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Laser-Induced In-Source Decay Applied to the Determination of Amyloid-Beta in Alzheimer's Brains

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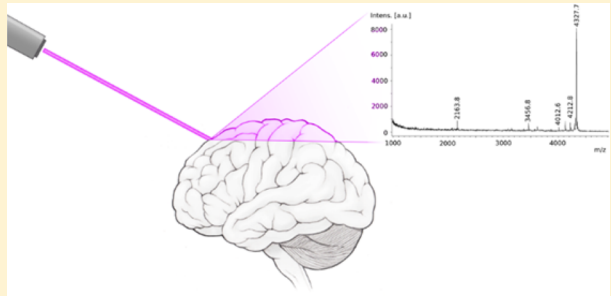
Supporting Information

ABSTRACT: A method for the analysis of amyloid-beta peptides in isolated plaques and intact tissue sections affected by Alzheimer's disease (AD) is presented. This method employs matrix-assisted laser desorption/ionization (MALDI) time-of-flight mass spectrometry and the inherent laser-induced in-source decay (ISD) that occurs coupled with imaging mass spectrometry (IMS) to investigate the composition of these samples eliminating the need for other confirmational MS/MS techniques. These results demonstrate this technique's usefulness for the identification of amyloid-beta peptides in tissue and isolated senile plaques from AD patients using the reproducible fragmentation pattern demonstrated via the laser-induced ISD of synthetic amyloid-beta peptide clips (1−40, 1−42). Clear differences between the hippocampal AD tissue and the control hippocampal tissue regarding the presence of amyloid-beta have been identified. These are based on laser-induced ISD of standard amyloid-beta clips as controls as well as the analysis of isolated senile plaques as a confirmation before tissue analysis. Using the resulting observed peptide clip masses from the control data, we present mass spectrometry based identification of the amyloid-beta peptides in both isolated plaques and hippocampal regions of those patients diagnosed with AD.



KEYWORDS: *MALDI, mass spectrometry, imaging, amyloid-beta, Alzheimer's disease*

Alzheimer's disease (AD) is the number one cause of dementia[1] with roughly 40 million people with AD worldwide.[1] Hippocampal formations from patients with AD show neuritic plaques and neurofibrillary degeneration.[2] Although the amyloid cascade hypothesis is broadly accepted as a relevant pathogenic mechanism in AD, the failure of therapeutic targeting of amyloid-beta to affect clinical outcome indicates a level of complexity that is as yet unresolved. The concept of low-*n*-mers of amyloid-beta (i.e., oligomers) has been raised as an ad hoc explanation for an increasingly variable relationship between pathology and clinical disease in AD, as has synaptic pathology; such concepts, however, are in their infancy. The relationship between amyloid-beta and phospho-tau in neurofibrillary degeneration is likely also incompletely resolved, given the differences in brain regions and topographical progression over time between the two proteins. A better understanding of the molecular composition of diseased tissue compared to control, specifically in human brain tissue in vivo, is therefore needed.

Amyloid-beta may be the number one studied species in regards to AD. Because of its ease of isolation as well as the readily available synthetic peptide clips, amyloid-beta is the first

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choice as many begin research in the field of AD. Amyloid-beta is widely known to make up the majority of the senile plaque buildups in the brains of individuals with AD and while many studies have been completed revolving around this peptide stemming from the amyloid precursor proteins (APP), much is still unresolved involving the localization of the peptide clips in the brain and their role in the onset and progression of the disease. In 2009, Spoto et al. reported the potential photofragmentation of amyloid-beta peptides when analyzed via mass spectrometry (MS).[3] Matrix-assisted laser desorption/ionization (MALDI), atmospheric pressure (AP) MALDI, and electrospray ionization (ESI) sources were used for the analysis of synthetic amyloid-beta peptides. The group was able to identify minimal fragments that they accounted for due to photoinduced dissociation from the laser within the MALDI and AP MALDI. Hence, they concluded that, for the analysis of monomeric amyloid-beta in solution, ESI was the best method for analysis to avoid this fragmentation.[3] However, here we propose that a more complete understanding of the laser-induced fragmentation of both Aβ1−40 and Aβ1−42, two of the most prominent amyloid-beta peptide clips in AD pathology, will aid in the analysis of isolated senile plaques and in-tact formalin-fixed paraffin-embedded (FFPE) tissue sections from AD patients.

Prior studies with a focus on mass spectrometry have been used to investigate the molecular composition of tissue from the human AD brain, most of which involve tissue homogenates.[4] The seminal work relating to Alzheimer's has been done with liquid chromatography coupled to mass spectrometry (LCMS).[4] While this is a useful technique which can be used to qualitatively, and even relatively quantitatively, determine the chemical makeup of the tissue, using tissue homogenates for analysis has drawbacks. The primary shortfall arises from the fact that homogenizing the tissue sample eliminates any information to be gained from the cellular topology and morphology of the tissue. While an overview of the chemical makeup of the tissue is important, recent developments in mass spectrometry have made it possible to investigate in-tact tissue, therefore retaining both the spatial and the relative molecular information.

Numerous investigations into the roles of the amyloid-beta peptides in AD using mass spectrometry have been reported. In 2004, Wu et al.[5] reported specific proteins that took part in the aggregation of amyloid-beta in the brain. It was determined that a particular 16 kDa protein band corresponding to α- and β-hemoglobin chains played a role in the aggregation. The hemoglobin was found to be at the highest levels in the hippocampus of AD patients. An LCQ ion trap MS coupled with high performance liquid chromatography (HPLC) was used to identify this protein band from the PAGE gel separation.[5] Rufenacht et al., in 2004, developed a method to collect single plaques from brain tissue for analysis through laser-dissection microscopy followed by analysis using MALDI MS. Both mouse and human plaques were analyzed (in homogenate form). Through this method, it was discovered that the amounts of amyloid in plaques isolated from transgenic mice and from humans were similar.[6] In 2005, Portelius et al.[7] analyzed human cerebrospinal fluid (CSF) for C-terminally truncated amyloid-beta peptides. Eighteen different peptides were identified via mass spectrometry which could be used as a diagnostic test for AD in the future.[7] In 2006, Soederburg et al. were able to analyze intact plaque cores from human AD brain samples by LCMS. Amyloid-beta was found to be the only peptides in the plaques. This indicates the possibility that no other proteins copolymerize with amyloid-beta in plaque cores.[8]

In 2002, Stoeckli et al. published the first of several papers on the distribution of amyloid-beta in mouse brain tissue by MALDI imaging mass spectrometry (IMS). Large mass differences were observed between theoretical and experimental data which were the result of uneven tissue surfaces and possible charging effects of the tissue.[9] In 2006, Stoeckli et al. then obtained images of mouse brain with a relatively high contrast of colocalized amyloid-beta peptides (particularly Aβ1−40 and Aβ1−42).[10] Only a year later in 2007, Stoeckli et al. imaged whole-body sections of mice which were dosed with α- and β-peptides. While the α-peptide degraded over time, there was retention of the β-peptide.[11] In 2008, Seeley et al. obtained images from MALDI IMS for amyloid-beta data and then subsequently stained the tissue in order to see the plaques on tissue.[12] In 2005, Rohner et al.[13] imaged mouse brain tissue as a model of AD. Recently, tissue preparation protocols have significantly improved making it possible to map human tissue the same way. In 2015, Kim et al. published an investigation into the makeup of human hippocampal tissue from control and AD brains and found several upregulated and downregulated genes in AD brains compared to the control in both FFPE and fresh frozen tissue.[14] These works, as well as the work presented here, may shine further light into mapping amyloid-beta in intact tissue sections from human patients.

Several prior studies have investigated the MALDI IMS of FFPE tissue.[15] All recognize the difficulties and discuss potential strategies to make the analysis and protein/peptide identification easier and more reliable. Cross-linking of proteins by formalin-fixation causes modifications and adducts including methylol adducts, Schiff bases, and methylene bonds.[15] Many researchers recommend an antigen retrieval step in which the tissue is heated in a buffer in order to facilitate delinking of proteins before an enzymatic digest is done.[15,16] While this seems to address the problem in the sense that you do indeed see more fragments after digest, the buffer identity, time of experiment, and temperature all widely vary depending on tissue type and age. According to Gorzolka and Walch in their review article on the MALDI imaging of FFPE tissue, even upon antigen retrieval, which accounts for the methylene bonds produced by fixation, you still must account for possible mass shifts due to Schiff bases and methylol adducts. These two groups may be enough to disallow for optimal enzymatic digest by blocking protein cleavage sites. Accounting for this reduces the accuracy from the database searches due to missed cleavages, Gorzolka and Walch insist.[15]

We report on developing a method for identifying amyloid-beta peptide clips in isolated senile plaques and human hippocampal tissue samples by laser-induced ISD fragmentation patterns. This technique allows for the identification of analytes in human diseased and control samples by MALDI fragmentation of their synthetic counterparts. We extend this method to map laser-induced amyloid-beta peptide clips localized in various regions of human AD and control hippocampal tissue. This technique reduces the need for confirmational MS/MS experiments requiring database searches, thus allowing for identification via a reproducible source of fragmentation via laser-induced in-source decay.

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RESULTS AND DISCUSSION

We began with the analysis of the synthetic amyloid-beta peptides as controls. N-terminal and C-terminal residue losses from the amyloid-beta synthetic peptides were observed due to laser-induced ISD. Figures 1 and 2 show representative mass spectra from the synthetic Aβ1−40 and Aβ1−42. We were not only able to observe the intact mass of the peptide, but we were also able to observe the +2 charge state as well as many of the N- and C-terminal residue losses. It is important to note that all masses reported are for the monoisotopic mass of each peptide clip (Tables 1 and 2). The ability to observe these fragments in the synthetic Aβ1−40 and Aβ1−42 by laser-induced ISD presented an opportunity for the correlation between these observed amyloid-beta fragments and the identification of amyloid-beta, and its fragments, in isolated senile plaque samples and FFPE tissue sections. This would eliminate the need for confirmational MS/MS experiments using LIFT to generate the peptide fragments.

The fragmentation from the laser-induced ISD observed in the synthetic samples was also observed in the isolated senile plaques from post-mortem AD patients. It was used to confirm the presence of amyloid-beta in the sample (Figure 3 and Table 3). While the samples were cell-sorted, the plaque samples may not have been completely pure and thus were not expected to contain only amyloid-beta. Therefore, having a MS/MS like technique to confirm the location of amyloid-beta in these samples is critical. Mass spectrometric analysis of several different senile plaque samples spotted onto ITO-conductive glass slides showed some of the same fragments that were observed in the synthetic peptides. Figure 3 shows the mass spectrum obtained from one such sample. The isolated senile plaque sample spotted, Figure 3, consisted of roughly 300 senile plaque cores in 5 μL of sample. The plaque cores isolated were amyloid-specific and therefore the majority of the cores are made up of the amyloid-beta peptides. Therefore, we can say that the sample was saturated with amyloid-beta peptide. The +1 charge state and the +2 charge state of Aβ1−40 can be seen along with several fragments due to residue losses from the ISD process. Not all fragments observed in the synthetic samples are seen in the senile plaque sample, but sufficient fragments were observed to confirm the assignment of Aβ 1−40.

Previously, tissue homogenate work, described in the intro, has been done primarily on the amyloid-beta peptides which, in AD cases, is the major component of senile plaques in AD and aging. Peptide fragments, Aβ1−40 and Aβ1−42, showed exaggerated amounts of amyloid-beta in the brains of those diagnosed with AD. The procedure that we have developed for the preparation of the tissue through modifications of previously published methods[10−12,18] such as matrix identity, sublimation parameters, omission of an antigen retrieval step, and so on, demonstrates the ability of IMS to differentiate between AD and control tissue giving both molecular and spatial information. Because of the availability, the tissue type, and the stability, we investigated the feasibility of using FFPE brain tissue samples. Formalin-fixation works to cross-link the proteins within the tissue sample. Because of this, upon deparaffination and rehydration, enzymatic digest yields peptide clips that are not typical of the in-tact proteins. This makes it difficult to determine protein identification by standard MS/MS database searches. Aβ1−40 and Aβ1−42 are known to be the most common and abundant amyloid-beta peptides in the brains of AD patients. Since similar fragments were observed in the human isolated senile plaques as in the synthetic samples, this identification procedure for amyloid-beta was applied to in-tact FFPE tissue samples from the human hippocampal region of the brain.

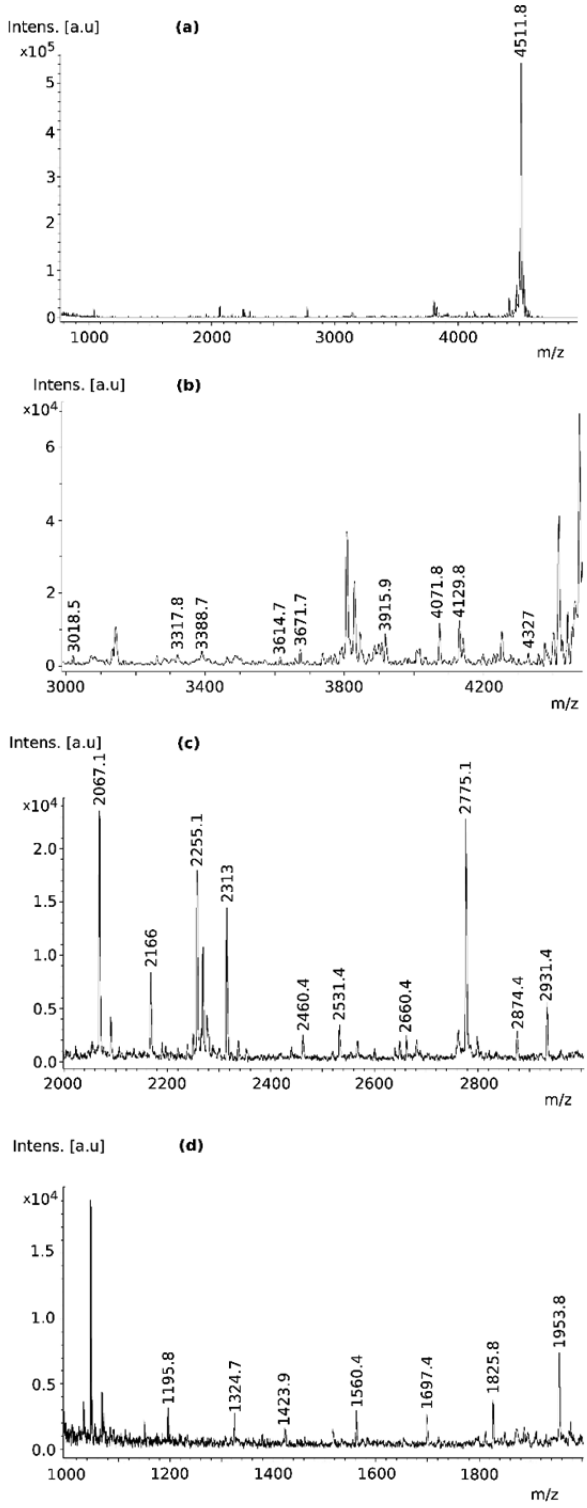

Figure 1. (a) Reflectron positive MALDI mass spectra of synthetic Aβ1−42. (b) Expanded region of (a) in the mass range of m/z 3000−4400; (c) expanded region of (a) in the mass range of m/z 2000−3000; and (d) expanded region of (a) in the mass range of m/z 1000−2000.

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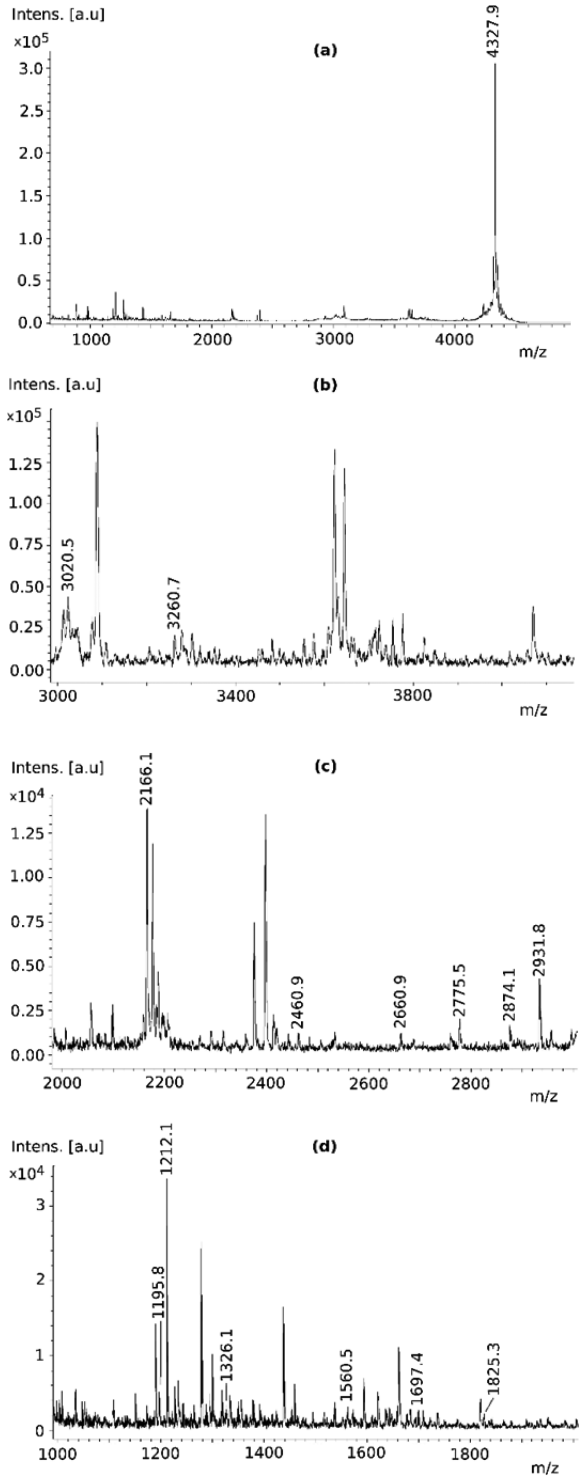

Figure 2. (a) Reflectron positive MALDI mass spectra of synthetic Aβ1−40. (b) Expanded region of (a) in the mass range of m/z 3000−4200; (c) expanded region of (a) in the mass range of m/z 2000−3000; and (d) expanded region of (a) in the mass range of m/z 1000−2000.

These peptide fragments are small enough that they may not have been cross-linked in the FFPE process. For each peptide fragment observed from the synthetic Aβ1−40 and Aβ1−42 peptides, a mass filter was added to both the AD and the control tissue in order to determine the localization of these peptide fragments in the tissue (Figures 4 and S2). A scale, seen to the left of the scanned image, shows the intensity of several of these peptide fragments from Aβ1−40 and Aβ1−42, shown by different colors (black and dark blue corresponding to the lowest intensity and pink and white corresponding to the highest intensity). Sample spectra from high intensity regions of each of the five masses can be found in the Supporting Information (Figure S3). Ideally, if all fragments belong to the amyloid-beta peptides, all mass filters should show maps of the fragments in the same region of the tissue. Some fragments were more pronounced than others, but for the most part, all of the fragments were localized in the same region, strengthening the argument that the fragments come from amyloid-beta peptides Aβ1−40 and Aβ1−42. Furthermore, mass maps of

Table 1. Peptide Clip Identification of m/z Values Observed from Synthetic Aβ1−42 (Figure 1)

Aβ 1−42 residue number	calcd mass (m/z)	obsd mass (m/z)
1−42	4511.3	4511.8
1−40	4327.2	4327.0
1−38	4129.0	4129.8
1−37	4071.9	4071.8
1−35	3915.9	3915.9
1−33	3671.7	3671.7
1−32	3614.7	3614.7
1−30	3388.6	3388.7
1−29	3317.5	3317.8
1−26	3018.4	3018.5
1−25	2931.4	2931.4
1−24	2874.3	2874.4
1−23	2775.3	2775.1
1−22	2660.2	2660.4
1−19	2313.1	2313.0
Aβ^{2+}	2255.6	2255.1
1−18	2166.0	2166.0
1−17	2067.0	2067.1
1−16	1953.9	1953.8
1−15	1825.8	1825.8
1−14	1697.7	1697.4
1−13	1560.7	1560.4
1−12	1423.6	1423.9
1−11	1324.5	1324.7
1−10	1195.5	1195.8

Table 2. Peptide Clip Identification of m/z Values Observed from Synthetic Aβ1−40 (Figure 2)

Aβ 1−40 residue number	calcd mass (m/z)	obsd mass (m/z)
1−40	4327.2	4327.9
1−28	3260.5	3260.7
12−40	3020.6	3020.5
1−25	2931.4	2931.8
1−24	2874.3	2874.1
1−23	2775.3	2775.5
1−22	2660.2	2660.9
1−20	2460.2	2460.9
1−18	2166.0	2166.1
1−15	1825.8	1825.3
1−14	1697.7	1697.4
1−13	1560.7	1560.5
27−40	1326.8	1326.1
28−40	1212.7	1212.1
1−10	1195.5	1195.8

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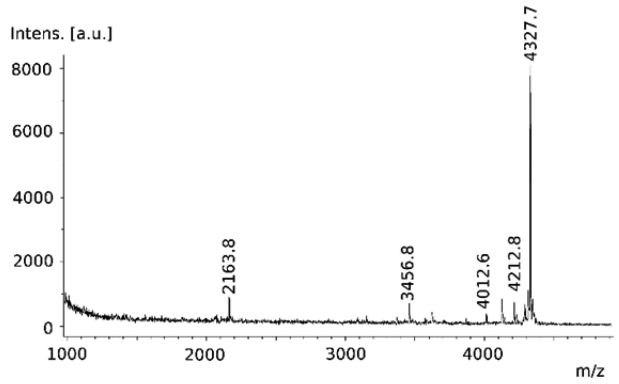

Figure 3. Reflectron positive MALDI mass spectra of isolated senile plaque.

Table 3. Peptide Clip Identification of *m/z* Values Observed from Isolated Senile Plaque (Figure 3 and Figure S1)

Aβ residue number	calcd mass (*m/z*)	obsd mass (*m/z*)
1−42	4511.3	4511.3
1−40	4327.2	4327.7
2−40	4212.1	4212.8
4−40	4012.0	4012.6
8−40	3456.8	3456.8
Aβ1−40^{2+}	2163.6	2163.8

these fragments on control tissue showed no intensity corresponding to these fragments. This demonstrates that IMS coupled with laser-induced ISD can be used to differentiate between AD tissue and non-AD brain tissue. Our modified imaging method paired with the in-source dissociations of known compounds has been shown to be an accurate and meaningful way in which to analyze human brain tissue without altering the morphology of the tissue through homogenization and to obtain a full mass map of the tissue in question.

In order to be able to compare relative intensities of two different tissues run at different times, it is imperative that certain experimental controls be maintained. All tissue sections were prepared under the same conditions for analysis. In addition, before each run, the instrument was calibrated with the peptide standard discussed in the experimental section. The relative intensities for these calibrations were comparable before each tissue sample was run. This makes it possible to relate the intensities of the mass spectra from the different imaging runs. Side-by-side comparison of undigested tissue with the tissue digested with trypsin showed none of the peaks in the undigested tissue. In fact, the spectra collected from the undigested tissue showed no distinguishable peaks. This is most likely due to the heavy cross-linking from the formalin-fixation. The peaks assigned to the amyloid-beta and its fragments were not the only masses that were observed. There are obvious differences in the mass spectra of the control tissue when compared to the diseased tissue. However, these amyloid-beta related fragments were good as a means of comparison and demonstrated the usefulness of using laser-induced fragmentation for identification purposes in tissue samples.

The overall distribution of the amyloid-beta peptide fragments shown in the images in Figure 4 can be related to AD pathology. Amyloid-beta is most commonly found within the gray matter of the brain and is not found in the white matter of the brain. The parts of the tissue where the amyloid-beta peptide fragments are localized in the mass spectrometry based images are gray matter-rich portions of the section. In addition, the tissue sections are from the middle gray matter layers of the brain in which one would most likely find amyloid-beta build-up in AD patient brains. These are very anterior cuts, as stated before, and the AD tissue involves slightly more tissue than just the hippocampus. The hippocampus is located toward the bottom of the tissue slice. It is known that the hippocampus is less amyloid-beta rich than the adjacent cortex of the brains of AD patients.[19] That section of the tissue has very limited amounts of the matched compounds which would be expected in the hippocampus. By comparing known anatomical progression of the disease in in-tact tissue, we were able to confirm our method more confidently than by isolated protein alone.

CONCLUSIONS

Laser-induced in-source decay and imaging mass spectrometry, coupled with the analysis of synthetic peptides and isolated senile plaques, has allowed for the direct observation of multiple peptide fragments of amyloid-beta in isolated senile

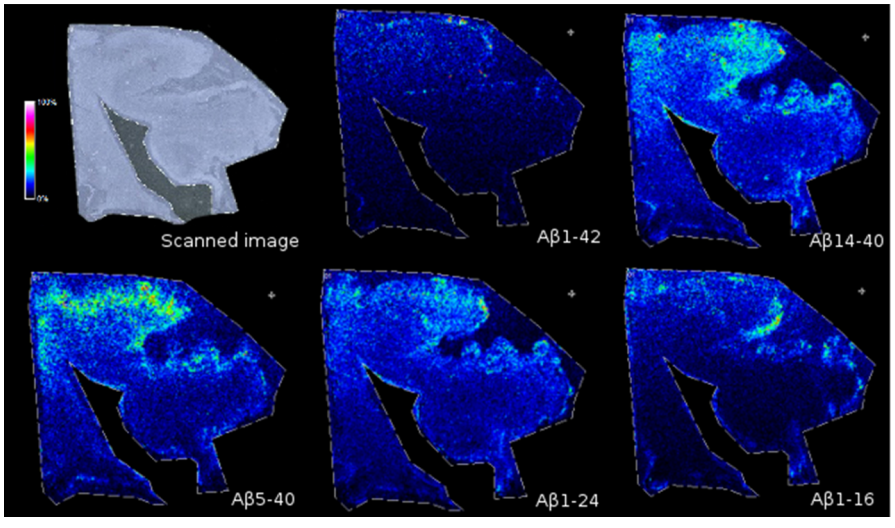

Figure 4. Mass-based images generated by MALDI IMS of AD human hippocampal tissue for five amyloid-beta fragments.

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plaques and FFPE tissue. There are other molecules observed in both the isolated plaques and the tissue. The identification of these peaks will be the focus of future work. This is the first step in coupling molecular information and the anatomical aspects of this devastating disease. We have clearly demonstrated the utility of our laser based ISD amyloid identification method. This approach has then been successfully applied to senile plaques and the imaging of FFPE tissue for the analysis of human brain tissue. This type of topological information lays the groundwork for a better understanding of AD and how AD brains differ from normal brains on a molecular level when amyloid-beta is considered. We were able to observe peptide fragments resulting from nearly every N- and C- terminal residue loss from Aβ1−40 and Aβ1−42 at significant intensities in the synthetic standards, plaques, and tissue without the need for a MS/MS step.

That the localization of the observed laser-induce amyloid-beta peptide fragments are within senile plaques and the regions of the FFPE tissue that relate directly to AD pathology and not found in any abundance in other regions of the tissue alone demonstrates that this technique is applicable for the eventual molecular mapping of brain tissue with anatomical relevance. We have clearly demonstrated this method to be an accurate and meaningful way in which to analyze human brain samples while maintaining the morphology of the tissue and to obtain an eventual full mass map of the tissue. There are several distinct advantages to using this technique instead of, or in conjunction with, immunohistological tagging. The methods developed in this manuscript may be applied to other potential biomarkers for different diseases, which may or may not have specific antibodies. This technique also allows for undeniable identification of the analyte in question, whereas some antibodies may cause false positives. In addition, further development of this technique will allow for the observation and identification of post-translation modifications, potential amyloid-beta binding to metals (as well as other analytes) and structural differences (i.e., fibrillar vs oligomeric amyloid-beta) which may not be readily seen by immunohistological analysis. For verification purposes, however, since this technique does not destroy samples, immunohistological analysis may be done after IMS on the same tissue section. This work opens the door to more projects involving AD and human tissue in general in order to relate anatomical and molecular information for a better understanding of disease processes.

METHODS

Synthetic Peptide Preparation and Analysis. *Reagents.* Aβ1−40 and Aβ1−42 were purchased from Anaspec, Fremont, CA.

Peptide Preparation. Methods were the same for Aβ1−42 and Aβ1−40 peptides. The amount of 1 mg of peptide was reconstituted with 80 μL of 1.0% NH_4OH. The reconstituted peptide was diluted with 1 mL of 1× PBS. A solution of PBS buffer was prepared by combining 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPH_4, and 0.24 g of KH_2PO_4 in 1 L of water at a pH of 7.4. The Aβ stock solution was separated into 10 μL aliquots which were then diluted with another 1 mL of 1× PBS. Samples were kept at a temperature of −20 °C. Different peptide concentrations versus spotting techniques were investigated, and the method that yielded the best resolved mass spectra with the highest peak intensity is as follows: 3 μL of synthetic peptide was spotted on an ITO-coated conductive glass slide. The slide was washed in nanopure H_2O and then 100% EtOH for 2 min each, and the slide was allowed to dry under vacuum. A supersaturated solution of HCCA matrix was prepared and spotted directly on top of the peptide and the slide was once again allowed to dry under vacuum. Before analysis, a standard peptide mixture obtained from Bruker (part #206195) was also spotted onto the slide with HCCA matrix as a calibration standard. It contained angiotensin II ($M + H^+ = 1047$), angiotensin I ($M + H^+ = 1297$), substance P ($M + H^+ = 1348$), bombesin ($M + H^+ = 1620$), ACTH clip 1−17 ($M + H^+ = 2094$), ACTH clip 18−39 ($M + H^+ = 2466$), and somatostatin 28 ($M + H^+ = 3149$).

MALDI Analysis. Mass spectrometric analysis was done in reflectron, positive ion mode on a time-of-flight mass spectrometer (Ultraflextreme; Bruker Daltonics, Bremen, Germany). Various parameters were optimized to maximize signal intensity and mass resolution. These parameters include the pulsed ion extraction and the laser intensity, as well as the number of shots, frequency and the smartbeam parameter set. The pulsed ion extraction was optimized and set at 190 ns with a laser power at ∼30% with a global attenuator offset of 65%. The number of shots was set to 300 shots at 1 kHz. The smartbeam parameter was set to large, ion source 1 at 25 kV, ion source 2 at 23.7 kV and the lens at 6.5 kV. There is a 100 nmol detection limit for the synthetic peptide using this technique. Roughly 1000 μmols of sample were spotted for the acquisition of each spectra (Figures 1 and 2).

Senile Plaque Preparation and Analysis. *Amyloid Plaque Isolation.* Isolation was completed at Case Western Reserve University.[17]

Reagents. Isolated senile plaques were provided by Case Western Reserve University. α-Cyano-4-hydroxycinnamic acid (HCCA) was purchased from Bruker.

Cleaning and Washing Procedure. Because we were interested in investigating the utility of using laser-induced ISD in human tissue samples and coupling this with a mass map with a well resolved spatial resolution of the isolated senile plaques, we optimized a method of cleaning and washing the senile plaque sample directly on an ITO-coated glass microscope slide for MALDI TOF analysis. Senile plaques were isolated in 0.1% SDS, 50 mM Tris buffer with 150 mM NaCl and 0.02% NaN_3. In order to run these samples on the MALDI TOF mass spectrometer, detergents and salts must be removed from the sample beforehand so as to minimize ion suppression and adducts. An amount of 5 μL of senile plaque was spotted onto the slide and allowed to dry under vacuum. A series of two Petri dishes were set up and filled with nanopure H_2O and 100% ethanol, respectively. The slide with the senile plaque was placed into each dish and allowed to sit for a previously optimized amount of time in order to remove the detergents and salts (2 min in the H_2O and 2 min in the 100% ethanol). The slide was then allowed to dry again under vacuum. A matrix solution of HCCA was prepared at a concentration of 10 mg/mL in TA solvent (0.1% trifluoroacetic acid in 50% acetonitrile/water). An amount of 2 μL of the matrix solution was pipetted onto the dried senile plaque on the slide and was once again allowed to dry under vacuum. Before analysis, a standard peptide mixture obtained from Bruker (#206195) was also spotted onto the slide with HCCA for calibration.

MALDI Analysis. Mass spectrometric analysis was done in reflectron, positive ion mode on a time-of-flight mass spectrometer (Ultraflextreme; Bruker Daltonics, Bremen, Germany). The various parameters were held the same as mentioned above.

Tissue Preparation and Analysis. *Brain Tissue.* Human brain tissue was obtained post-mortem from the National Institutes of Health Neurobiobank at the University of Maryland. All tissues received from the Neurobiobank are approved for use by the local Institutional Review board. The Alzheimer disease tissue was obtained from an 80 year old man with a long history of dementia, and advanced Alzheimer's disease pathology according to National Institute on Aging-Alzheimer Association 2012 guidelines. No comorbid processes such as Lewy body dementia, vascular dementia, or frontotemporal lobar degeneration were present. Control tissue was obtained from a 48 year old man who died suddenly of a non-neurologic process. Brain tissue was analyzed for apolipoprotein E genotype using standard methods (PCR and restriction enzyme analysis) and was determined to be APOE 2/3, a genotype considered "protective" against the development of AD. Tissue selected for examination was hippocampal formation at the level of the lateral

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geniculate nucleus, which also included subiculum and inferior temporal neocortex, although the control sample is slightly more anterior in the coronal plane, which accounts for the differing architecture in the dark field microscopic image (Figure 4). Immunostains for amyloid-beta (4G8) and phospho-tau (AT8) of formalin-fixed, paraffin embedded tissue show no amyloid-beta plaques and no significant neurofibrillary degeneration (data not shown).

Reagents. Ethanol, chloroform, acetic acid, xylenes, ammonium bicarbonate, and acetonitrile were purchased from Fisher Scientific. 2,5-Dihydroxybenzoic acid (DHB) was purchased from Sigma-Aldrich. MS grade trypsin was purchased from Pierce.

Trypsin Preparation for Digest. An amount of 100 μg of trypsin powder was reconstituted by addition to 200 μL of 50 mM glacial acetic acid which produced a stock solution of 0.5 μg/μL. The solution for digest was prepared by the addition of 100 μL of the stock solution to 500 μL of 100 mM ammonium bicarbonate and 60 μL of acetonitrile in water.[16]

Tissue Preparation for MALDI Analysis. Tissue sections were cut and mounted on indium tinoxide (ITO) conductive glass slides (Delta Technologies part no. CG-81IN-S115) at The University of Maryland School of Medicine at a thickness of 12 μM. All slides were stored at room temperature until ready to be analyzed. The depariffination procedure was completed by washing the slide in xylene followed by an additional fresh xylene wash for 3 min each. The slide was then washed in 100% ethanol twice for 1 min each, 95% ethanol for 1 min, 70% ethanol for 1 min, and water twice for 3 min each. Next, the slides were washed with a series of solvents in order to remove the salts and lipids from the tissue facilitating protein/peptide analysis via MALDI. Sections were washed systematically in plastic Petri dishes with 70% and 100% ethanol for 30 s each, Carnoy's fluid for 2 min, and 100% ethanol, water, and 100% ethanol for 30 s each.[18] Upon completion of the washing step, a trypsin (MS-grade) digest was performed on the intact tissue by hand pipetting the trypsin solution onto the tissue and allowing it to dry twice.

Matrix Deposition. Prior to application of matrix, an image of the slide was taken with an Epson Perfection V37 flatbed scanner to be used in the imaging process. Matrix deposition was carried out by sublimation. Oil was heated to 110−120 °C before sublimation was carried out.[18] The slide was taped to the apparatus with conductive copper tape. The amount of 0.300 g of DHB was deposited into the apparatus and the sublimation was carried out under vacuum for 14−16 min in order to achieve a matrix coating of optimal thickness and homogeneity.

Rehydration. Immediately before MALDI IMS analysis, the sublimated slides were subject to a rehydration step in order to draw the analytes into the matrix layer. Sublimation, a dry deposition technique, requires rehydration for optimal analysis. The slide was taped to the top portion of a Petri dish with conductive copper tape and a piece of paper towel was put in the bottom portion with 1 mL of water and 50 μL of acetic acid.[18] The Petri dish was taped closed and put in an oven at 85 °C for 3.5 min. The time seemed to be a very important consideration in this step. Too long in the oven and the slide would become far too wet, resulting in the delocalization of the analyte, and too little time in the oven would not allow for enough of the analyte to be drawn out.

MALDI Imaging. Mass spectrometric analysis was done in linear, positive ion mode on a time-of-flight spectrometer (Ultraflextreme; Bruker Daltonics, Bremen, Germany). Various parameters were optimized to maximize signal intensity and mass resolution before any imaging experiments were conducted. Some of these parameters include the pulsed ion extraction and the laser intensity, as well as the number of shots at what frequency and the smartbeam parameter set. The pulsed ion extraction setting was optimized and set at a constant 300 ns with a fairly constant laser setting at roughly 24% with a global attenuator offset of 70%. The number of shots was set to 500 shots at 1 kHz. The smartbeam parameter was set to large, ion source 1 was held at 25 kV and ion source 2 was kept at 23.7 kV with the lens at 6.5 kV. A peptide mixture obtained from Bruker was used as a calibration standard which contained the same as before. Mass spectral data was acquired over the entire human hippocampus sections using FlexImaging software (Bruker Daltonics, Bremen, Germany) in the mass range between m/z 1000 and 18 000 Da with a raster step size of 100 μm and 500 laser shots per spectrum.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschemneuro.5b00295.

Mass spectra of isolated senile plaque highlighting Aβ1−40 and Aβ1−42, mass-based images generated by MALDI IMS of control human hippocampal tissue, MALDI IMS generated mass spectra representative of each AD mass image (PDF)

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Author Contributions

A.R.K. was responsible for carrying out all experiments, compiling data, and assembling the manuscript. G.P. was responsible for supervision and guidance regarding the direction of the project, information regarding AD, and editing of/additions to the manuscript. R.J.C. provided the tissue samples, information regarding the pathology of AD, and editing of/additions to the manuscript. S.B.H.B. provided supervision and guidance as well as final editing and additions to the manuscript.

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Notes

The authors declare no competing financial interest.

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