Solvent-free MALDI-MS for the Analysis of β-Amyloid Peptides via the Mini-Ball Mill Approach: Qualitative and Quantitative Advances

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Manual and automated solvent-free mini-ball mill (MBM) matrix-assisted laser desorption/ionization (MALDI) analysis of mixtures of β-amyloid peptides (**1-11**), (**33-42**), (**1-42**) and non-β-amyloid component of Alzheimer's disease peptide yielded interpretable spectra for all of the peptides present regardless of their relative amounts in the samples. This was not the case for solvent-based MALDI analysis using traditional acidic aqueous/organic solvent conditions, which resulted in severe over-representation of hydrophilic peptide (**1-11**) and provided no spectra for insoluble amphiphilic peptide (**1-42**) even when present at 50% relative molar amount. Less accurate representation of components in mixtures by the traditional method appears to be a combination of poor dissolution of peptides in the solvent and preferential ionization of more hydrophilic peptides in the mixture. Consequently, only MBM provided a complete tryptic map of β-amyloid (**1-42**) compared to 67% coverage by traditional MALDI. Acetonitrile (0.1% TFA) led to improved coverage only at a 50% molar ratio of peptide (**1-42**), but also to a side product of (**1-42**), Met oxidation (amino acid 35), a phenomenon not observed in MBM MALDI analysis. Traditional MALDI analysis resulted in over-representation of hydrophilic soluble β-amyloid (1-11) in defined mixtures and autoproteolytic peptides of trypsin. In contrast, over-representation and under-representation were less pronounced in solvent-free MALDI in all of the investigated cases. Analysis of defined peptide and tryptic peptide mixtures showed that MBM MALDI yielded greater qualitative reliability, which also improved quantitative response relative to the solvent-based approach. (J Am Soc Mass Spectrom 2007, 18, 1533–1543) © 2007 American Society for Mass Spectrometry

Evading solvent-mediated changes, e.g., segregation and side-reactions, to the sample while achieving excellent sensitivity and resolution using the solvent-free matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) method are important advantages for the analysis of synthetic polymers and other large synthetic macromolecules even when solubility restrictions apply [1–3]. However, the method is potentially as important for the analysis of peptide molecules that in many cases are intractable, such as peptides derived from membrane proteins. The current study seeks to determine if the mini-ball mill (MBM) MALDI-MS method [4] can representatively analyze sample mixture components as well as or better than the conventional solvent-based MALDI method. A common case where mixture analysis is important is in analyzing components of a tryptic digest. β-Amyloid (**1-42**), (Scheme **1**), comprises a particularly good test since its tryptic digests are expected to contain a mixture with components of widely varying hydrophobicity [5]. This solubility-restricted, aggregating biopolymer [6, 7] is amphiphilic (Scheme **1**), and has a hydrophilic N- and a hydrophobic C-terminus. β-Amyloid (**1-42**) is an enzymatic cleavage product of the amyloid-precursor protein (APP). APP processing and the role of cerebral amyloid β accumulation in common forms of Alzheimer's disease (AD) have been extensively studied and reviewed; for brevity only one reference is cited [5].

Experimental

Materials

α-Cyanohydroxycinnamic acid (HCCA) was purchased from Sigma-Aldrich (St. Louis, MO). β-Amyloid (**33-42**; $MW_{average}$ (average molecular weight) 915.2 Da) **1**, β-Amyloid (**1-11**; $MW_{average}$ 1325.3 Da) **2**, non-β-amyloid component of Alzheimer's disease (**NAC**; $MW_{average}$ 3260.6 Da) **3**, and β-amyloid (**1-42**; $MW_{average}$

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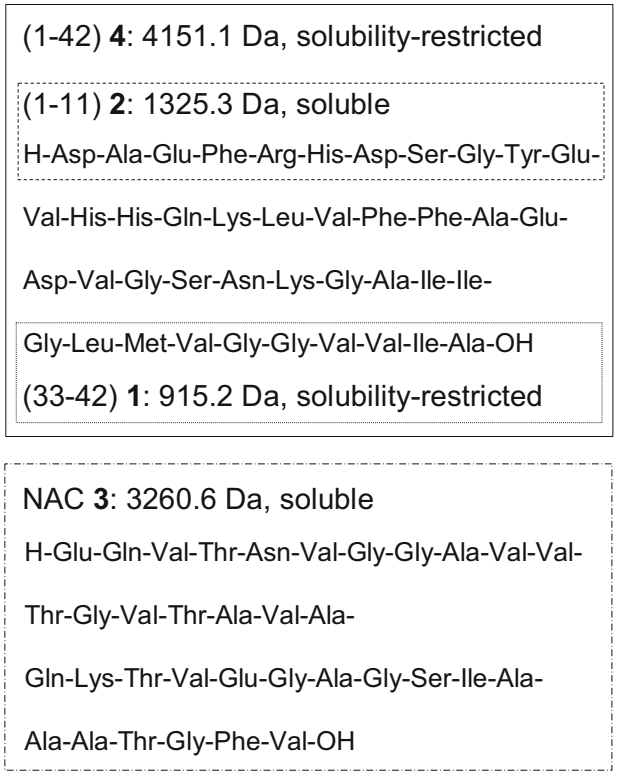

Scheme 1. Primary amino acid sequences of amyloid-precursor protein (APP) peptides **1** to **4**: primary amino acid (aa) sequences, average molecular weight ($MW_{average}$), and solubility in aqueous solution.

4515.1 Da) **4** were obtained from American Peptide Company (Sunnyvale, CA). Sequencing-grade trypsin along with its suspension buffer (Promega, Madison, WI) was used for digestion experiments. High purity water (Burdick and Jackson, Muskegon, MI), acetonitrile (ACN) (J. T. Baker, Philipsburg, NJ), and trifluoroacetic acid (TFA) (Sigma Chemicals Co., St. Louis, MO) were used for dissolving samples.

MALDI-TOF Analysis

MALDI-time-of-flight-mass spectrometry (TOF-MS) was performed on an ABI4700-TOF/TOF mass spectrometer (Applied Biosystems, Framington, MA) with an accelerating voltage of 20 kV. Samples were placed on a standard 192-well stainless steel sample plate, dried if needed (solvent-based approach), and excess powder removed using a difluoroethane duster canister (Dust-Off, Williston, VT). Data were acquired in the reflectron mode. Mass spectra were calibrated using standards supplied with the Applied Biosystems ABI4700 CalMix. Experimental conditions (e.g., laser power) were kept constant throughout to ensure relevant comparisons within specific sets of experiments. The conditions were set so that individual peptide signals with good intensities but minimum detector saturation resulted for as many mixture components as possible. However, sample preparation (solvent) and laser power conditions were altered in solvent-based MALDI to determine whether or not analysis can be improved in cases of detection failure under the previous set of conditions. For automated mass measurement analysis, an inclusion list was used for both preparation methods. The list determines which signal should be used for MS/MS analysis if detected in the initial mass spectrum.

Sample Preparation

I. Solvent-free based on the mini-ball mill (MBM) method. A general solvent-free protocol was used [4]. Mixing of the analyte and matrix powder was performed using a mini-beadbeater (Biospec Products, Bartlesville, OK) with a mixture of beads in plastic tubes (0.2 mL PCR tubes; Molecular Bioproducts, San Diego, CA). In Exp. 1 and 2, three metal beads (1.2 mm size; Biospec Products) were used. The rationale for the use of metal beads was to avoid any metal cation sources. Alternatively, in Exp. 3, glass beads in 0.5 mm and 1.0 mm sizes (Biospec Products), were employed. The amount of beads used corresponded roughly to the volume of the MALDI sample already present in the tube. If drying procedures were involved, the matrix was added to the analyte that was dried over the beads. The capped tube was placed in the original MBM container and placed in the two-dimensional shaker arm of the MBM and shaken for the desired length of time. A minimum amount of the ground powder was applied to the MALDI plate and spread carefully over several spots and affixed by pressing with the flat edge of a spatula. Estimate amounts of sample transferred to the MALDI plate are ~1/20th of the finely distributed MBM material in the 200-μL vial providing coverage of about two to five MALDI spots.

II. Solvent-based method. For the solvent-based method, 2 mg α-cyano-4-hydroxycinnamic acid (HCCA) was dissolved in 1 mL 50:50 acetonitrile (ACN):water 0.1% trifluoroacetic acid (TFA), a solvent system generally used in traditional solvent-based MALDI analysis; 1.5 μL of the matrix solution were combined with 0.5 μL of the dissolved sample and mixed in the vortexer for 10 s; 0.5 μL was placed on the MALDI plate. Solvents used to dissolve the model peptide mixtures were 50:50 ACN: water 0.1% TFA or 99.9% ACN 0.1% TFA; the tryptic digested sample was used as is. Dissolved sample was placed on the MALDI plate and evaporated.

Experimental Details

Experiment 1. For tryptic digestions, 1 to 5 μL of 1 μg μL^{-1} trypsin in a suspension buffer were added to 100 μg β-amyloid **1** dissolved in 100 to 200 μL water and the digestion mixture was placed on a 37 °C shaking air bath overnight. Tryptic peptides were generated (**A**) overnight for solvent-based MALDI- and electrospray ionization (ESI)-quadrupole-time-of-flight (Q-TOF)-MS and MS/MS analyses; 100 μg were dissolved in 100 μL water, 5 μL trypsin solution was added for digestion or

Table 1. Response factors of β-amyloid peptides **1:2:3:4**:(molar ratios) in defined mixtures: (1) 1:1:1:1, (2) 1:1:1:1, and (3) 1:1:1:3 in MALDI-TOF mass spectra using solvent-based and solvent-free MBM MALDI-MS preparation methods

		Peptide ratios		Solvent-based			Solvent-free		
Mix. no.	Peptides	Molar	Percent	Signal intensity[1]	Percent of total signal	Response factor[2]	Signal Intensity	Percent of total signal	Response factor[2]
1	**(33–42) 1**	0.99	24.6	0.22	5.47	0.22	0.52	12.9	0.52
	(1–11) 2	1.05	26.1	3.59	89.3	3.42	2.93	72.9	2.79
	NAC 3	0.98	24.4	0.21	5.22	0.21	0.18	4.48	0.18
	(1–42) 4	1	24.9	n.s.	n.s.	n.a.	0.39	9.7	0.39
2	**(33–42) 1**	3	30	0.1	1	0.03	2.11	21.1	0.7
	(1–11) 2	3	30	9.19	92	3.07	6.75	67.5	2.25
	NAC 3	2.99	29.9	0.7	7	0.23	0.79	7.9	0.26
	(1–42) 4	1	10	n.s.	n.s.	n.a.	0.35	3.5	0.35
3	**(33–42) 1**	0.9	14.8	0.12	1.97	0.13	0.76	12.5	0.85
	(1–11) 2	1.18	19.4	5.66	92.9	4.8	4.44	73	3.76
	NAC 3	1.01	16.6	0.31	5.09	0.31	0.4	6.58	0.4
	(1–42) 4	3	49.3	n.s.	n.s.	n.a.	0.48	7.89	0.16

n.s. No signal. The signals were either too weak, non-descript or non-existent to be measured.
n.a. Not applicable.
[1]Signal intensities are the sum of the signal cluster areas of protonated, sodiated, potassiated, and doubly charged ions. Total ion current for each mass spectrum (Supplementary Information) is: **(A)** solvent-based: Mix. 1: 1.7 e^{+4}; Mix. 2: 1744.6; Mix. 3: 5572.2; **(B)** solvent-free: Mix. 1: 3384.9; Mix. 2: 1.8 e^{+4}; Mix. 3: 1.3 e^{+4}.
[2]Response factor is the ratio of the percent of total signal for each peptide over the percent of peptide present (by weight).

(**B**) for 3 or 24 h periods for solvent-based and solvent-free MALDI- and ESI-Q-TOF-MS and MS/MS analyses; 100 μg were dissolved in 200 μL water, 1 μL trypsin solution was added for digestion. In general, more dilute solutions of **4** produced improved digestion with less autoproteolysis of trypsin. Additionally, **4** was denatured by incubation at 100 °C for 5 min. After cooling, trypsin was added and digestion was carried out for 3 and for 24 h.

General. After proteolytic digestion of **4**, an aliquot was taken for solvent-based MALDI analysis. The remaining sample was frozen by liquid N_2 and then freeze-dried to obtain MBM analysis. MS and MS/MS spectra of the digest of **4** were recorded.

Experiment 2. A model complex mixture of insoluble peptides **1** and **4**, and soluble peptides **2** and **3** were mixed in a molar ratio of about 1:1:1:1 (Mix. 1) for peptides **1:2:3:4** and investigated by solvent-free and solvent-based MALDI methods. Additional mixtures, 3:3:3:1 (Mix. 2) and 1:1:1:3 (Mix. 3), were prepared accordingly. Exact values of molar peptide ratios of defined peptide mixtures are provided in Table 1. Peptide mixtures were transferred on a weighing foil to the MBM plastic tube (0.2 mL) and vortexed with three metal beads to homogenize the peptide sample within the tube. In both steps solvent was omitted to avoid preferential dissolution of soluble peptides. Matrix was initially left out when homogenizing the four peptides to prevent adduction with alkali metal cations to the peptides, which had previously been shown to increase with increasing grinding time when matrix is present [4]. Homogenized peptide samples were then individually mixed with HCCA matrix according to the MBM procedure (1 min) described above. Sample preparation might have introduced small systematic error(s) if not all of the powder came off of the foil when homogenizing solely the peptides or subsequently peptides with the matrix. Nevertheless, the homogeneity achieved for a given peptide mixture will be the same in solvent-free and solvent-based MALDI experiments; so that a comparison of results between the traditional solvent-based and solvent-free MALDI analysis for any given sample is assumed to be accurate. Mixtures 2 and 3 were vortexed longer to insure homogeneity before the HCCA matrix was added. Resulting MALDI samples were (1) applied directly to the MALDI plate (solvent-free MBM MALDI), or (2) completely dissolved in 50:50 ACN:water 0.1% TFA and applied to the MALDI plate (solvent-based MALDI), or (3) dissolved in ACN 0.1%TFA solution in an effort to dissolve the in aqueous solution insoluble peptides **1** and **4** for solvent-based MALDI analysis.

Experiment 3. A 6.0 pmol μL^{-1} solution of peptide (**1-11**) **2** was serially diluted to make solutions of 3.0, 1.5, 0.75, 0.375, and 0.187 pmol μL^{-1}. 100 μL of each solution was used with the MBM solvent-free MALDI method to determine the lowest detectable peptide amount required per vial; the amount transferred to the MALDI plate is estimated to be 1/100th of the total.

LC-ESI-MS

LC-MS/MS employed a Waters (Millford, MA) CapLC connected to a Waters Q-TOF Ultima Global. 2-6 μl of sample were loaded onto a Waters Symmetry C18 trap at 4 μL min^{-1}, then the peptides were eluted from the trap onto the 10 cm × 75 μm lab packed Phenomenex

Table 2. MASCOT ion scores of data obtained in the automated MS/MS measurements of the individual peptides from model peptide mixtures **2** and **3** studied by two different MALDI methods; solubility in aqueous solution, and hydropathicity index obtained°from°http://us.expasy.org/tools/protparam.html°are°provided

	Average molecular weight in Da	Solubility in aqueous solution[1]	GRAVY score[2]	Mix. 2[3]		Mix. 3[4]	
				Solvent-based	Solvent-free	Solvent-based	Solvent-free
Peptide **1**	915.2	Low	2.133	n.a.	82	n.a.	95
Peptide **2**	1325.3	High	–1.782	74	65	75	56
Peptide **3**	3260.6	High	0.726	40	74	45	56
Peptide **4**	4515.1	Low	0.162	n.a.	12	n.a.	18

[1]Solubility according to suppliers' information and e.g., [16].
[2]Grand average of hydropathicity (GRAVY) index.
[3,4]Compositions: Molar ratios of peptides **1:2:3:4** of 3:3:3:1 (Mix. 2) and 1:1:1:3 (Mix. 3).

Jupiter C5 analytical column (Phenomenex, Torrance, CA) at 300 nl min^{-1}. The precursor ions of peptides eluting from the analytical LC column were monitored using 0.6 s scans from *m/z* 500 to 1990. Up to three parent ions per survey scan that had sufficient intensity and had up to three positive charges were chosen for MS/MS. The MS/MS scans were 2.4 s from *m/z* 50 to 1990. The mass spectrometer was calibrated using the MS/MS spectrum of glu-fibrinopeptide. Masses were corrected during each run using a lock mass scan every 30 s of glu-fibrinopeptide.

Data Analysis

MASCOT (Matrix Science, London, UK) was used to score and interpret the tandem mass spectral data for the°peptides°[8].°The°following°restrictions°were°used: precursor and MS/MS tolerance of 0.5 Da, peptide charge +1 for MALDI, in general +2 and +3 for ESI, and modifications were limited to the parameters sodiated (amino acids D, E), sodiated (C-terminus of a peptide), and oxidation (amino acid M).

Computational Analysis of Peptide Properties

Grand°average°of°hydropathicity°(GRAVY)°scores°(Table°2)°of°peptides°**1** to°**4** were°determined°using°the database supplied by the Swiss Institute of Bioinformatics°at°http://us.expasy.org/tools/protparam.html.

Results and Discussion

Tryptic Peptide Mapping Investigations of Peptide (1-42) Using Solvent-Based MS Methods

Solvent-based MALDI analysis of the tryptic peptides of **4** yielded MS and MS/MS spectra (Exp. 1A) with good signal intensity. However, only the more soluble tryptic peptides of **4** could be recorded and identified (Table°3).°None°of°the°hydrophobic°C-terminal°tryptic peptides, $T_{6\text{-}42}$, $T_{17\text{-}42}$, or $T_{29\text{-}42}$, were detected. This result indicates that the hydrophobic tryptic peptides from tryptic digestion of (**1-42**) might be detected less favorably under conditions (50:50 ACN:water 0.1% TFA) commonly employed in traditional solvent-based MALDI analysis. Sequence coverage of **4** was 66.7%, which corresponds to the coverage of just the soluble N-terminal region amino acids (aa) 1 to 28. Abundant additional signals were observed at *m/z* 2211 Da, corresponding to peptides formed in trypsin self-digestion, and at *m/z* 2543 Da and *m/z* 4512 Da corresponding to doubly and singly charged undigested **4**, respectively. These latter signals suggest that a large amount of peptide **4** remained undigested, which, from a biological perspective, is not unexpected considering its poor solubility, high aggregation tendency, and ability to inhibit° trypsin° digestion° [6];° however,° this° cleavage specific protease was used throughout this work to keep a maximum degree of confidence in data interpretation. The detection of the large insoluble peptide **4** coupled with the failure to observe the smaller insoluble peptides $T_{17\text{-}42}$ and $T_{29\text{-}42}$ was unexpected.

Quantitative representation of peptides in the complex mixture, in particular peptides with limited solubility, was not obtained using the solvent-based MALDI method. In the tryptic digestion of **4**, peptides are produced that show very different solubilities (Scheme **1**,° Table° 2).° When° molar° ratios° in° the° solutions° are shifted towards the soluble peptides, the characterization of the less soluble ones becomes increasingly difficult. Thus, if solvent-based MALDI is employed, approaches to keep the less soluble peptides in solution may need to be involved. In earlier work, detergents were employed to keep the hydrophobic and/or solu-

Table 3. Trytpic peptides of amyloid (**1–42**) **4** according to their MS signal intensities using solvent-based MALDI as well as MASCOT ion scores derived from MS/MS data (all data available in the Supplementary Information section). Tryptic autoproteolysis has been observed to <2% (*m/z* 2211)

	m/z [Da]	z=1	Signal intensity	MASCOT ion score
$T_{1\text{-}16}$	1954.9	1	4140	88
$T_{1\text{-}42}$	4512.1	1	1863	n.a.
$T_{6\text{-}16}$	1336.6	1	1035	78
$T_{1\text{-}42}$	2257.6	2	517	n.a.
$T_{6\text{-}28}$	2642.2	1	310	36
$T_{1\text{-}16}$	977.9	2	207	n.a.

n.a. not applicable.

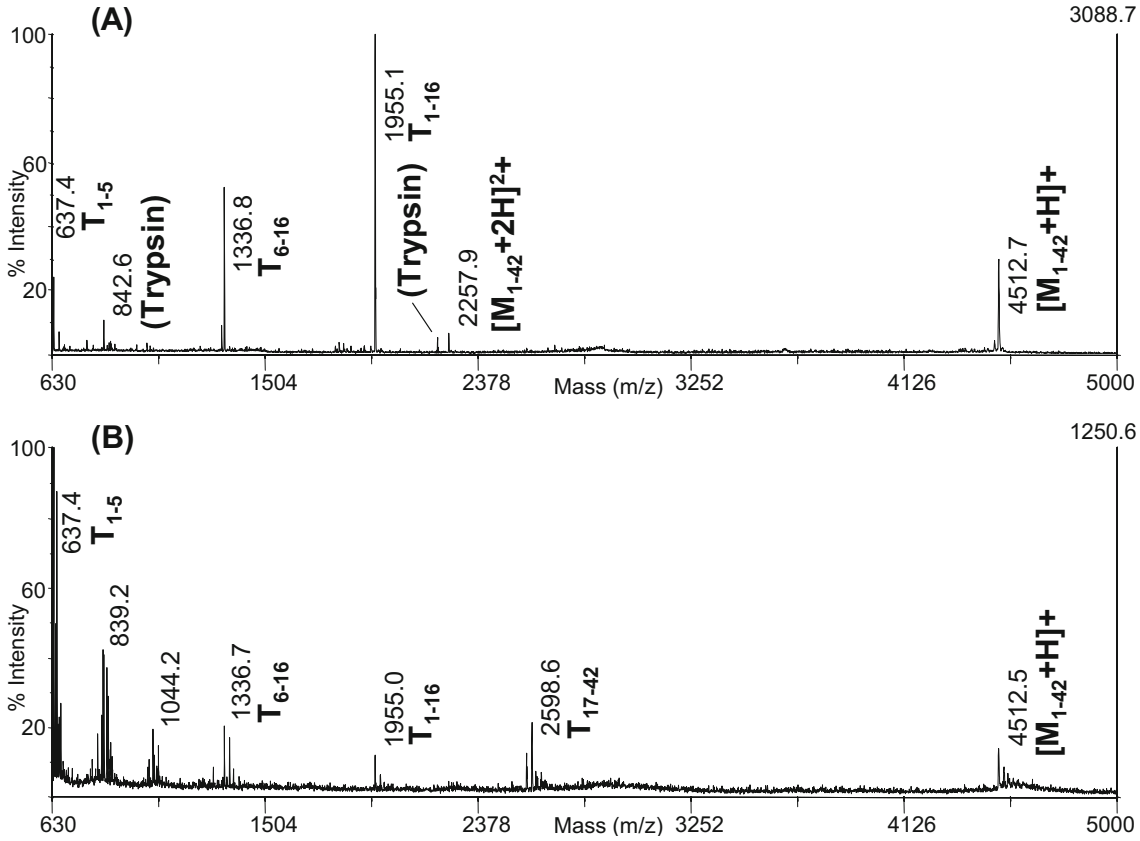

Figure 1. Mass spectra of tryptic digest of **4**: (**A**) solvent-based, (**B**) solvent-free MBM MALDI analysis. Only the MBM approach detects the hydrophobic, solubility limited C-terminal, protonated and metal adducted, peptide T_{17-42} (*m/z* 2598). In the solvent-based spectrum, *m/z* 2211 Da, a trypsin auto-proteolysis product, is observed, but not in the solvent-free sample preparation method.

bility-restricted peptides in solution [9–12]. Mass spectrometric analysis is in general very sensitive to additives like detergents, therefore, are more likely to be removed by, for example, hyphenated liquid chromatography (LC)-MALDI or LC-ESI approaches.

LC-ESI (see Supplementary Information section, which can be found in the electronic version of this article) of the tryptic digest of **4** also showed peptide peaks, thus confirming that some digestion had occurred, but their relatively low signals strongly suggests that **4** was largely undigested. In addition, the MS and MS/MS data interpreted by MASCOT showed only signals for peptides within aa residues 6 to 28 with peptide T_{6-28} and peptide T_{17-28} giving MASCOT scores of 51 and 84, respectively, thus providing high confidence peptide identification (expectation values of up to $1.7e^{-6}$) with a sequence coverage of 54.8%.

Peptides that contain aa residues 29 to 42 were not MS accessible and therefore the entire sequence of β-amyloid peptide **4** could not be identified manually or through MASCOT interpretation. The poor detection of T_{17-42} is probably a combination of this peptide not being protonated in sufficient amounts when either solvent-based MALDI or ESI is used and its poor solubility. To the best of our knowledge there is no MS data available on tryptic digested β-amyloid peptide (**1-42**) but there is for β-amyloid peptide (**1-40**) [13]; β-amyloid peptide (**1-42**) has a higher tendency of aggregation than β-amyloid peptide (**1-40**) [7]. These previous tryptic peptide mapping results of (**1-40**) revealed the ESI-MS detection in the following chromatographic order: T_{6-16}, T_{1-5}, T_{1-16}, T_{17-28}, T_{1-40}, T_{6-40}, and T_{6-40} in which the latter three peptides were not chromatographically resolved.

The results of the solvent-based MS investigations sought to study in-depth the differences in solvent-based and solvent-free MALDI by varying digestion procedures of β-amyloid peptides (**1-42**) **4** and by preparing defined mixtures of β-amyloid peptides (**33-42**) **1**, (**1-11**) **2**, (**1-42**) **4**, and non-β-amyloid component of Alzheimer's disease peptide **(NAC) 3**.

Direct Comparison of Tryptic Peptide Mapping Investigations of Peptide (1-42) Using Solvent-Based ESI and MALDI and Solvent-Free MALDI Methods

To be certain that all peptides were formed in sufficient amounts, the digestion procedure for **4** was altered to include a denaturation step. After 3 h, the solvent-based MALDI mass spectra showed one intense signal at *m/z*

1955.9 Da corresponding to sequence T_{1-16}, one signal with low intensity at *m/z* 1336.6 Da corresponding to sequence T_{6-16}, and little else. After 24-h (Figure 1a, Exp. 1B), digestion had proceeded further, but undigested **4** was still detected. The solvent-based MALDI MS method again yielded 66.7% sequence coverage, confirming the previous solvent-based results. The relative signal intensities in the solvent-based MALDI mass spectrum were in the order T_{1-16}, followed by T_{6-16}, T_{1-5}, and a very weak T_{17-28}. The signal intensity of undigested **4** (z = 1, 2) is significantly smaller than observed in the sample that was not denatured before proteolysis (Table 3), indicating that the digestion degree had increased. LC-ESI results detected peptide T_{17-28} with MASCOT ion scores up to 84 providing sequence coverage of 28.6% (see Supplementary Information section for chromatograms, mass spectra, and MASCOT ion scores under various conditions); trypsin autoproteolysis was also observed. Overall, LC-ESI analysis was more involved in sample handling, e.g., column plugging, and results were based on sequence coverage of less quality than solvent-based MALDI.

The solvent-free MALDI method has been shown to be capable of analyzing solubility-limited macromolecules [1–3], and therefore this approach might be expected to provide spectra for the insoluble amyloid peptides. Solvent-free MBM (Figure 1b, Exp. 1B) not only displayed intense signals for all the tryptic peptides of **4** recorded by solvent-based MALDI, but in addition displayed a T_{17-42} signal with high intensity that provided 100% sequence coverage. Undigested **4** (z = 1) was also detected, but trypsin autoproteolysis products were not observed. This seems to be in accord with the 1:500 trypsin to β-amyloid **4** ratio used for proteolysis. Based on these MALDI results as well as the LC-ESI result discussed above, the solvent-based analysis over-represents the trypsin self-digestion peptides. A high degree of sodiated parent ions accompanied the solvent-free analyses; the higher metal adduction tendency of the solvent-free versus the solvent-based approach was described previously [4, 14]. Generally, the most intense signal for the individual peptides were those of the protonated parent ion, e.g., $[T_{6-16} + H]^+$; only the C-terminal peptide appeared predominantly as the sodiated ion $[T_{17-42} + Na]^+$. The source(s) of sodium ions has not been fully established but the matrix may be a major contributor [4].

Qualitative Assessment of the MS and MS/MS Analyses of Defined Peptide Mixtures

To better understand the differences observed between the solvent-based and solvent-free approaches, sets of defined peptide mixtures were studied. The peptide mixtures, Mix. 1 to 3, employed the same β-amyloid peptides (**33-42**) **1**, (**1-11**) **2**, (**1-42**) **4**, and (**NAC**) **3** in different molar ratios. Aqueous soluble peptides, **2** ($MW_{average}$ = 1325) and **3** ($MW_{average}$ = 3261), and the less insoluble peptides, **1** ($MW_{average}$ = 915) and **4** ($MW_{average}$ = 4515) (Exp. 2) were mixed in a 1:1:1:1 molar ratio (Mix. 1, Table 1). A portion of the solvent-free MBM sample was dissolved in 50:50 ACN:water 0.1% TFA and used for solvent-based MALDI.

Only soluble peptides, **2** and **3** (Figure 2, Inset 2A and 3A), and low molecular weight hydrophobic peptide **1** (Figure 2, Inset 1A, were detected by the solvent-based method while all peptides were detected in the solvent-free MALDI spectra (Figure 2, Inset 1–4B) with excellent shot-to-shot reproducibility. The higher molecular weight soluble peptide **3** was observed in lower abundance compared to the soluble lower molecular weight peptide **2** (Figure 2, Inset 2, 3B).

The degree of alkali metal adduction and exchange were high in both MALDI methods for the most hydrophobic peptide **1**. The solvent-free method, as noted above, tends to promote metal adduction (Figure 2) for all peptides, giving a ratio of about 10:8:3 (proton:sodium:potassium cations) compared with 10:1:3 using the solvent-based method for peptide **3**. Cation adduction was advantageous in the case of peptide **1**, which has no hydrophilic sites for protonation and yet a signal could be observed because of the adduction. However, the molecular ion signal for the peptide is distributed over different ions, including protonation and various different metal cation adductions, all of which are of low abundance.

The sample preparation method does not seem to matter in the case of peptide **1**; the most intense ion signals are metal adduct ions (Figure 2, Inset 1) having relative intensities about the same for the two MALDI methods. The adduction extent in solvent-based MALDI is unexpectedly high, which may be rationalized by the use of the MBM method before desolution of the analyte and matrix, although it is not obvious why this should be the case. An additional weak signal $[M + 16]^+$ was observed when either method was employed. Pure peptide **1** was examined using MALDI and ESI (MS and MS/MS). The results indicated that peptide **1** is oxidized at Met^{35} (Supplementary Information). The analyses reveal that this modification may not have been caused during sample preparation but was already present in the purchased sample.

Overall, the MS/MS study (Supplementary Information) of the peptides in Mix. 1 gave equivalent mass spectra for peptides **2** and **3** by both methods. Fragmentation of **3** was of somewhat poorer quality in the solvent-free approach. Peptide **4** in Mix. 1 was readily observed using the solvent-free method (Figure 2, Inset 4B) and gave interpretable MS/MS fragment ions, but was not detected using the solvent-based method (Figure 2, Inset 4A). Pure **4** could be analyzed by either method using MS/MS, but the results were not notable, as might be expected for such a large molecule. Interestingly, only N-terminal fragment ions, b_7 and b_{23}, were observed, which precludes C-terminal information being obtained.

The response factors for model mixtures of peptides **1**:**2**:**3**:**4** (Table 1) in molar ratios 3:3:3:1 (Mix. 2) and

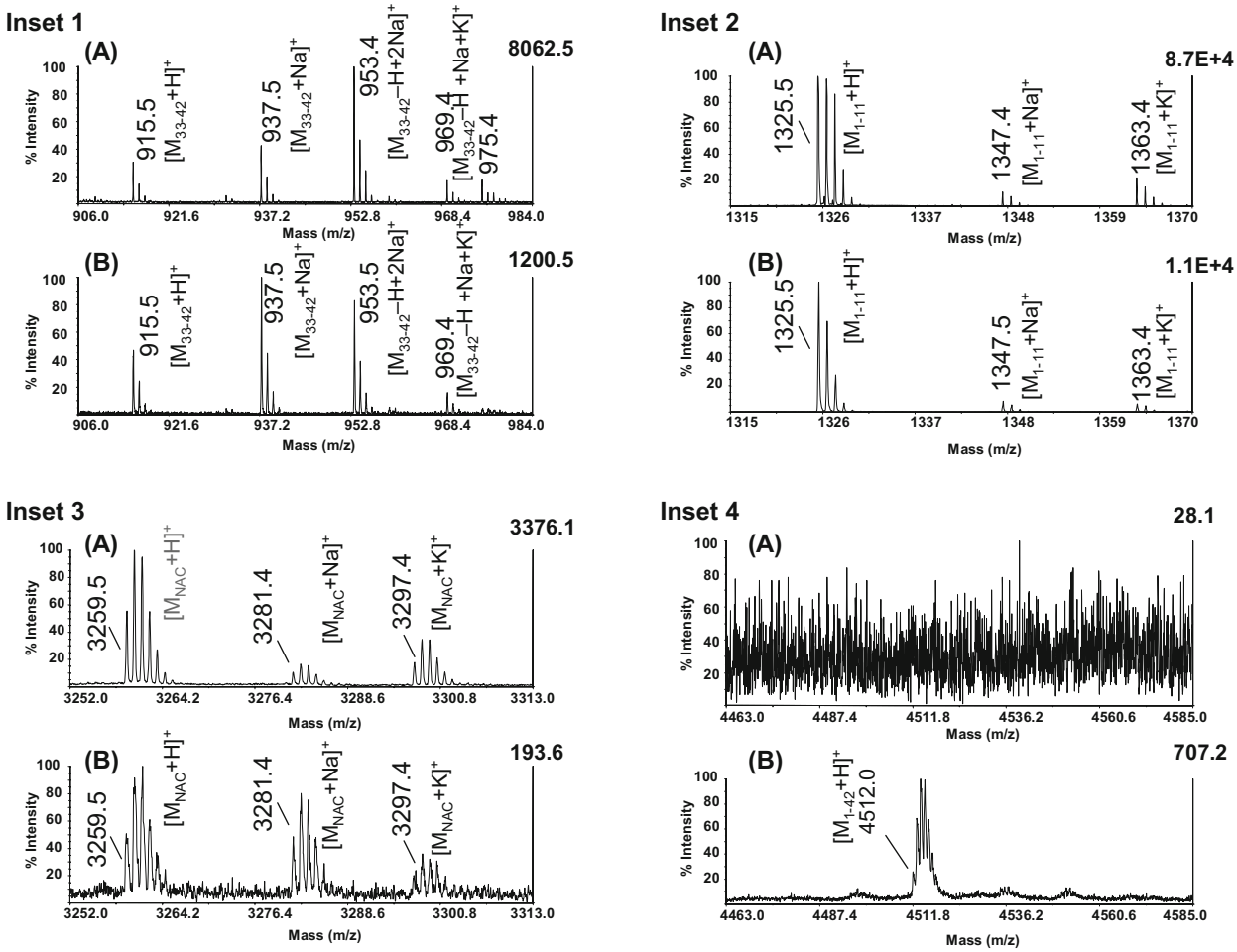

Figure 2. Inset mass spectra of a molar 1:1:1:1 model peptide mixture with inherently different solubilities of the components: Soluble (in aqueous solution), hydrophilic β-amyloid (**1-11**; MW_{theor} 1325.3 Da) and non-β-amyloid component of Alzheimer's disease (NAC; MW_{theor} 3260.6 Da) and insoluble (in aqueous solution), hydrophobic peptides β-amyloid (**33-42**; MW_{theor} 915.2 Da) and β-amyloid (**1-42**; MW_{theor} 4515.1 Da); Inset spectra 1, peptide **1**; Inset spectra 2, peptide **2**; Inset spectra 3, peptide **3**; Inset spectra 4, peptide **4**; by (**A**) solvent-based and (**B**) solvent-free MALDI-MS (full scale mass spectra in the Supplementary Information section).

1:1:1:3 (Mix. 3) show that peptide **2** is again over-represented in both methods. The mass resolution was good and both protonation and metal adduct formation were evident. Peptide **4** showed no interpretable signals when the solvent-based preparation method was used for either of these model mixtures (Table 1). In addition, peptide **1**, where it represented 16.7% or 30% in Mix. 2 and 3, respectively, was not appreciably detected in the solvent-based MALDI approach (Table 1), which is in contrast to Mix. 1 where peptide **1** is present in 25% and readily observed. Significantly higher detection of **1**, along with the significant increase of the total ion current in one case (Supplementary Information) and the absence of notable signal and lower total ion current in others, seem to support a hot-spot phenomenon that arises in the solvent-based MALDI method. Solvent-free MALDI-MS and MS/MS analyses, on the other hand, characterized low molecular weight peptide **1** and high molecular weight peptide **4** in all the mixtures (Table 1). The cumulative results from this study indicate that the dry (solvent-free) MALDI approach is generally more reliable provided sufficient material is available.

Solvent-free MALDI requires more analyte than the solvent-based method. For this reason it was important to evaluate the sensitivity of the MBM MALDI method (Exp. 3). This was followed through by determining the amount of β-amyloid (**1-11**), the most soluble of the peptides studied, required to obtain an MS/MS spectrum with good MASCOT scores. By using a dilution method that continuously halved the peptide concentration, it was determined that solvent-free MBM method yielded readily interpretable MS/MS spectra with 750 fmol but not 375 fmol of peptide (**1-11**) **2** (Supplementary Information). It was therefore concluded that the MBM method was sufficiently sensitive for many problems relating to the analysis of peptides and proteins.

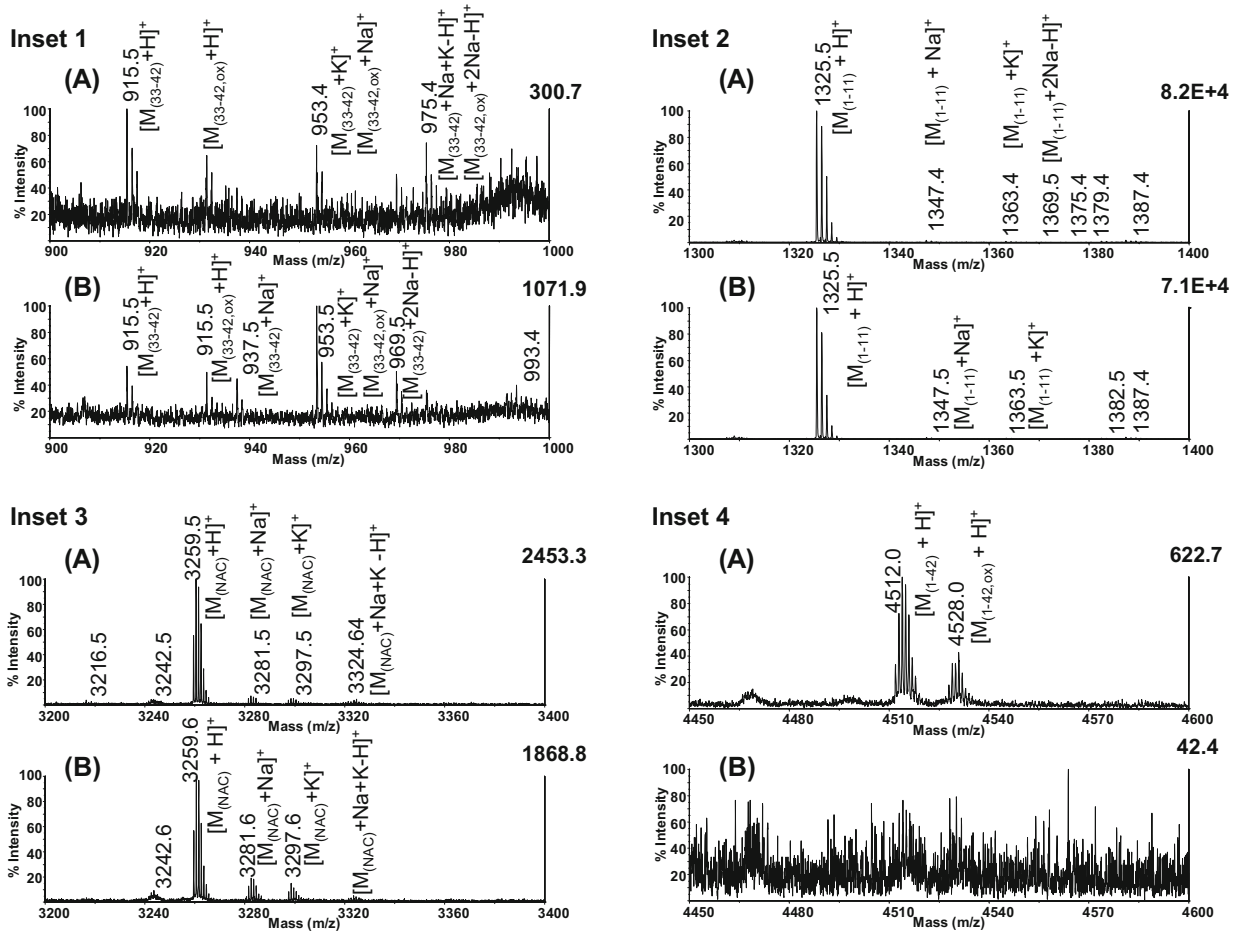

Figure 3. Mass spectra (full scale mass spectra in the Supplementary Information section) of peptide mixtures with molar ratios for peptides **1:2:3:4** of (**A**) 1:1:1:3, and (**B**) 1:3:3:3 using solvent-based MALDI-MS and ACN 0.1% TFA. Inset spectra 1, 2, 3, and 4 correspond to peptides **1, 2, 3,** and **4,** respectively. Contrary to predictions, only **4** was detected in mixture 1:1:1:3, where **4** equals 50% of the composition, and then only with low signal intensity. A signal at M + 16 Da is also observed indicating that **4** underwent oxidation during solvent-based MALDI-MS. Even though **4** is present at 50% and **1** at 16.7%, these peptides were barely detected. When analyzing mixture 1:3:3:3, which equals 10% **1** and 30% **3**, peptide **4** was not detected even when applying very high laser power.

Optimization Trials for the Solvent-Based MALDI Analysis of Defined Peptide Mixtures

In principle, the analytical performance of the solvent-based MALDI approach should improve by using a solvent that dissolves the solubility-limited peptides **1** and **4** along with **2** and **3**, however, finding such a universal solvent is problematic, especially for β-amyloid peptides. For example, β-amyloid peptide (**1-42**) has very low solubility and, thus, is only soluble in solvents with low MS compatibility such as 1,1,1,3,3,3-hexafluoro-2-propanal (HFIP) and sonication (10 min) to a concentration of 1 mg mL^{-1} [15]. A very recent paper [16] evaluated the solubility degree of (1-42) to be 95% in HFIP and 83% in dimethyl sulfoxide compared with MS compatible solvents with 6% in water (pH 7.4), 0% in ACN, 29% in 50% ACN:water, and 20% TFA. The solubility degree was determined by dissolving 10 mg of purified **4** in 1 mL of the respective solvent followed by centrifugation, supernatant removal, and repetition till constant weight was achieved.

Dissolving Mix. 2 (10% of **4**) and Mix. 3 (50% of **4**), in 99.9% ACN 0.1% TFA makes peptide **4** modestly accessible only in Mix. 3 (Figure 3, Inset 4A). Although according to study [16], **4** is not soluble in ACN; this solvent nevertheless improved the detection. However, before dissolving **4**, it was homogenized with matrix to give a representative MBM sample as is evident by the successful solvent-free MALDI analysis. Possibly because of the prior homogenization, the HCCA matrix, which is readily soluble in ACN, aided the desolution of the β-amyloid peptide. These factors may account for the successful detection of **4**. In Mix. 3, the overall signal intensity was not as abundant as it was for any of the mixtures using solvent-free MALDI. Mix. 2, however, gave no appreciable signal for peptide **4** (Figure 3, Inset 4B). A mass spectrum was not obtained when the molar

amount of **4** was 10%, even when applying high laser power. Peptide **4** is possibly lost through adhesion to the wall of the tube, however, insufficient homogeneity (segregation) might be a cause, too.

In addition to the molecular ion signal for peptide **4**, a signal was also observed for $M_{(1-42)}$ + 16 Da in the ACN-dissolved sample (Figure 3, Inset 4A) suggesting that **4** underwent oxidation during solvent-based sample preparation or analysis. The most likely site of oxidation is at Met^{35} located in the hydrophobic membrane-containing C-terminal region of the protein. MS/MS fragment ions from peptide **4** do not include amino acid position Met^{35} so that it was not possible to confirm oxidation at this residue. Further studies, but on pure peptide **1**, revealed that Met oxidation increases in either pure water or pure ACN (Supplementary Information), however the effect was greater in ACN.

Detection of **1** in the 3:3:3:1 mixture corresponding to 30% **1** by solvent-based MALDI was not significantly better than in the 1:1:1:3 mixture corresponding to 17% **1** (Figure 3, Inset 1). The signal intensity increased, but predominantly for the $[M + K]^+$ ion and not the protonated ion. Overall, the small solubility-limited peptide **1** was still barely detected in either of the two mixtures. In addition, the mass spectra of **1** dissolved in water at 37 °C for 24 h was also of poorer quality, based on a significantly reduced signal intensity of the non-oxidized **1**, in comparison with the mass spectra obtained right after dissolving the sample. This shows that pure sample **1** is sensitive to solvent in the vial and not only to sample conditions used in solvent-based MALDI analysis. These results on pure **1** assist in explaining the solvent-based results of peptide **1** within Mix. 1 to 3, suggesting that, in addition to the hotspots on the MALDI plate, sample loss and undesired reaction in the vial also occur. These results indicate that optimization trials in solvent-based MALDI are also limited for low molecular weight peptides.

For both **1** and **4** there is evidence for sample loss and side-reactions occurring upon use of solvent. Adsorptive sample losses of extracts can also be a problem with even soluble analytes [17, 18]. Problems such as insufficient homogeneity and sample loss are not encountered when the dry MBM MALDI method is used because the dry analyte is effectively homogenized with the matrix in the vial to give a more representative sample on the MALDI plate (Table 1). Both the higher signal intensity and the absence of side-products formed during MALDI sample preparation of **1** and **4** show clear advantages of the solvent-free approach relative to a solvent-based method. Similar results were previously observed [3].

Automated MALDI-MS and MS/MS Analysis of Defined Peptide Mixtures

Automated MALDI-MS and MS/MS studies of the four peptides, Mix. 2 and 3, were used to compare the solvent-free MBM and solvent-based (50:50 ACN:water 0.1% TFA) methods. The data were interpreted using the MASCOT program (Table 2). All four peptides scored high based on solvent-free MBM MALDI data from both mixtures, but only the soluble peptides **2** and **3** scored high enough to identify them using data from the solvent-based experiment. The observed ion scores for the soluble peptides **2** and **3** were similar for both MALDI methods. In the solvent-based MALDI method, the most hydrophilic low molecular weight peptide **2** (grand average of hydropathicity (GRAVY) score −1.782, Table 2) is easily detected and, in fact, greatly over-represented in comparison with all the other more hydrophobic peptides. The detection of the β-amyloid peptides **2** and **3** coupled with the failure of detecting **1** and **4** using the traditional solvent-based MALDI correlates well with the solubility of each peptide but not with the GRAVY score. The results from the solvent-free MALDI analysis followed the same trend but differences in signal intensities were not as pronounced, indicating that solubility and hydrophobicity are less significant in non traditional MALDI. These results are in accord with studies of bacteriorhodopsin [19], in which the two most hydrophilic peptides (GRAVY scores −0.265 and −0.038) are generally the base signal in any of the mass spectra; the observed over-representation is less significant in solvent-free results.

Response Assessment for MALDI Analysis of Defined Peptide Mixtures

The solvent-free MBM method not only detected intractable analyte **4** but also showed an increase in the signal intensity with increasing molar ratio of this peptide in the mixtures (Supplementary Information). The ion signal cluster areas of the individual peptides inclusive of H^+, Na^+, K^+ ions were summed and their contributions determined relative to the four peptides in each Mix. 1 to 3, and reported as response factors (Table 1). The response factors, which are the individual peak areas divided by the sum of peak areas of all of the peptides in the mixture, represent departures from the expected response. For example, a response factor of 1 would be the expected response; response factors >1 are considered over-representation and <1, under-representation in the analysis. In the case of Mix. 2 (Table 1), the molar ratio corresponds to 10% of **4** and 30% each of the other three peptides. The ratio 0.1:9.19:0.7:0 from solvent-based MALDI data corresponds to response factors of 0.03:3.07:0.23:n.d. By solvent-free MALDI, a ratio of 2.11:6.75:0.79:0.35 was observed that corresponds to response factors of 0.73:2.25:0.26:0.25. Hence, the small insoluble peptide **1** is under-represented by ~97% in the solvent-based approach and 27% in the dry MALDI experiment. The small soluble peptide **2** is greatly over-represented by ~207% (solvent-based) and 125% (solvent-free). The soluble high-molecular weight peptide **3** is under-represented by ~77%

(solvent-based) and 74% (solvent-free), and the insoluble high molecular weight peptide **4** is underrepresented by ~75% (solvent-free) but not observed in solvent-based. If detected, both soluble and insoluble high molecular weight peptides are under-represented between 77% and 74%, indicating that both methods underlie similar desorption/ionization processes if proper sample preparation had been employed, which is not the case for the combination insoluble **4** and solvent-based MALDI.

Comparing the results of solvent-based and solvent-free MALDI analytical approaches, it becomes clear that the same trends are observed, however, the results from the solvent-based approach are more extreme, e.g., not all peptides are detected and the response factors for detected peptides range between ~9% and 207%. In contrast, the values obtained for the solvent-free method are within the range ~75% to 125% and all peptides were detected. Similar trends are observed for Mix. 1 and 3 (Table 1).

Response Assessment for MALDI Analysis of Insoluble Peptides

Clearly, the use of solvent-free MALDI analysis for the small hydrophobic peptide **1** is more reliably represented than when the solvent-based procedure is used, which is also evident by the lower variability in total ion current. The different behavior in the analysis of **4** exemplifies the problem of quantitation by MALDI-MS (Table 1). An increase in the individual peak areas of **4** are observed correctly (0.35, 0.39, and 0.48) in that they follow the weight percentage trend of **4** (10%, 24.9%, 49.3%), but the response factors (0.35, 0.39, and 0.16) are influenced by the response factors of *all* the peptides present in a mixture. The inaccuracy of response factors of peptide **4** is probably related to a combination of the total ion current (Table 1, Figure in Supplementary Information) and the sample preparation strategy of assuring identical comparison between both sample preparation methods, which is achieved at the expense of small systematic errors comparing results between Mix. 1 to 3 within each method. Despite variations in the response factors, the signal increase with increasing amounts of **4** in the solvent-free spectra (Table 1) is advantageous since solvent-based MALDI did not allow the detection of **4** at all. The variable response factors demonstrate that quantitation in MALDI experiments are complicated as noted previously, e.g., [20], although relative responses (Table 1) of peptides in the solvent-free method deviate less, which is a positive attribute for this approach.

Conclusions

Metal cation adduction was shown to be advantageous as it provides reliable detection of the more hydrophobic peptides (**33-42**) **1** (model peptide mixtures) and T_{17-42} (tryptic digest of peptide **4**); shorter, very insoluble amyloid peptides encompassing aa residues 17 to 42 have been observed in amyloid deposits related to AD in addition to β-amyloid peptides (**1-42**) and (**1-40**) [21]. The solvent-free approach increases metal adduction, which can also be a disadvantage because it tends to complicate the interpretation of the mass spectra as well as distributing the ion signal over several mass-to-charge values. The MBM method holds promise for analyzing insoluble peptides such as those obtained from membrane proteins and digests in which digestion is limited or incomplete. In this study, it is demonstrated that both hydrophobic and hydrophilic peptides over a wide mass range can be analyzed by the solvent-free MALDI-MS approach, thus expanding the repertoire of the MALDI method to protein/peptide analyses for which ESI-MS has traditionally been better suited [22–24]. It is shown that the ability to detect the peptides used in this study is more closely related to solubility than to the GRAVY index. This is particularly the case for the amphiphilic peptide **4**, which had the second highest hydrophilic GRAVY score of the four peptides used in this study, but very low solubility and high aggregation propensity [5–7, 13, 15, 16, 25].

The lower degree of discrimination between hydrophilic and hydrophobic peptides by the MBM MALDI method is an obvious advantage in obtaining quantitatively more representative spectra of all peptides present in the sample. In contrast to the solvent-based method, peptides from trypsin autoproteolysis essentially were not observed with the solvent-free MBM method, which correlates with the fact that this method gives more reliable quantitative results since the protease is only employed in trace amounts compared to the peptide **4**. These studies show that the MBM MALDI approach can produce more representative information about peptides present in a sample regardless of whether they are hydrophobic, hydrophilic, soluble, or insoluble, and present in large or small relative amounts. The result, as shown in this study, can be improved sequence coverage. In addition, without the use of solvents, chemical modifications promoted in situ, such as oxidation, are less likely. For example, the reaction with APP at Met^{706}, corresponding to Met^{35} of Aβ (**1-42**), plays a critical role in induced oxidative stress and neurotoxicity related to Alzheimer's disease [25] and is therefore an analytical challenge as it can occur in vivo and during sample preparation for MS analysis. Clearly, when analyzing peptide samples, oxidized methionine(s) should not arise from the sample preparation procedure, and as observed here, the dry MALDI approach circumvents this problem. This characteristic in particular may be important for studying the progression of AD.

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References

1. Przybilla, L. M.; Brand, J. D.; Yoshimura, K.; Räder, H. J.; Müllen, K. Characterization of Large Synthetic Polycyclic Aromatic Hydrocarbons by MALDI- and LD-TOF Mass Spectrometry. *Anal. Chem.* **2000,** *72,* 4591–4597.
2. Trimpin, S.; Grimsdale, A. C.; Räder, H. J.; Müllen, K. Characterization of an Insoluble Poly(9,9-Diphenyl-2,7-Fluorene) by Solvent-Free Sample Preparation for MALDI-TOF Mass Spectrometry. *Anal. Chem.* **2002,** *74,* 3777–3782.
3. Trimpin, S.; Keune, S.; Räder, H. J.; Müllen, K. Solvent-free MALDI-MS: Developmental Improvements in the Reliability and the Potential of MALDI Analysis of Synthetic Polymers and Giant Organic Molecules. *J. Am. Mass Spectrom.* **2006,** *17,* 661–671.
4. Trimpin, S.; Deinzer, M. L. Solvent-free MALDI-MS for the Analysis of Biological Samples Via a Mini-Ball Mill Approach. *J. Am. Mass Spectrom.* **2005,** *16,* 542–547.
5. Turner, P. R.; O'Connor, K.; Tate, W. P.; Abraham, W. C. Roles of Amyloid Precursor Protein and Its Fragments in Regulating Neural Activity, Plasticity, and Memory. *Prog. Neurobiol.* **2003,** *70,* 1–32.
6. Soderberg, L.; Dahlqvist, C.; Kakuyama, H.; Thyberg, J.; Ito, A.; Winblad, B.; Naslund, J.; Tjernberg, L. O. Collagenous Alzheimer Amyloid Plaque Component Assembles Amyloid Fibrils Into Protease Resistant Aggregates. *FEBS J,* **2005,** *272*(9), 2231–2236.
7. Tsubuki, S.; Takaki, Y.; Saido, T. C. Dutch, Flemish, Italian, and Arctic Mutations of APP and Resistance of Abeta to Physiologically Relevant Proteolytic Degradation. *Lancet* **2003**, *361*(9373), 1957–1958.
8. Perkins, D. N.; Pappin, D. J.; Creasy, D. M.; Cottrell, J. S. Probability-Based Protein Identification by Searching Sequence Databases Using Mass Spectrometry Data. *Electrophoresis* **1999,** *18,* 3551–3567.
9. Redeby, T.; Emmer, A. Membrane Protein and Peptide Sample Handling for MS Analysis Using a Structured MALDI Target. *Anal. Bioanal. Chem.* **2005,** *381*(1), 225–232.
10. Tummala, R.; Green-Church, K. B.; Limbach, P. A. Interactions Between Sodium Dodecyl Sulfate Micelles and Peptides During Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS) of Proteolytic Digests *J. Am. Soc. Mass Spectrom.* **2005,** *16,* 1438–1446.
11. Zischka, H.; Gloeckner, C. J.; Klein, C.; Willmann, S.; Swiatek-de Lange, M.; Ueffing, M. Improved Mass Spectrometric Identification of Gel-Separated Hydrophobic Membrane Proteins After Sodium Dodecyl Sulfate Removal by Ion-Pair Extraction. *Proteomics* **2004,** *4*(12), 3776–3782.
12. Zhang, N.; Li, L. Liquid Chromatography MALDI MS/MS for Membrane Proteome Analysis. *Rapid Commun. Mass Spectrom.* **2004,** *18*(8), 889–896.
13. Kheterpal, I.; Williams, A.; Murphy, C.; Bledsoe, B.; Wetzel, R. Structural Features of the Abeta Amyloid Fibril Elucidated by Limited Proteolysis. *Biochemistry* **2001,** *40*(39), 11757–11767.
14. Wang, M. Z.; Fitzgerald, M. C. A Solid Sample Preparation Method That Reduces Signal Suppression Effects in the MALDI Analysis of Peptides. *Anal. Chem.* **2001,** *73,* 625–631.
15. Liu, R.; Barkhordarian, H.; Emadi, S.; Park, C. B.; Sierks, M. R. Trehalose Differentially Inhibits Aggregation and Neurotoxicity of β-Amyloid 40 and 42. *Neurobiol. Dis.* **2005,** *20*(1), 74–81.
16. Malavolta, L.; Pinto, M. R.; Cuvero, J. H.; Nakaie, C. R. Interpretation of the Dissolution of Insoluble Peptide Sequences Based on the Acid-Base Properties of the Solvent. *Protein Sci.* **2006,** *15*(6), 1476–1488.
17. Speicher, K. D.; Kolbas, O.; Harper, S.; Speicher, D. W. Systematic Analysis of Peptide Recoveries from In-Gel Digestions for Protein Identifications in Proteome Studies. *J. Biomol. Technol.* **2000,** *11,* 74–86.
18. Stewart, I. I.; Thomson, T.; Figeys, D. 18O Labeling: A Tool for Proteomics. *Rapid Commun. Mass Spectrom.* **2001,** *15,* 2456–2465.
19. Trimpin, S.; Deinzer, M. L. Anal. Chem. **2007,** *1, 79(1),* 71-78.
20. Wallace, W. E. Recent Advances in Quantitative Synthetic-Polymer Mass Spectrometry at NIST. *Proceedings of the 52nd ASMS Conference on Mass Spectrometry and Allied Topics*; Nashville, TN, May 2004.
21. Lalowski, M.; Golabek, A.; Lemere, C. A.; Selkoe, D. J.; Wisniewski, H. M.; Beavis, R. C.; Frangione, B.; Wisniewski, T. The "Nonamyloidogenic" p3 Fragment (Amyloid β 17–42) is a major constituent of Down's Syndrome Cerebellar Pre-Amyloid. *J. Biol. Chem.* **1996,** *271,* 33623–33631.
22. Stapels, M. D.; Barofsky, D. F. Complementary Use of MALDI and ESI for the HPLC-MS/MS Analysis of DNA-Binding Proteins. *Anal. Chem.* **2004,** *76,* 5423–5430.
23. Bigwarfe, P. M., Jr.; Wood, T. D. Effect of Ionization Mode in the Analysis of Proteolytic Protein Digests. *Int. J. Mass Spectrom.* **2004,** *234,* 185–202.
24. Tummala, R.; Limbach, P. A. Serum Protein Profiling Using Surfactant-Aided Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry. *Anal. Chim. Acta* **2005,** *551,* 137–141.
25. Butterfield, D. A.; Boyd-Kimball, D. The Critical Role of Methionine 35 in Alzheimer Amyloid β-Peptide (1–42)-Induced Oxidative Stress and Neurotoxicity. *Biochim. Biophys. Acta* **2005,** *1703,* 149–156.