kg/L. A comprehensive analytical protocol has been developed for the analysis of these lipoprotein subclasses based on their density [9–11]. Isopycnic fast ultracentrifugation coupled with a novel metal-ion EDTA density-forming solute is used to resolve the lipoprotein subclasses [11]. A high-resolution lipoprotein particle density profile is generated using fluorescence image analysis [9,12]. In the study reported here, our objective was to determine whether the pattern of post-translational modifications previously observed for apoA-I are the same throughout the HDL density profile. This information is part of an ongoing program to better understand features of the population of circulating lipoproteins at the molecular level.

## 3. Experimental

### 3.1. Chemical reagents

The fluorophore 6-((*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino)-hexanoyl) sphingosine, NBD ($c_6$-ceramide), was obtained from molecular probes (Eugene, OR). EDTA, ferulic acid, and cesium carbonate were from Sigma Aldrich (St. Louis, MO). Sodium bismuth EDTA was obtained from TCI America (Portland, OR). De-ionized water used in all experiments was from a Milli-Q water purification system (Millipore, Bedford, MA).

### 3.2. Serum/plasma collection

The serum for this study was obtained from a normolipidemic subject and an individual with documented cardiovascular disease following a 12 h fast via blood draw into a 9.5 mL Vacutainer treated with polymer gel and silica activator (366510, Becton Dickinson Systems, Franklin Lakes, NJ). The serum was separated from the red blood cells by centrifugation at 3200 rpm for 20 min at 5 °C and separated into 250 μL aliquots. These samples were stored at −85 °C until used.

### 3.3. Synthesis of $Cs_2$CdEDTA

The $Cs_2$CdEDTA complex was synthesized from $H_4$EDTA, cesium carbonate, and cadmium carbonate using a procedure similar to that described elsewhere [13,14]. The reagents were combined stoichiometrically in 100 mL of DI $H_2O$, followed by a 2 h reflux, yielding a clear solution. The cesium carbonate was then added to the clear solution to bring the final pH range to 6–7. The final solution volume was reconstituted to 100 mL to account for evaporation during reflux to give stoichiometric solutions with a final concentration of 0.300 M.

### 3.4. Preparation of serum samples for an ultracentrifuge (UC) spin

Into a 1.5 mL Eppendorf tube, 1100 μL of a solution of the metal-ion EDTA salt, an aliquot of the serum sample, and 10 μL of 1 mg/mL NBD ($c_6$-ceramide) in DMSO were added and vortexed at 1400 rpm for 1 min and allowed to stain for a period of 30 min. For the density-profiling measurement, 0.200 M NaBiEDTA was the density-forming solute and 6 μL of serum was used for the analysis. For the study where fractions of the HDL subclasses were recovered for MALDI analysis, 0.300 M $Cs_2$CdEDTA was the solute and 200 μL of serum was used.

### 3.5. Ultracentrifugation

A 1000 μL volume of the metal-ion EDTA salt and NBD-ceramide stained serum was transferred to 1.5 mL, thick-walled, polycarbonate ultracentrifuge tube. These solutions were spun for 6 h at 120,000 rpm and 5 °C in a Beckman Optima™ TLX-120 Ultracentrifuge equipped with a 30° fixed angle TLA 120.2 rotor.

### 3.6. Imaging

A custom-built fluorescence imaging system was used to measure the distribution of the lipoprotein particles in the ultracentrifuge tube after the spin. The light source used was a Fiber-Lite MH-100 Illuminator, (MH100A, Edmund Industrial Optics). The camera used was a digital color microscope camera (S99808, Optronics, Goleta, CA). The camera and light source were placed orthogonally to each other on an optical bench and a slit (1 cm × 4 cm) was placed 8 cm away from the tube holder and suspended by a post/post-holder in order to collimate the excitation beam. A gain of 1.0000 and an exposure time of 15.8 ms were chosen using the accompanying MicroFire camera software. A blue-violet excitation filter (BG-12, Schott, Edmund Industrial Optics) with a bandwidth centered at 407 nm and a yel-

low emission filter (OG-515, OEM, Edmund Industrial Optics) with a bandwidth centered at 570 nm was used, chosen to match the NBD ($c_6$-ceramide) excitation and emission.

### 3.7. Generation of the lipoprotein density profile

Following an ultracentrifuge spin at the previously described settings, 200 μL of DI $H_2O$ was carefully layered atop of freshly spun solutions in order to provide a separation of the VLDL band from the meniscus at the top of the solution. The Microfire camera system was used to record images of the ultracentrifuge tube and Origin software was used to convert the 1024 × 1024 pixel map of the image into a lipoprotein density profile [9]. The coefficient of variance (CV) of the density profile, based on the fluorescence intensities of the lipoprotein subclasses, was determined to be on the order of 7%. The CV for the position of the lipoprotein subclasses in the density profile is on the order of 0.5%.

### 3.8. Recovery of HDL fractions

HDL subfractions were obtained by first freezing the ultracentrifuge tube in liquid nitrogen after the spin. The tube was then sliced into fractions at accurately defined positions along the tube coordinate within a known density range. These fractions, ∼200 μL volumes were thawed and stored for subsequent MALDI analysis.

### 3.9. MALDI analysis

A 5 μL aliquot of the HDL fraction was desalted and delipidated using a C4 Zip tip and eluted with a solution consisting of 60% ACN, 0.1% TFA, and 40% water (v/v) The effluent was then mixed with a 5 μL volume of a solution consisting of ferulic acid (15 mg/mL) in 40% $H_2O$, 60% ACN, 0.1% TFA (v/v) and evaporated to dryness. MALDI spectra were recorded using Voyager DE, STR Biospectrometry Workstation (Applied Biosystems, Foster City, CA). The system was operated in the linear mode at an acceleration voltage of 25 kV using a 92% transmission acceleration grid. Delay time was set at 750 ns. Mass ranges were acquired between 5000 and 50,000*m/z* with a laser intensity of 2500 (arbitrary units). The system was initially calibrated externally with bovine insulin (MW 5733.6), bovine serum albumin (MW 66,341), and *Escherichia coli* thioredoxin (MW 34,622). For the final calibration, two-naturally occurring components of HDL, apoC-I (MW 6630.6) and apo-C-$III_1$ (MW 9421.3) were used as internal standards.

A key feature of the MALDI analysis is the identification of isoforms of the major apolipoproteins. The reproducibility of the relative intensities of the isoform patterns was determined to be on the order of 4%. The coefficient of variance for the *m/z* for repeated measurements of a given subject was 0.08% and was determined by comparing the mean *m/z* for the most highly ionized peak in each replicate measurement of a given sample. Observations of results obtained from

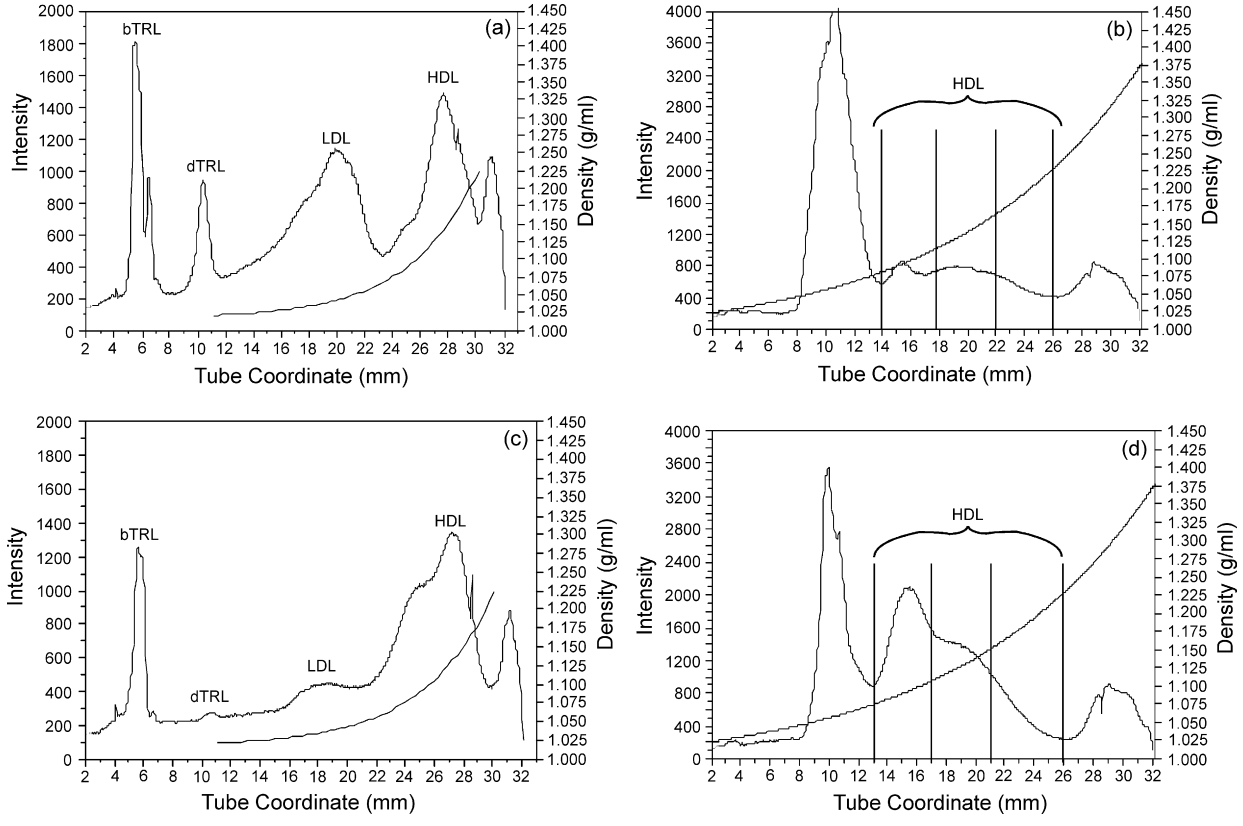


Fig. 1. (a) Total lipoprotein profile for subject 1-control; (b) isolated HDL profile for subject 1-control; (c) total lipoprotein profile for subject 2-CVD; (d) isolated HDL profile for subject 2-CVD.

density gradient ultracentrifugation prior to MALDI analysis indicated no signs of variability among replicate sample measurements.

## 4. Results and discussion

### *4.1. Lipoprotein density profiles for two subjects (comparing no CVD and with CVD)*

In establishing a protocol for clinical studies on the role of lipoproteins in the detection of cardiovascular disease, MALDI is an integral part of the analysis, providing detailed information at the molecular level on the structural features of an individual's apolipoproteins. We have chosen to present an example of the application of our protocol in a manner that follows its application in a clinical study. The two subjects who provided serum for the results reported here is part of an ongoing clinical study to determine why some individuals with a "healthy" lipid profile still develop cardiovascular disease. Measurement of the lipoprotein density profile is the first measurement that is made, carried out under highly controlled conditions that give optimal performance in terms of reproducibility and resolution. Fig. 1 shows the density profiles obtained for the two subjects. The lipoprotein classes are identified based on literature values for the density ranges for these classes. The four major lipoprotein classes are labeled as buoyant triglyceride-rich lipoprotein (bTRL), dense triglyceride-rich lipoprotein (dTRL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL). Free proteins (P) sediment to the bottom of the tube because of their higher density. In comparing the two density profiles on the left panels of Fig. 1, it is apparent that a major difference in the two profiles is a shoulder on the low-density side of the HDL distribution for the subject with CVD (Fig. 1c) compared to the control (Fig. 1a). The next step is to determine what is different about this more buoyant HDL subclass. MALDI is one of the methods used to determine whether the apolipoproteins are different; however, we cannot recover a fraction from this profile for two reasons. At the 6 μL serum level, only picomole levels of apolipoproteins are present. Further, under conditions used to obtain the density profiles shown in Fig. 1, the HDL fraction is compressed into a 6 mm long segment of the ultracentrifuge tube, making it difficult to split into separate fractions.

To solve these two problems, we first increased the volume of serum analyzed from 6 μL to 200 μL to enhance sensitivity. Then, taking advantage of the versatility of the EDTA metal-ion complex system, we altered the density gradient in the ultracentrifuge tube to spread out the HDL distribution. We did this by changing the solute to a higher MW complex ($Cs_2CdEDTA$) and increased the initial concentration of the solute from 0.2 M to 0.3 M in order to generate an expanded profile. Fig. 1b and d shows the new profiles obtained with this solute system. Comparing these two profiles with what was obtained using 0.2 M NaBiEDTA (Fig. 1a and c), the HDL distribution now occupies the central portion of the ultracentrifuge tube in a segment 13 mm long. When comparing the expanded profiles (Fig. 1b and d) with the compressed profiles (Fig. 1a and c), the LDL distribution coalesces with the dTRL fraction at the meniscus. Interestingly, the HDL distribution, originally presented as a narrow distribution with evidence for a shoulder on the buoyant side in the compressed profile, is shown in the expanded profile to be a broad distribution with the small shoulder prominently featured in the buoyant HDL region (Fig. 1b). The influence of the buoyant HDL component for the CVD subject is dramatically accentuated in the extended density profile. Three HDL fractions were recovered from the compressed density profile for MALDI analysis. Vertical lines inserted into Fig. 1b and d are the locations for the cut-points for recovery of the HDL fractions using the freeze/cut/thaw protocol as described above. The buoyant HDL region covers a density range from 1.075 to 1.100; intermediate density HDL, 1.100–1.175; dense HDL, 1.175–1.225 kg/L. At this point in the study, the objective was to determine whether there are differences in the apolipoprotein component of the three subclasses based on particle density, particularly those associated with post-translational modifications.

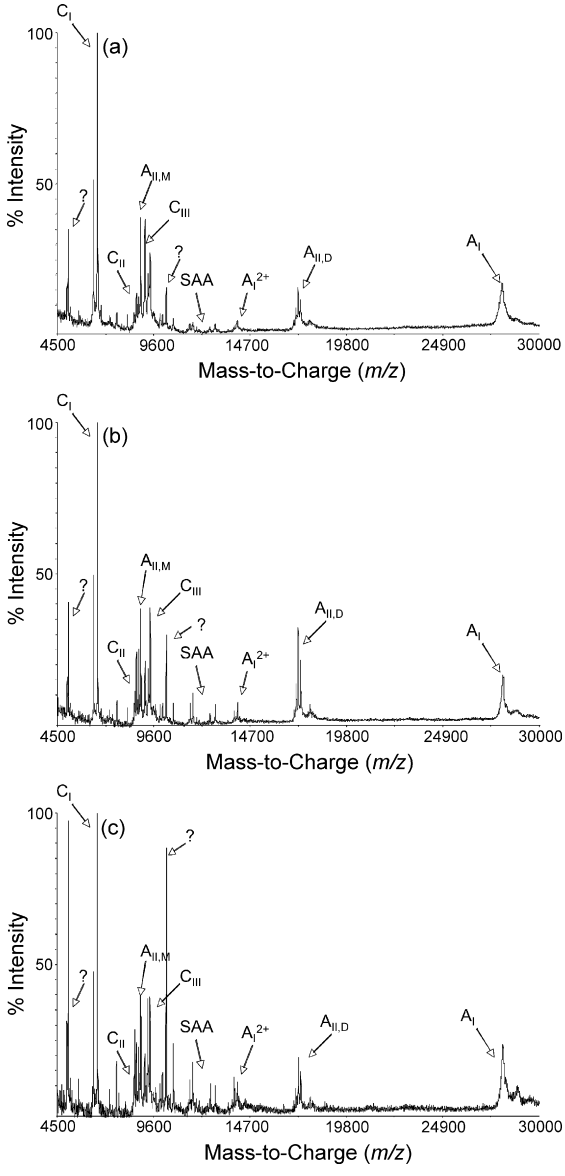


Fig. 2. Mass spectra for subject 1 from range 4500 to 30,000*m/z* for (a) buoyant, (b) intermediate and (c) dense HDL subclasses.

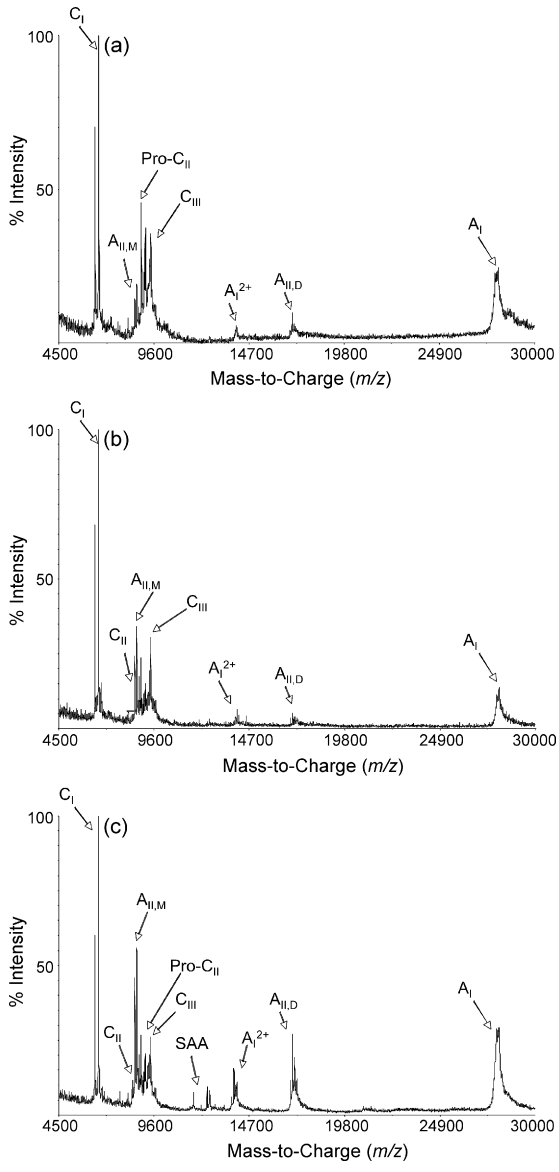


Fig. 3. Mass spectra for subject 2 from range 4500 to 30,000*m/z* for (a) buoyant, (b) intermediate and (c) dense HDL subclasses.

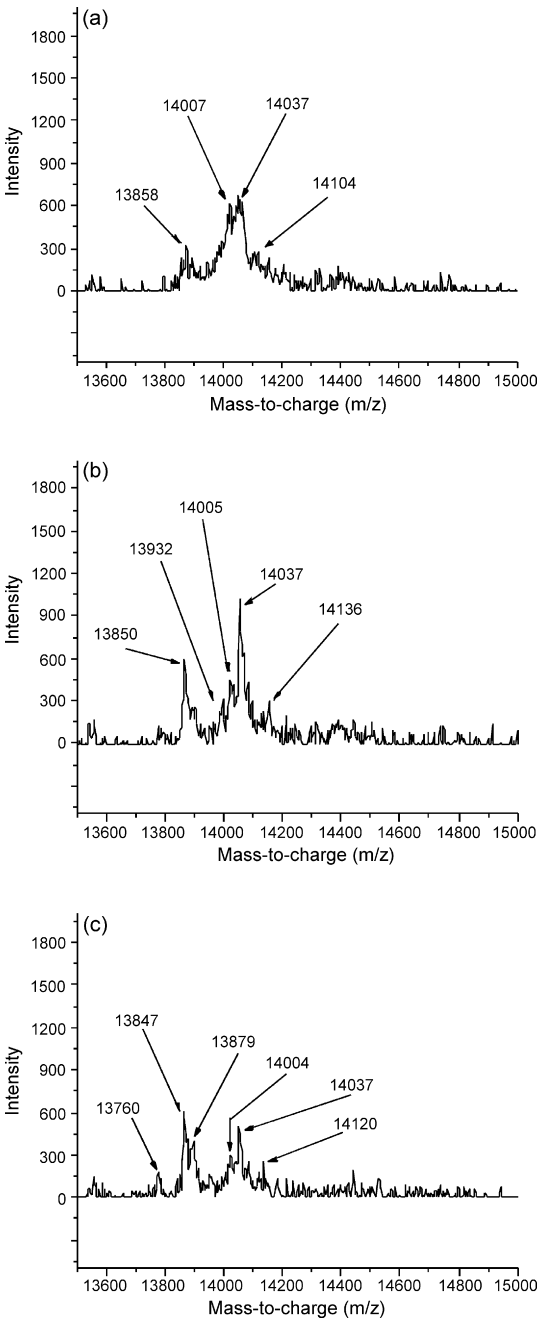


Fig. 4. Mass spectra for subject 1 from range 13,500 to 15,000*m/z* for (a) buoyant, (b) intermediate and (c) dense HDL subclasses.

### *4.2. MALDI analysis of the HDL subfractions*

MALDI spectra were recorded for the three HDL subfractions. Fig. 2 shows the spectra for the three regions for the control sample. For the most part, the spectra are qualitatively the same and are close to what we observed in previous studies, with the dominant peaks coming from: apoA-I,II, and apoC-I,II,III and its isoforms. Two proteins were observed that had not previously been detected at 5079 and 10,277*m/z*. These proteins have yet to be identified.

Fig. 3 shows the set of MALDI spectra obtained for the three segments of HDL for the subject with CVD. Again, the major apolipoproteins were observed as with the control subject. The proteins at 5079 and 10,277*m/z* were not present in this sample. Examining these spectra in greater detail reveals some significant differences. Three questions of current interest are: the role of oxidative processes in modifying the HDL apolipoproteins, inflammatory markers, and the possibility of post-translational modifications of the major protein, apoA-I.

First, by examining the spectra obtained for the control sample, we found that greater detail could be obtained for doubly protonated apoA-I species because of the higher resolution in the lower *m/z* region. Fig. 4 compares the set of spectra obtained for the buoyant, intermediate, and dense components of the HDL distribution for the control. What is remarkable about these spectra is that the pattern of post-translational modifications shows significant differences for all three regions. A similar result was

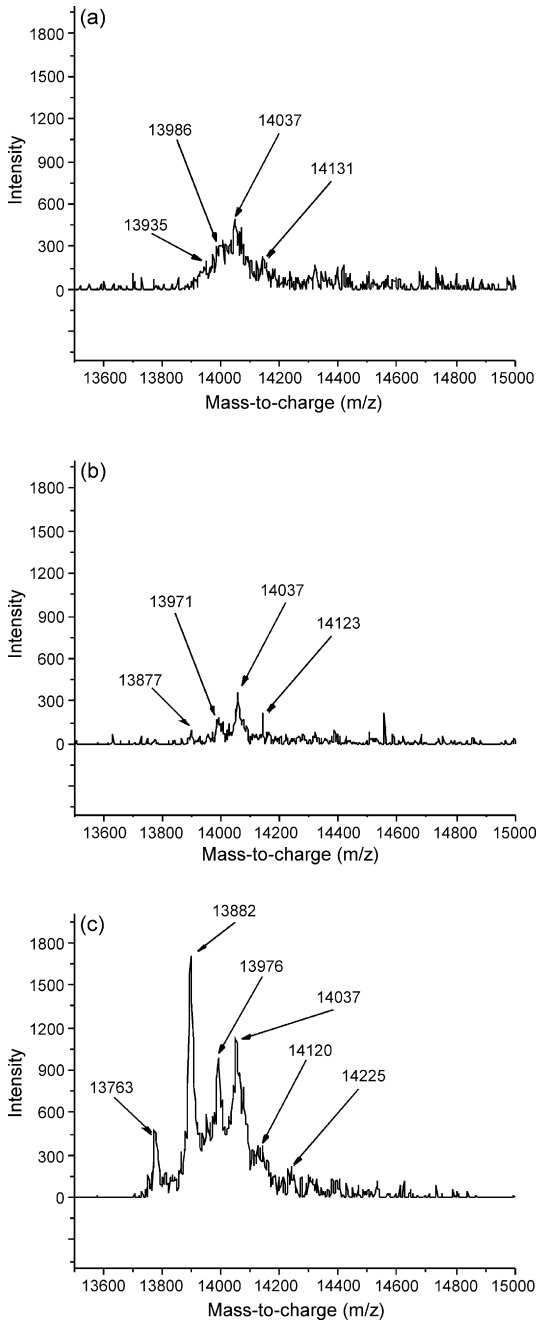


Fig. 5. Mass spectra for subject 2 from range 13,500 to 15,000$m/z$ for (a) buoyant, (b) intermediate and (c) dense HDL subclasses.

obtained for the HDL from the subject with CVD (Fig. 5). The accuracy of the mass measurements was not sufficient to identify the nature of the products of the post-translational modifications, but significant loss of structure (100 to 500$m/z$) has taken place, particularly in the denser HDL fraction. We can only postulate that different proteases are resident on the HDL particles dependent on the density of the particle. There appears to be more protease activity in the dense HDL component for the CVD subject. It is clear from these results that the nature of post-translational modifications is linked somehow to the density of the HDL particle.

## 5. Summary remarks

The results reported here is part of a long-range program to characterize serum lipoproteins using an array of modern analytical methods. MALDI has been an integral component of this characterization leading to the identification of new isoforms of the major apolipoproteins. By introducing the system of metal-ion EDTA complexes, we now have the capability of spatially distributing the major lipoprotein subclasses for a more significantly detailed study of their composition as a function of density. The objective of this study was to determine whether the nature of post-translational modifications was different for the HDL subclasses. Most revealing are the results obtained for apoA-I, the major protein of HDL where the isoform profiles are not only density-dependent but vary for the two subjects studied. The implication of these findings is that the kinds of processes that are modifying HDL *in vivo* are different depending on the density (size) of the HDL particle.

This study represents a new direction for the characterization of the isoforms of the major apolipoproteins. Little is known about post-translational processing and where it occurs in the circulatory system. Most likely, the isoform pattern will be found to be variable for healthy individuals as well as those with CVD. These issues can only be resolved with a more extensive study involving healthy controls and those with documented CVD. MALDI mass spectrometry is providing a window of opportunity to address these issues, important not only for a better understanding of the fundamental processes but also providing an opportunity to approach the "chemistry of CVD" from a new perspective.

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