methods

Novel mass spectrometric immunoassays for the rapid structural characterization of plasma apolipoproteins

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Abstract **Novel mass spectrometric immunoassays (MSIAs) for the isolation and structural characterization of plasma apolipoprotein A-I (apoA-I), apoA-II, and apoE have been developed. The assays combine selective isolation of apolipoprotein species via affinity capture with mass-specific detection using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. In application, plasma (from 50 μl of whole blood drawn from individuals, using finger lancet) was addressed with affinity pipette tips derivatized with antibodies toward the specific apolipoprotein. The time required for each assay was ~15 min, less if assays on multiple individuals were performed in parallel. In a brief study of five individuals, several recently reported apoA-II variants were identified and observed consistently in all individuals. Additionally, the apoE phenotype of E3/E3 was observed in three of the individuals, and E2/E3 and E3/E4 observed in the remaining two individuals, the latter of whom suffers from Alzheimer's disease. Overall, the MSIA approach offers a rapid, sensitive, and highly accurate means of profiling apolipoproteins from small volumes of plasma.**—Niederkofler, E. E., K. A. Tubbs, U. A. Kiernan, D. Nedelkov, and R. W. Nelson. **Novel mass spectrometric immunoassays for the rapid structural characterization of plasma apolipoproteins.** ***J. Lipid Res.*** **2003.** 44: **630–639.**

Supplementary key words MALDI-TOF MS • affinity capture • apoE • apoA-II • posttranslational modifications • point mutations • phenotype

The apolipoproteins represent a significant class of proteins whose functions range from enzyme activation (e.g., LCAT and lipoprotein lipase) to lipid/cholesterol transport and clearance. Although well studied over the past two decades, it has only recently become clear that mutations present within the apolipoproteins may be responsible for, or assist in, the progression of disease. **Table 1** gives a brief overview of some diseases correlated with specific mutations present within apolipoproteins. Universally, these mutations are commonly studied on the DNA level using a variety of genotyping assays. For instance, apolipoprotein E (apoE) genotyping usually involves PCR amplification of short subsequences of exons 2 and 3 (containing both polymorphic sites) and analysis of the PCR products by: *1*) dot blot hybridization with allele-specific oligonucleotide probes (1); *2*) oligonucleotide ligation assay (2); *3*) single-stranded conformational polymorphisms (3); or, *4*) restriction fragment-length polymorphism analysis using restriction enzymes recognizing the sites of polymorphism (4). More advanced approaches involve competitive amplified single mutation detection by selective probe hybridization immunoassay (5) or primer-extension mass spectrometry assays directed at the polymorphic sites within the gene (6). This latter assay brings to the analysis the direct detection of indicators (extended primers containing the base complement of the polymorphism) at exact mass values, thus enabling an assay that is free of artifacts encountered in other assays using, e.g., electrophoretic gel shifts or differential hybridization.

As with the nucleic acid mass spectrometry assays, direct mass spectrometric analysis of proteins (or proteolytic fragments) brings an unprecedented degree of analytical accuracy to the analysis of point mutations. However, whereas the polymorphism analyses generally target a single-nucleotide polymorphism (SNP) in each analysis, direct analysis at the protein level makes possible (in a single assay) the detection of mutations throughout an entire protein, and thus an entire gene. Direct analysis of heterogeneous proteins (mix of wild-type and mutant) will produce split signals differing in molecular weight by the mass difference between the mutated residues. A second value inherent to direct mass analysis of the protein is the ability to evaluate proteins for posttranslational modifications (PTM). This aspect of biochemical structure is

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Abbreviations: CDI, carbonyldiimidazole; CMD, caboxymethyl dextran; HBS, Hepes-buffered saline; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MSIA, mass spectrometric immunoassay; PTM, posttranslational modifications; SNP, single-nucleotide polymorphism; TCEP, tris-2-carboxyethyl phosphine hydrochloride.

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TABLE 1. Major plasma apolipoproteins, their isoforms, and associated diseases

Apolipoprotein	MW (Wild-Type)	Plasma Concentration	Mutations (Δ MW)	Associated Diseases
ApoA-I	28,078.6 Da	1.0–1.5 g/l	Lys107–del (−128 Da)	Possible amyloidosis
			Pro90–Leu (16 Da)	Amyloid cardiomyopathy
			Gly26–Arg (99 Da)	Amyloidosis
ApoA-II	8,707.9 Da	0.5–0.7 g/l	None known	Alzheimer's Disease (AD)
In plasma as a dimer	17,415.8 Da			
ApoC-I	6,630.6 Da	0.05–0.10 g/l	Thr-Pro–del (−198 Da)	None known
ApoC-II	8,203.9 Da	0.03–0.05 g/l	Glu 55–Lys (−1 Da)	Hypertriglyceridemic, not associated to cause
			Lys55–Gln (0 Da)	Lowered cholesterol
			Met22–Val(−32 Da)	Hyperlipidemia
			Glu38–Lys (−1 Da)	Hyperlipidemia
			oxidized apoC-II (16 Da)	None known
			Pro-apoC-II (711 Da)	None known
			sialo-pro-apoC-II (1,002 Da)	None known
			disialo-pro-apoC-II (1,293 Da)	None known
ApoC-III	8,764.7 Da	0.07–0.12 g/l	Deficiency	ApoA-I deficiency and low plasma triglycerides
			asialo-ApoA-III (−948 Da)	None known
			sialo-proapoC-III (291 Da)	None known
			disialo-ApoC-III (582 Da)	ApoA-I deficiency
			Thr74–Ala (−30 Da)	Nonglycosylated mutant
			Lys58–Glu (1 Da)	Hyperlipidemia
ApoE	36,236.6 Da	0.04–0.14 g/l	Arg158–Cys [E2] (−53 Da)	Protective for AD and hyperlipidemia
			Cys112–Arg [E4] (53 Da)	Atherosclerosis, myocardial infarcation, and AD

Apolipoprotein name, molecular weight (MW), concentration in plasma, amino acid sequence modification, and clinical significance of polymorphism (if reported) are given.

largely out of the realm of DNA-based analyses and allows changes brought on by side-chain modifications and/or truncations and extensions to be viewed in the same manner as mutations derived from genetic polymorphisms.

An axiom of mass spectrometrically analyzing proteins for point mutations/modifications, however, is that the protein under investigation must be readily purified in sufficient quantity from relatively small volumes of the biological sample in which it resides. Regarding the apolipoproteins, previous work by Bondarenko et al. has demonstrated the ability to use ultracentrifugation in subfractioning lipoproteins from plasma/serum, followed by solid-phase extraction and delipidation of lipoproteins in preparation for direct matrix-assisted laser desorption/ionization time-of-flight mass spectrometric (MALDI-TOF MS) analysis (7, 8). Although able to provide high-quality data, the assay was undertaken using milliliter volumes of blood (drawn intravenously) and required prolonged centrifugation (~4 h). Both of these aspects detract from a sensitive, high-throughput assay. More recently, Dayal and Ertel have reduced the time required to isolate and purify apolipoproteins prior to mass analysis to roughly 1 h (9). Using chemically active (cationic or anionic exchange) surface-enhanced laser desorption/ionization targets, these investigators were able to demonstrate the simple extraction and mass analysis of apolipoproteins apoA-I and apoA-II. However, the relatively poor quality of the data (as evidenced by low mass resolution and accuracy), and presence of albumin interferences generally precludes using the approach for screening apoA-I and apoA-II for mutations/modifications (of which none were identified), or for apoE phenotyping (the signal of which would lie under a broad doubly-charged signal of albumin), respectively.

In this study, the use of mass spectrometric immunoassay (MSIA) (10–12) for structural characterization of apolipoproteins, apoA-I, apoA-II, and apoE was investigated. Important to the success of the MSIA approach were: *1*) the development of devices and methods for the selective retrieval and preparation of the apolipoproteins for MALDI-TOF MS analysis; *2*) the generation of high-quality mass spectrometric data capable of discerning variants of the apolipoproteins; and *3*) the use of the approach in screening a small number of individuals in order to determine apolipoprotein profiles and, critically, in the phenotyping of apoE.

MATERIALS AND METHODS

Materials

Aminopropyltriethoxysilane and ethanolamine were purchased from Aldrich (Milwaukee, WI); caboxymethyl dextran (CMD), carbonyldiimidazole (CDI), chloroform, sinapinic acid, and tris-2-carboxyethyl phosphine hydrochloride (TCEP) were obtained from Fluka (Milwaukee, WI). Sodium acetate, ammonium citrate, ammonium phosphate, and Tween 20 were purchased from Sigma (St. Louis, MO). Acetonitrile (HPLC grade) and nano-pure water were purchased from American Bioanalytical (Natick, MA). Polyclonal antibodies to the apolipoproteins were purchased from Academy Bio-Medical Co., Inc. (Houston, TX). Affinity pipette tips were manufactured at Intrinsic Bioprobes, Inc. (Tempe, AZ). Hepes-buffered saline (HBS) was prepared using 0.01 M Hepes (pH 7.4), 0.15 M NaCl, 0.005% (v/v) polysorbate 20, and 3 mM EDTA.

Affinity tip manufacture

Affinity pipette tips were prepared as previously described (12). In brief, frits were produced in bulk by loading soda lime glass beads into stainless steel annealing molds and baking them

to form a solid, yet porous frit. The frits were then removed and acid conditioned prior to a 12 h treatment with 10% aminopropyl-triethoxysilane. The amine-functionalized frits were equilibrated in a phosphate buffer, followed by a mixture of 15 kDa molecular mass CMD and CDI to produce frits with surfaces covered with carboxyl groups. The carboxyl groups were activated prior to antibody coupling by vigorously rinsing away any free CMD with phosphate buffer and activating the carboxyl surface with an additional volume of CDI. The activated frits were loaded into wide-bore P-200 pipette tips, and the tips were subsequently loaded onto a 96-format robotic pipetting workstation (Beckman Coulter, Multimek 96, Fullerton, CA) for antibody coupling (i.e., the covalent attachment of antibody to the activated surface of the frits). Antibody was coupled to the frits by repetitively flowing (aspirating and dispensing 400 times) 50 μl volumes of antibody solution (100 μl/well; 0.01 mg/ml in 10 mM sodium acetate, pH 4.8) through each of the tips. The remaining (underivatized) active sites on the frits were blocked with ethanolamine (1 M, pH 8.5, 50 aspirations and dispensing, 100 μl each) and the tips were equilibrated in HBS buffer prior to their use. This process yielded affinity pipette tips that were stable and active for a period of at least 1 month following the antibody coupling (by storing at 4°C in HBS).

Plasma collection

Plasma from a healthy 26-year-old male (subject 1) was primarily used in the development of the assays. For the phenotyping assays, another four human subjects were recruited: a 28-year-old male (subject 2), a 32-year-old male (subject 3), a 38-year-old male (subject 4), and a 70-year-old male (subject 5). At the time of blood collection, all subjects were healthy except subject 5, who was diagnosed with Alzheimer's disease.

Plasma sample collection was performed using procedures and protocols approved by Intrinsic Bioprobes Inc.'s Institutional Review Board, and after each participant had signed an Informed Consent Form. In short, human blood was drawn under sterile conditions from a lancet-punctured finger using two heparinized glass capillaries (45 μl each; Drummond Scientific Co., Broomall, PA). Immediately after collection, the blood was diluted with 400 μl HBS containing 0.1% Tween 20. The diluted blood was mixed gently to avoid the rupturing of the red blood cells and then centrifuged for 5 min (at 7,000 RPM, 2,600 *g*) to pellet the red blood cells. The supernatant was aliquotted into wells of a 96-well microtiter plate, providing 200 μl sample volumes of diluted plasma (~10-fold dilution) for each analysis.

Extraction/isolation of plasma apolipoproteins

Apolipoproteins were analyzed from a single plasma sample by sequentially extracting the apoA-I, followed by the apoA-II, and finally the apoE (in each case using the appropriate antibody-derivatized affinity pipette). To eliminate any variability in protocol, the plasma samples from each of the five subjects were processed in parallel (for each of the apolipoprotein assays) using an octa-pipette (Thermo Labsystems, Finnpipette, Helsinki, Finland). For each analysis, the specific apolipoprotein was extracted by repeatedly passing (aspirating and dispensing 25 times) the plasma through an antibody-derivatized affinity pipette tip, after which the affinity pipette tip was rinsed with five 200 μl aliquots of HBS, five 200 μl aliquots of nano-pure water, five 200 μl aliquots of a mixture of chloroform-acetonitrile-HBS (4:8:1, v/v/v), and once again with five 200 μl aliquots of nano-pure water. Retained species were eluted by drawing 3.5 μl of MALDI matrix solution [sinapinic acid saturated in 33% (v/v) aqueous acetonitrile and acidified with 0.4% (v/v) trifluoroacetic acid] into the affinity pipette tips and depositing it directly onto a 96-well formatted MALDI-TOF target that had been treated as previously described (10). Samples were allowed to air dry prior to insertion of the MALDI target into the instrument. The time required to extract and prepare the three apolipoproteins from the five individuals (i.e., 15 samples) was ~30 min.

On-target reduction of apoA-II

ApoA-II was isolated from plasma using the protocol listed above. The elution from the affinity pipette tips was achieved with an acid solution (0.3% trifluoroacetic acid) (in place of the MALDI matrix solution), and the eluates were applied directly to sites on the MALDI target containing 4 μl of dried 100 mM citrate-phosphate buffer (pH 6.5). Immediately afterwards, 2 μl of 50 mM TCEP was added to the eluates to reduce the disulfide bonds. The reducing reactions were allowed to proceed for 5 min at 40°C in a high-humidity enclave before termination by the addition of 3 μl of matrix solution. Samples were air dried prior to insertion of the MALDI-TOF target into the mass spectrometer.

Mass spectrometry

MS analyses were performed on a Bruker Biflex III MALDI-TOF mass spectrometer operating in linear delayed-extraction mode with 19.00 kV full accelerating potential. Typically, five spectra were acquired for each sample by averaging 200 laser shots (10 Hz) per spectrum and gauging the quality of the spectra for high levels of resolution ($M/\Delta m$; measurement of the full width of the peak at half the maximum height of the peak, FWHM) and high signal-to-noise (S/N) ratios.

RESULTS

Optimizations of the protocol in sample preparation and extraction

Initial efforts were directed toward the optimization of protocols used in apolipoprotein MSIA. Factors that can potentially affect the overall results of the MSIA are: *1*) the liberation of apolipoproteins from existing complexes formed in plasma (i.e., disruption of noncovalent interactions between the apolipoproteins and other serum proteins) such that immobilized antibodies have free access to target proteins, and *2*) delipidation of apolipoproteins once isolated from plasma (i.e., once retrieved using antibodies) without interrupting antibody-antigen interaction. Studies began with analyzing plasma apoA-I. In the initial trials, whole blood was simply diluted with HBS prior to antigen extraction, and affinity pipette tips were rinsed only with HBS followed by nano-pure water. These simple protocols resulted in the detection of apoA-I [molecular weight (MW) = 28,078.6 Da], which, although observed prominently in the spectra, was accompanied by relatively strong signals from other apolipoproteins (**Fig. 1A**). Subsequently, detergents (Triton X-100, SDS, etc.) were added at various concentrations to the plasma samples in an attempt to liberate apolipoproteins (before extraction) by disrupting the HDL. Ultimately, HBS buffer fortified with 0.1% Tween 20 was found to be most adequate for this task, resulting in an increase in the apoA-I signal (both single- and double-charged, $m/z \cong 28{,}079$ Da and 14,039 Da, respectively) and a relative reduction in other apolipoprotein signals (Fig. 1B). In a final effort to eliminate the detection of extraneous proteins, various rinse protocols were tested. A mixture of chlo-

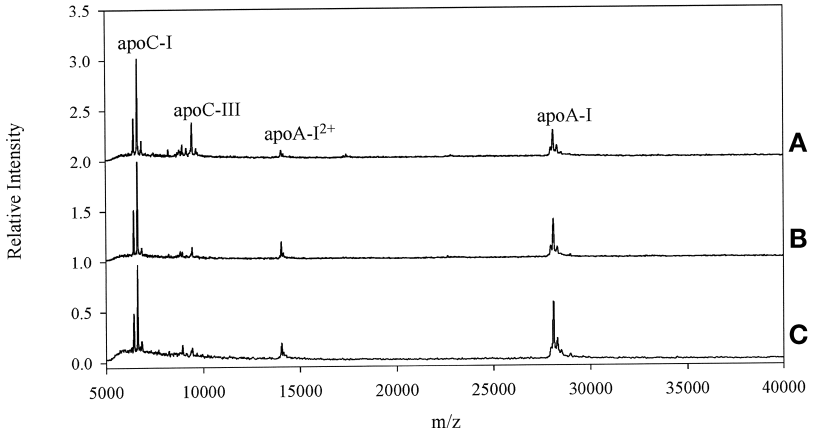

Fig. 1. Optimization of the apoA-I assay. A: Mass spectrum obtained from the interrogation of plasma sample diluted with Hepes-buffered saline (HBS) using an anti-apoA-I affinity pipette tip, and rinsing the tip with HBS and nano-pure water. B: Mass spectrum obtained from the interrogation of plasma sample diluted with HBS containing Tween 20 (0.1% (v/v)), using an anti-apoA-I affinity pipette tip, and rinsing the tip with HBS and nano-pure water. C: Mass spectrum obtained from the interrogation of plasma sample prepared as in B, and rinsing the tip with HBS, nano-pure water, a mixture of chloroform-C AN-HBS (4:8:1, v/v/v), and nano-pure water. ApoA-I (MW_{calc} = 28,078.6 Da; MW_{obs} = 28,081.3 Da) is observed most prominently using the pre- and posttreatment.

roform, acetonitrile, and HBS (4:8:1, v/v/v) was found optimal in reducing nontargeted binding without sacrificing apoA-I signal. Using both the Tween-fortified buffer and the post-capture chloroform rinse, high quality spectra were produced in which apoA-I was observed as the predominant species (Fig. 1C). [Note: The residual detection of the C apolipoproteins is attributed to nonspecific interactions between free C apolipoproteins and the solid supports of the affinity frits, as has been observed previously during the MSIA of other plasma proteins (10, 13)].

These pre- and postisolation protocols were then tested for use in the assay of apoA-II and apoE. **Figures 2** and **3** show comparative results obtained during the analysis of apoA-II and apoE, respectively. In both cases, the protocols found optimal for the apoA-I assay were found preferential. Importantly, the sample/rinse protocols were able to eliminate spectral contributions from doubly-charged albumin, which, due to its presence at $m/z \sim 33$ kDa, could potentially interfere with the accurate analysis of apoE (Fig. 3, inset). Overall, the optimized protocols resulted in spectra largely free of nontargeted species and with generally high S/N ratios (>15) and resolutions (800–1000 M/Δm; FWHM) for the targeted apolipoproteins.

Application of the apolipoprotein assays in the analysis of plasma samples from five individuals

In a brief study, plasma apoA-I, apoA-II, and apoE were analyzed in five male individuals. Results for the analysis

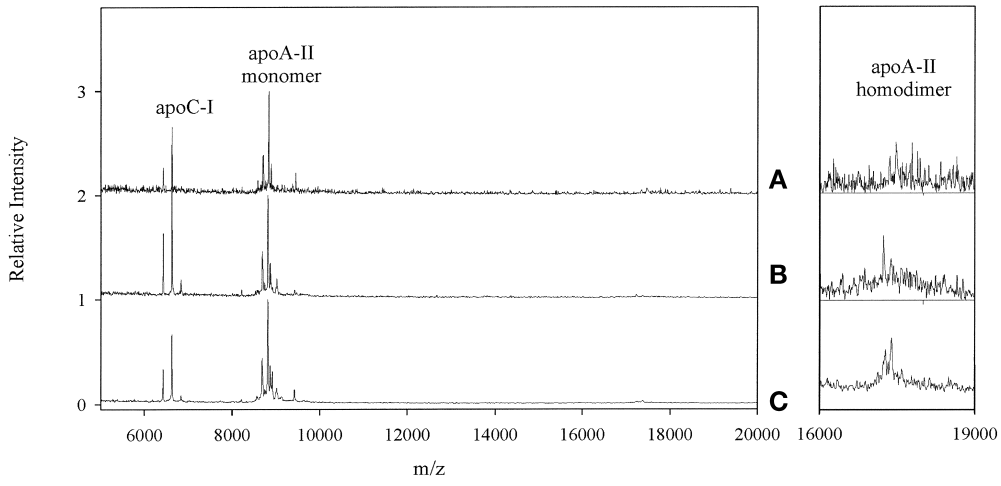

Fig. 2. Optimization series applied to apoA-II. A–C show spectra resulting from the same treatments listed in Fig. 1 A–C, except using anti-apoA-II affinity pipette tips. Also shown is an expanded view of the apoA-II homodimer peaks. Spectral quality is best using the pre- and postincubation treatment (C). Assignments for the signals are given in Fig. 6. With the exception of the apoC-Is [nonspecifically retained by affinity pipette supports (10)], no other apolipoproteins were observed.

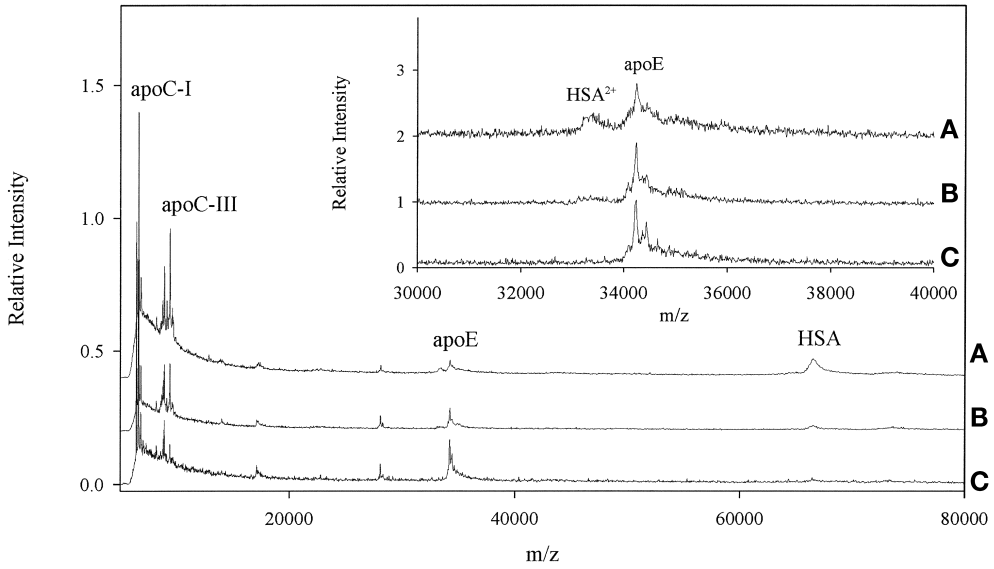

Fig. 3. Optimization series applied to apoE. A–C show spectra resulting from the same treatments listed in Fig. 1A–C, except using anti-apoE affinity pipette tips. Spectral quality is best using the pre- and postincubation treatment (C). ApoE (E3/E3; single signal, MW_{calc} = 34,236.6 Da) is observed at MW_{obs} = 34,239.4 Da with good signal-to-noise (S/N) ratio (inset).

of apoA-I are shown in **Fig. 4**. ApoA-I was detected in each of the five individuals with no significant difference from one individual to another. The quality of the spectra is reasonably good, with a resolution of ~1,000 M/Δm (FWHM) and little existence of peaks from nonspecifically bound species. Mass accuracies were on the order of ~400 ppm using external calibration ($m/z_{(5\ samples)}$ = 28,068–28,090 Da). The presence of a peak shifted in mass by ~−128 Da from the wild-type apoA-I was identified as an isoform of apoA-I formed by the cleavage of the C terminus glutamine amino acid residue (a matrix adduct peak at +224 Da was also observed). This modification is present in all five individuals and therefore is likely to be caused by the degradation of the protein as it ages in the circulatory system. Although there are numerous mutations known for apoA-I (14), none of resolvable mass was found present (as would be observed by split or mass-shifted signals) in the five subjects participating in this study.

Interrogation of the same five samples with anti-apoA-II affinity pipette tips resulted in the spectra presented in **Fig. 5**. Each of the spectra contains peaks in the region of 8,500 Da to 9,000 Da ($MW_{apoA\text{-}II\ monomer}$ = 8,707.9) and in the region of 17,300 Da and 17,500 Da. These signals are identified as forms of the apoA-II monomer and the dimer (formed via a disulfide bridge between two apoA-II monomers), respectively. Closer inspection of the signals (**Fig. 6**, Subject 1) shows the existence of a cysteinylated version (Δm = +120 Da) of the monomer and two forms of the homodimer associated with the loss of end terminal glutamines (Gln, Δm = −128 Da and −256 Da; note: both termini of the monomer are glutamines). In support

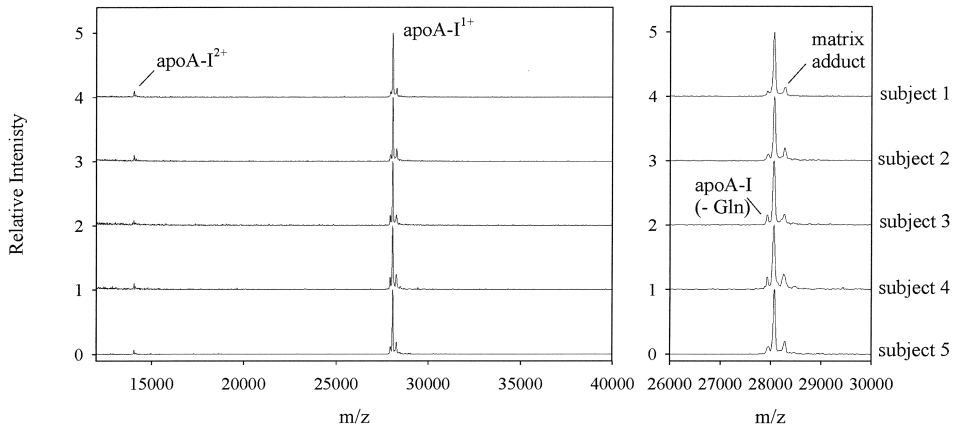

Fig. 4. Mass spectra resulting from the application of the apoA-I assay in the analysis of human plasma samples from five subjects. The spectra have high S/N ratio and signals with good resolutions. MWs determined for the apoA-I ranged from 28,073–28,084 Da.

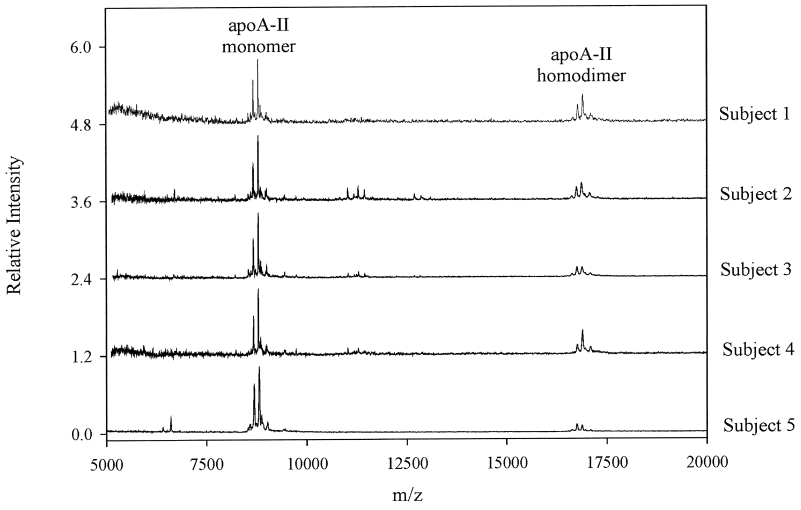

Fig. 5. Mass spectra resulting from the application of the apoA-II assay in the analysis of human plasma samples from the five human subjects. The spectra demonstrate high consistency between individuals.

of these assignments, a second analysis was performed in which the eluates of the apoA-II analyses were reduced on the MALDI target using TCEP. The spectra acquired from the reduced samples are shown in **Fig. 7**. Upon 5 min reduction, the peaks in the homodimer region and the peak identified as the cysteinylated isoform of apoA-II are noticeably absent from the spectra, consistent with reduction of disulfide linkages. The remaining signals at m/z = 8,701.2 Da and 8,578.3 Da are identified as the apoA-II monomer and the monomer minus an end-terminal glutamine, respectively (**Fig. 8**).

Further analysis of the five original samples with anti-apoE affinity pipette tips resulted in the spectra shown in **Fig. 9**. Signals due to apoE isoforms are observed in each spectrum, with resolution (~800 M/Δm) that enabled the identification of several apoE isoforms. All spectra share a common signal at m/z = 34,239, which can be attributed to the apoE3 isoform (MW_{apoE3} = 34,236.6). One spec-

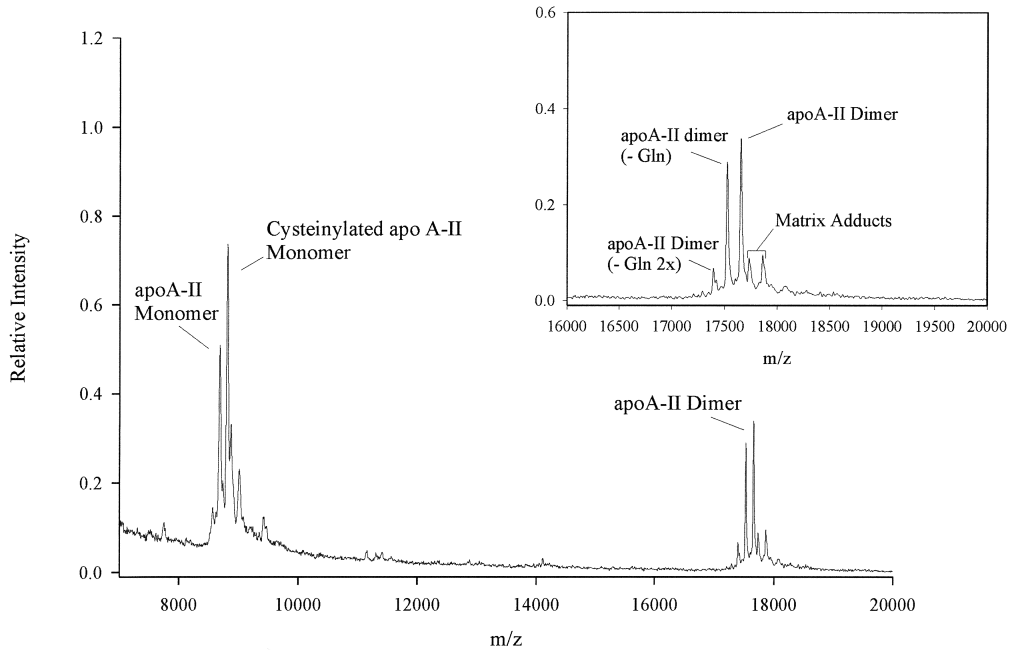

Fig. 6. Expanded view of the mass spectrum from subject 1 shown in Fig. 5. Signals for the apoA-II$_{(monomer)}$ species were observed with the following MWs: apoA-II$_{(monomer)}$ (MW_{calc} = 8,707.9 Da, MW_{obs} = 8,701.2 Da), cysteinylated apoA-II$_{(monomer)}$ (MW_{calc} = 8,827.9, MW_{obs} = 8,823.4 Da); signals for the homodimer species (inset) were observed at: apoA-II$_{(dimer)}$ (MW_{calc} = 17,415.8 Da, MW_{obs} = 17,421.3 Da), apoA-II$_{(dimer)}$ without an end terminal Gln (MW_{calc} = 17,287.9 Da, MW_{obs} = 17,293.4 Da), and apoA-II$_{(dimer)}$ without two end terminal Gln (MW_{calc} = 17,159.9 Da, MW_{obs} = 17,166.2 Da).

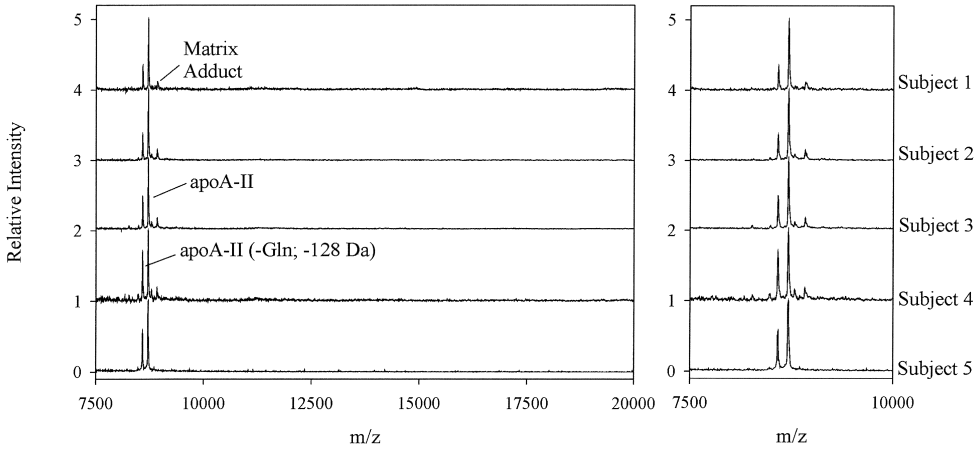

Fig. 7. Mass spectra resulting from the application of the apoA-II assay in the analysis of human plasma samples from the five subjects, with on-target reductions of the eluates from the anti-apoA-II affinity pipette tips. The absence of the 17 kDa species is consistent with the reduction/break up of the homodimer resulting in the formation of two independent apoA-II monomers. Also shown is an expanded view of the apoA-II monomer peaks.

trum also contained a signal mass shifted by −55.04 ± 0.68 Da from the apoE3 signal (Subject 4), and another spectrum contained a signal mass shifted by +52.33 ± 0.69 Da (Subject 5). These mass-shifted signals correspond well with the existence of the apoE2 and apoE4 isoforms, respectively, (i.e., the gene for human apoE is highly polymorphic, containing two major alleles that result in the production of three protein isoforms of MW = 34,183.6 (apoE2: Cys_{112}, Cys_{158}), MW = 34,236.6 (apoE3: Cys_{112}, Arg_{158}), and MW = 34,289.6 (apoE4: Arg_{112}, Arg_{158}).

DISCUSSION

Protocol development and speed of analysis

The use of MSIA in this work allowed for the isolation and detection of plasma apolipoproteins. As little as 50 μl of whole blood was required for the sequential analysis of all three apolipoproteins from a single sample, with each assay taking as little as 15 min to perform (from sample collection to acquisition of spectra). The speed of the method relies on the development of affinity pipette tips (12), which utilize antibodies immobilized to a porous frit, for the isolation of the apolipoproteins from plasma. The availability of affinity pipette tips with large surface areas and high flow characteristics contribute to short incubation times without sacrificing sensitivities (detection on the order of femtomoles to picomoles). Additionally, the protocols developed in this work (for sample preparation and postcapture rinses) enabled the efficient capture and delipidation of the apolipoproteins, which improved the spectral quality of the data without greatly sacrificing sensitivity. Given high-quality data (i.e., high S/N, resolution, and accuracy), it is possible to apply the approach in screening individuals to accurately establish apoA-I, apoA-II, and apoE phenotypes.

ApoA-I and apoA-II profiling

The mass spectra resulting from the apoA-I assays did not reveal any obvious differences between the individuals participating in this study. Signals from the wild-type apoA-I, its matrix adduct (Δm = t224 Da), and a post-

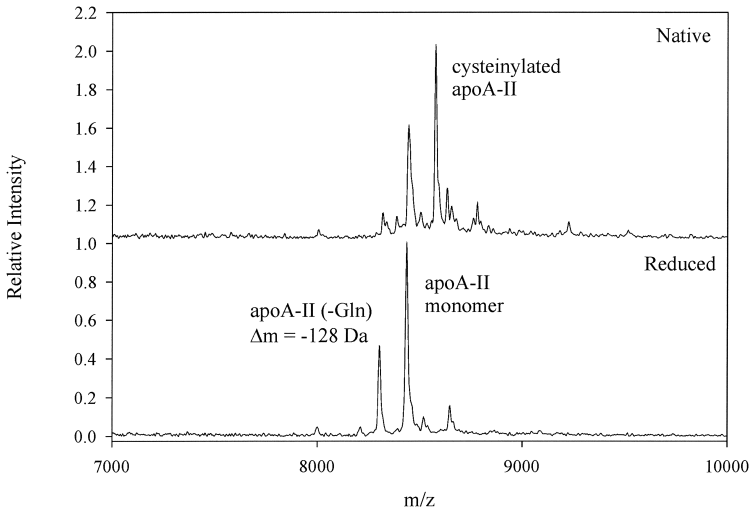

Fig. 8. Comparison of the mass spectra resulting from the application of the apoA-II assay in the analysis of the plasma sample from Subject 1, without (top trace) and with (bottom trace) on-target reduction. The absence of the cysteinylated species is consistent with the reduction of the disulfide linkage between the cysteine of apoA-II and a free cysteine. In addition, the increase in the intensity of the species mass-shifted (Δm = −128 Da) from the monomer is also consistent with the reduction of both the homodimer and the cysteinylated monomer.

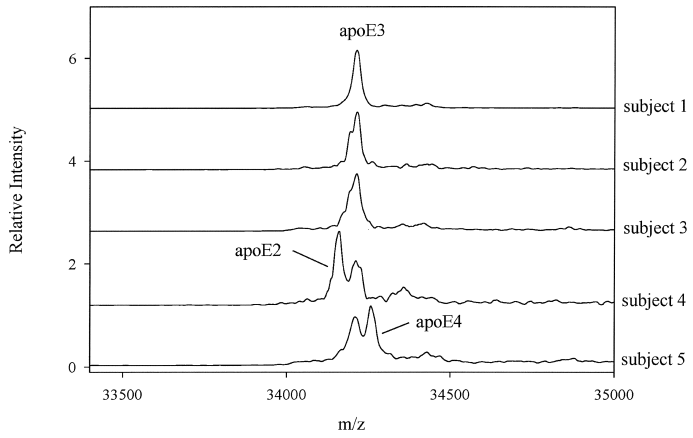

Fig. 9. Mass spectra resulting from the application of the apoE assay in the analysis of human plasma samples from the five subjects. Signals for apoE are observed in three distinct regions reasonably corresponding to the three major isoforms (E2, MW_{calc} = 34,184.6 Da; E3, MW_{calc} = 34,236.6 Da; E4, MW_{calc} = 34,289.6 Da).

translationally modified form (created by the removal of the C terminus glutamine) are observed in all five spectra (Note: The absence of pro-apoA-I, the mature form of apoA-I containing its propeptide, is most likely a consequence of the anti-apoA-I antibody lacking affinity for this isoform). The existence of the truncated apoA-I isoform in all five individuals suggests that this modification occurs naturally in plasma and is perhaps part of the natural metabolism of apoA-I. No other resolvable differences were observed between the individuals. However, the possibility of a heterozygous point mutation resulting in a mass difference of less than ~15 Da cannot be ruled out (because of resolution limitations), and the use of postextraction mass mapping [as previously described (13)] would be warranted if the identification of point mutations within the individuals were the focus of this study. (Note: In this manner, and when using high-performance mass spectrometry, mutant forms of any of the apolipoproteins differing in mass by as little as 1 Da may be discovered/detected.) Interestingly, the apoA-II assay revealed that apoA-II exists as several forms in plasma: apoA-$II_{(dimer)}$, apoA-$II_{(dimer)}$ minus the C-terminal glutamines, apoA-$II_{(monomer)}$, cysteinylated version of the apoA-$II_{(monomer)}$, and the apoA-$II_{(monomer)}$ minus its C-terminal glutamine.

Most recently, various isoforms of apoA-I and apoA-II have been observed independently by two other groups investigating apolipoproteins using mass spectrometry. Using commercially available apolipoprotein standards, Deterding et al. observed the C-terminally truncated form of apoA-I and additional oxidized isoforms of apoA-I (presumably, oxidation at methionines present in apoA-I) (15). Additionally, apoA-$II_{(dimer)}$ isoforms reduced in mass by 128 Da were observed, which were subsequently determined by proteolytic digestion/tandem mass spectrometry to be C-terminally truncated variants (i.e., minus glutamine amino acid residues). Likewise, Bondarenko et al., using the ultracentrifugation-solid phase extraction approach, have observed the presence of the C-terminally truncated isoform of apoA-I and the truncated apoA-II isoforms (16). In addition, Bondarenko et al. have cataloged the apoA-$II_{(monomer)}$ and apoA-$II_{(monomer)}$ minus the C-terminal glutamine, the pro-apoA-I species, and several oxidized isoforms of apoC, apoA-I, and apoA-II.

These studies, and the present study, collectively illustrate the potential of direct mass spectrometric analysis of apolipoproteins to establish profiles of proteins. Importantly, the mass spectrometric analysis of full-length proteins is able to discover and differentiate isoforms of a given apolipoprotein. However, these analyses are of value only if the isoforms reflect true in vivo species. In the present study, the absence of oxidized apolipoprotein isoforms (that have been identified in these other studies) can be most easily rationalized as an effect of analyzing the apolipoproteins within minutes of collecting the plasma sample, thus reducing the time available for oxidization to occur. Accordingly, the MSIA approach may offer a specific advantage over these other approaches by allowing the apolipoproteins to be analyzed within minutes of blood collection, thereby eliminating chemical modifications that potentially occur over time (in vitro). Additionally, given a priori knowledge of the specific antibody, the MSIA approach is able to selectively isolate additional isoforms that are potentially obscured (from analysis) using less-selective extractions. A case in point is the observation of the cysteinylated isoform of apoA-$II_{(monomer)}$, which is observed during MSIA yet escapes detection in the previous studies. The existence of such cysteinylated variants of plasma proteins has been shown previously (13), and is characteristic of proteins having a free cysteine residue. With regard to a possible physiological effect from cysteinylating apoA-II, studies have shown that disulfide bridge formation between apoA-II and apoE reduces the level of β-amyloid in plasma, which in turn decreases the formation of amyloid plaques associated with neurodegenerative diseases (17–19). Thus, excessive cysteinylation of the apoA-II monomer could potentially interfere with the formation of the apoA-$II_{(monomer)}$-apoE heterogeneous dimer found to assist in the regulation of β-amyloid in plasma. In the present study, the ratio of cysteinylated apoA-II isoform to the apoA-II monomer appears fairly constant. However, as the study group in this investigation was small, further MSIA studies involving a much larger group of individuals are needed to determine the impor-

tance of variability of cysteinylation (or other apoA-II variants) and any possible links to neurodegenerative diseases.

ApoE phenotyping and possible applications

The MSIA approach readily allowed for accurate apoE phenotyping via direct detection of apoE species with high mass accuracy. The resolving power of the current instrumentation was sufficient to resolve the $\Delta m = \pm 53$ Da mass shifts expected for the Cys-Arg point mutations occurring in the major apoE phenotypes. As a note in passing, it was necessary to eliminate albumin contributions to the profile (specifically, the doubly-charged signal) in order to accurately phenotype apoE. In this manner, the elimination of nonspecific binding of the high-level plasma protein was critical, and, as demonstrated, was accomplished through the construction/development of optimized devices and associated analytical protocols. The sensitivity of the approach does not appear to be a limitation, as high S/N spectra were produced from relatively small volumes of blood. Moreover, the use of small sample volumes (50 μl of whole blood) allowed finger-prick blood-draw to be used in acquisition, which essentially requires no special training and is attractive when considering further applications of the apoE MSIA.

Previous to this report, the ability to phenotype apoE using direct mass spectrometric detection (of the intact protein) has not been demonstrated. Thus, we believe that MSIA provides a rapid and sensitive means to phenotype apoE (as homozygote and heterozygote individuals), which, at the very least, complements large-scale genotyping efforts directed at the analysis of SNPs. Given the ability to perform protein phenotyping assays rapidly and on small amounts of blood (with no PCR intermediate step), economically competitive MSIA (i.e., pennies per assay) can be constructed to screen populations for polymorphisms/mutations already correlated to disease. Presently, apoE phenotypes have been linked most significantly to Alzheimer's disease and coronary heart disease (20–22), and a limited number of apoA-I mutations have been linked to amyloidosis (14, 23–26). Assaying these and other mutations will find use in proteomic applications geared toward understanding the biological processes underlying the (protein) phenotype-related diseases.

Alternatively, the MSIA assays may find future use as point-of-care analytics used in personalized medicine. In this manner, phenotyping assays may be of assistance in the optimized administration of drug or application of diet therapy. Currently, one treatment of individuals possessing high lipid/cholesterol levels is the administration of lipid/cholesterol-reducing drugs (e.g., Lipitor, Zocor, Pravac, Mevacor). These drugs reach maximum effectiveness with the knowledge of specific apoE phenotype and when combined with modified diets, with such therapy being most effective for apoE2 phenotypes and least effective for apoE4 phenotypes (27). Such phenotyping versus effectiveness (of drug/diet administration) applications stand to gain even greater importance as drugs for combating Alzheimer's disease are developed and proceed through phase trials.

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