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Review

Analysis of post-translational modifications in Alzheimer's disease by mass spectrometry☆

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ABSTRACT

The roles of post-translational modifications (PTMs) in the onset and progression of disease have been extensively studied for decades. More specifically, various PTMs have been the focus of research in Alzheimer's disease (AD). The two most discussed hallmarks of the disease, senile plaques and tau tangles, are the result of PTMs of the amyloidβ protein precursor (AβPP) and the microtubule stabilizing protein: tau. While these modifications have been characterized indirectly by biochemical-based methods, the primary shortcoming to this research can be linked to a lack of a thorough molecular-based means of qualitative and quantitative analysis of many of these modifications within transgenic animal, and human samples. In this review, we discuss the important proteins and their associated PTMs linked to AD and the ways in which mass spectrometry has and will be utilized to analyze them. We also comment on novel ways in which molecular-based mass spectrometry methods should be employed going forward to resolve the interconnections of the PTMs involvement in various stages of AD pathology (preclinical, mild cognitive impairment, advanced-stage AD).

1. Introduction

Post-translational modifications (PTMs) are chemical modifications to proteins occurring at any point in the protein "life-cycle" and account for the vast complexity of the human proteome [1]. Modifications can occur anywhere on the amino acid backbone of a protein or on the N- or C-terminus [2]. There are many PTMs [3] that can occur individually, or in conjunction with each other including, but not limited to, regulated protein degradation, and the addition of functional groups to protein subunits [2]. Phosphorylation, acetylation, glycosylation, and ubiquitination comprise some of the most commonly observed PTMs and all play an integral role in pathogenesis [4]. PTMs are often related to aging and dementia and can be either reversible or irreversible [5]. While PTMs are essential to biological homeostasis, some PTMs contribute to disease progression as well [1,6–8]. This is particularly true for neurodegenerative disorders [9]. Due to this, it is imperative to develop methods for PTM analysis as it pertains to potential disease interventions and treatments.

PTMs characterize some of the most discussed players (amyloid-beta, α-synuclein, tau, etc.) in neurodegenerative disorders, such as Alzheimer's disease (AD) and Parkinson's disease (PD) [1,7,8]. Because of the ways in which PTMs can so drastically alter the functions of proteins, they are strictly controlled biologically. Any disruption in their regulation can result in abnormal pathology. Some of the most common PTMs in neurodegenerative diseases include phosphorylation [10–12], acetylation [13–16], glycosylation [17,18], ubiquitination [19–22], and PTMs associated with oxidative stress [23–27]. This review will focus on the PTMs associated with important proteins that have been linked to AD pathology. By no means is this an exhaustive list of all PTMs connected to the disease state. There are many thorough reviews [7,8,18,28–31] referenced herein that outline the known involvement of PTMs in AD, however, very few modification-centric studies have utilized mass spectrometry as a primary analytical tool. Here, we highlight the limited mass spectrometry-based modification work completed by ourselves and a few other groups that have taken advantage of the technique to elucidate structural and quantitative information as it relates to AD pathology.

Protein modifications have classically been investigated in a variety of biochemical/immunological approaches (ELISA, western blots, immunohistochemistry, protein assays, HPLC, mass spectrometry etc.) [2,32–34]. In recent years, mass spectrometry has played a greater role at the forefront of PTM analysis due to its sensitivity, specificity and

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robust nature [2,33,34]. Probably more significant, is the unbiased analysis mass spectrometry can bring to a field highly dependent on specific probes for identification. While there are still some difficulties associated with PTM analysis by mass spectrometry [35], which will be discussed herein, great strides have been made in the ability to qualitatively, and even quantitatively, analyze PTMs within various sample mediums (blood, CSF, brain homogenates, etc.). It may seem odd then that there are so few reports describing this technique as it pertains to AD. We will first give a general overview of the considerations associated with using mass spectrometry to characterize protein modifications and how many of the most prominent modifications, affecting proteins in AD, can be identified. Furthermore, we will examine the individual proteins, discuss briefly their documented roles in the disease state, and introduce how mass spectrometry has been, or can be, utilized, often in conjunction with other techniques, to develop a clearer molecular-based picture of PTM involvement in disease onset and progression.

2. Mass spectrometry of PTMs

According to Parker et al., there are four main aspects to utilizing mass spectrometry for PTM analysis that must always be considered: [35]

1. The mass shift (in the mass spectra) due to the modification
2. The abundance of the modified peptide within the sample (whether pure or complex)
3. The stability of the modified peptide both in solution and during MS analysis
   (proton affinity/ionization efficiency)
4. The effect of the modification on the peptides ionization efficiency

These four characteristics will be discussed throughout this section as they pertain to the proteins and modifications. While it is relatively easy to detect and identify a protein via tandem mass spectrometry (MS/MS) by the observation of a few fragments corresponding to highly abundant peptides, the identification of a PTM requires the detection of the modified sequence itself, along with fragments of the peptide also containing the modification [35] if structural information is to be elucidated. This is highly dependent on the specific peptide and ionization efficiency/proton affinity of the peptide which can be contingent on the modification [35]. Hence, protein purification has become an integral step in the determination of PTMs by mass spectrometry [33–35]. Often times however, particularly with exploratory work or with work focused on the characterization of unknown PTMs, protein purification and/or enrichment can be improbable. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is a common means of separating components of a sample prior to proteomic analysis, and is most useful for those components, or peptides, in relatively high abundance. It is not uncommon for a single SDS-PAGE band to contain multiple species. Therefore, other separation and purification techniques should be considered when analyzing PTMs such as liquid-chromatography coupled electrospray ionization mass spectrometry (LC-ESI MS).

While mass spectrometry is now routinely utilized to investigate PTMs, it is more often times used for the development of antibodies and antibody-enrichment strategies for the observation of PTMs with low site occupancy [36]. This is particularly important when the sample medium becomes complex, such as with tissue homogenates, blood, or cerebrospinal fluid (CSF). These enrichment strategies work well when it comes to modification specific analysis (for instance if all phosphorylation sites are of interest etc.). However, other approaches must be utilized when the target analyte is a specific protein or all potential modifications, even those of low occupancy, are to be analyzed.

Database searches are routinely utilized to identify proteins by their peptide fingerprint after MS/MS analysis. Enzymatically digested peptides and their fragments are analyzed for their amino acid sequence and the probability of protein identification is given at a user specified error [35]. Additionally, the probability of modifications can be specified within the search criteria to retrieve identifications containing the expected modification [35]. Typically, however, the modifications must be amino acid-specific and singular. Multiple modification sites on a single peptide, or a non-specific modification can be difficult to identify by database searches due to their reliance on probability and theoretical enzymatic peptides of the protein hits [35]. A work-around for this, is the manual analysis of MS/MS spectra with special attention to the peptide fragment mass shifts commonly reported for the modification. The intact parent mass (m/z) of the peptide will be shifted by a specific number of Da (as will all modification-containing fragments). One must therefore assume, that the modification containing peptide is stable enough to withstand the conditions of MS/MS analysis [35]. This becomes tricky when modifications such as glycosylation, which will be discussed in more detail later, are of interest. Table 1 lists some relevant PTMs and the corresponding average mass shifts commonly observed in the mass spectra.

Table 1
Relevant PTMs and corresponding observable mass shifts.

Post-translational modification	Observed mass shift - average (Da)
Acetylation	42.04
Formylation	28.01
Glycation	162.14 (as an example of one potential glycation modification)
Hydroxylation	16.00
Methylation	14.03
Phosphorylation	79.98
Sulfation	80.06

While antibody and western blot analysis are of great importance in PTM identification, quantitative information and localization are not generated by these means. Additionally, antibodies can be non-specific, expensive, and irrelevant if complex samples are being analyzed for non-specific PTMs. In the next sections, we will discuss the ways in which researchers have had success in characterizing PTMs by mass spectrometry, and how these methods would be beneficial to the better understanding of PTM roles in AD pathogenesis.

3. Amyloidβ protein precursor (AβPP)

The AβPP is an integral type 1 membrane protein, and while its exact function is debated, in the healthy state, it has roles in synapse formation and neuronal plasticity [37]. It is because of this that mutations of normal AβPP cleavage have been linked to AD. AβPP is cleaved in three different ways, by α-, β-, and/or γ-secretase. Intracellular cleavage occurs through α-secretase. AβPP is cleaved at the -carboxyl end of the protein near the cellular membrane by the α-secretase enzyme that results in the creation of a larger intracellular protein which is thought to be neuroprotective. AβPP is also cut by β-secretase and γ-secretase in the absence of α-cleavage to yield the small 40–42 amino acid sized Aβ peptide which accumulates into aggregates and plaques [37–39]. This section will focus on the modifications linked to AβPP and amyloid-beta peptides and the PTMs associated with them will be discussed in more detail in the following section.

Many AD studies have focused on the suspected neurotoxic amyloid-beta [40–42] species. It is therefore important to first better understand the mechanisms of AβPP modification. If variations surrounding AβPP processing are clarified, it is possible that preventative therapies could be developed. Several PTMs have been observed to occur during the translation of AβPP [1,5,7,40]. Some of these modifications contribute to normal AβPP pathology. However, a change in the homeostasis of the modifications can have either negative or positive effects that may then contribute to, or theoretically protect from, developing AD [40].

AβPP has two possible N-glycosylation sites [40]. It has been documented that an imbalance in the proper glycosylation form of the protein can inhibit the secretion of the neuroprotective form of the protein and facilitate the build-up of the cellular protein [40]. On the other hand, if an additional terminal sialic acid is added to the oligosaccharide chain of the protein, it has been reported that the secretion of the soluble, neuroprotective protein is increased [40].

Glycosylation is arguably one of the most difficult PTMs to analyze via mass spectrometry. Unlike with acetylation or phosphorylation, where the addition of a standard group can be accounted for due to expected mass shifts, glycosylation occurs through the addition of varying isobaric glycans [35]. Because the glycans are so large (typically several thousand mass units), often times, peptide masses are shifted by such a large number, that sequencing is no longer viable. Therefore, it is often required to first remove the glycans and subsequently digest the protein for analysis [35].

The documented methods of proteomic analysis of glycoproteins is highly dependent on the type of glycosylation present. For instance, N-glycoproteins are typically enriched through hydrazide chemistry [41]. This enrichment has been followed by digestion, derivatization and mass spectrometric analysis to shed light on N-glycoprotein characterization and quantification in human saliva [42]. These methods have been shown to elicit insight into the glycosylation sites on the protein, however, because of the isobaric nature of the glycans, elucidation of structure yields further difficulties. Because AβPP has been documented to undergo N-glycosylation, methods such as these may begin to, not only confirm or deny the glycosylation sites proposed in earlier works [40], but quantify the glycan occupancy for the individual sites and relate that to disease pathology. A secondary approach to glycan capture, comes with the application of lectin nanoprobes [43]. This technique has been coupled with LC-MS/MS analysis to qualitatively and quantitatively characterization glycan modifications in complex human samples [44] which would be of benefit to the analysis of samples such as isolated NFTs and senile plaques.

4. Amyloid-beta

As previously mentioned, amyloid-beta peptides ranging from 40 to 42 amino acids in length are thought to be the primary component of senile plaque aggregates in the brains of those with AD [7,45]. These extracellular protein aggregates are insoluble and although they have been thoroughly characterized by immunohistochemistry and fluorescent microscopy [7,46], there is still much left unknown about their mechanisms of aggregation and even their composition.

Ubiquitin, an 8 kDa protein that is expressed in most eukaryotic cells [28], has previously been detected within neurofibrillary tangles (NFTs) and senile plaques in AD brains [29]. Ubiquitination induces protein degradation, disrupts protein-protein interactions, and modifies the localization of proteins [47,48]. It occurs through the covalent attachment of one or more ubiquitin molecules to a target protein [1]. This modification occurs in three steps. First, a high-energy thiol ester intermediate is formed by an activated C-terminal glycine of a ubiquitin molecule. Next, the activated ubiquitin is transferred to a member of the ubiquitin-protein ligase family which then promotes the transfer of the ubiquitin molecule to a lysine residue of the target protein [1]. Defects in this pathway have been documented to be associated with the etiology of various neurological disorders [49]. Because the pathway is important in the removal of misfolded proteins and many neurological disorders are characterized by the build-up of misfolded proteins, it is logical that disruptions in the ubiquitination pathway could decrease the ability to transport and degrade misfolded aggregates.

Because of the focus on amyloid-beta in AD research, PTM analysis by mass spectrometry as it pertains to AD has been accomplished and success has been found in better understanding the roles of modifications in amyloid-beta peptides. Tay et al. (2012) characterized the PTMs associated with amyloid-beta by mass spectrometry [50]. Mass spectrometry methodology called precursor ion mapping (PIM) was described as a means of modification characterization. The synthetic amyloid-beta [1–40] monomer was cross-linked and subsequently digested, yielding three transglutaminase cross-linked species [50]. The undigested protein did not yield these fragments. This technique had previously been described as a means of phosphorylation, oxidation, and glycosylation analysis [51–53]. The use of the synthetic peptide as a model compound suggests that this method would also be of benefit for the analysis of endogenous human peptides and proteins. Inoue et al. (2013) described a covalent chiral derivatized ultraperformance liquid chromatography tandem mass spectrometry (CCD-UPLC-MS/MS) method for determining amino acid racemization (AAR) and amino acid isomerization (AAI) of N-terminal amyloid-beta [1–5] in human brain. Quantification was accomplished with a stable isotope (15 N) labeled amyloid-beta [1–40] as an internal standard [54]. We have developed mass spectrometry-based methods for the analysis of amyloid-beta [55,56] and modified amyloid-beta [57]. The matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF MS) methods that we have developed have the potential to be applied to the characterization of other forms of modified amyloid-beta.

5. Tau

NFTs are insoluble aggregates within neurons, thought to be primarily made-up of misfolded tau, and comprised of paired helical filaments (PHFs) [58,59]. These aggregates are associated with neuronal dysfunction and death and are at the center of the tau hypothesis of AD pathogenesis. Normal, adult tau is a soluble, naturally unfolded, microtubule-associated protein (MAP) that has 6 common isoforms (352–441 amino acids) [39]. All isoforms of tau can appear in the hyperphosphorylated form in PHFs of AD brains [39]. While normal tau is known to stabilize microtubule assembly, hyperphosphorylated tau does the opposite, by disassembling microtubules and aiding in the proliferation of insoluble PHFs, which interfere with axonal transport [60]. The longest isoform of tau (441 amino acids) contains 85 possible phosphorylation sites. In the normal case, 30 of these sites are phosphorylated [61]. In AD pathology however, additional phosphorylation has been documented to occur at specific sites [61]. What causes increased tau phosphorylation is intensively studied but still relatively unknown. Braak and Braak discovered that, unlike senile plaque localization, NFTs followed a consistent and uniform pattern while spreading throughout the brain [62]. Because tau pathology, alone, can cause neuronal loss, it has become a focus of AD pathogenesis research [38]. The accumulation of NFTs has been shown to occur primarily in the hippocampus (an area of the brain important in memory) [62] and NFTs have been shown to occupy much of the space within neurons which might lead to their eventual death [63]. However, because tau pathology does not resolve in the proliferation of senile plaques, yet AβPP and PSEN1/PSEN2 mutations have been shown to yield both senile plaques and NFTs, tau pathology is placed downstream from amyloid-beta pathology in relation to AD disease onset and progression [38,60]. Recently, some focus has shifted to the smaller soluble tau oligomers being the actual neurotoxic species rather than the insoluble NFTs (which have been theorized to be neuroprotective), thus allowing for modified tau to gain more focus in AD research.

Protein phosphorylation is the most common PTM [1]. This type of PTM is reversible and occurs primarily at serine residues but can also occur at threonine and tyrosine residues, albeit to a much lesser extent [64]. Phosphorylation takes place by the regulated transfer of the γ-phosphate group of ATP to the hydroxyl group of a target residue [65]. This occurs in conjunction with the hydrolysis of the newly formed phosphoester bonds and thus, if there is a shift in the equilibrium of these two enzymatic activities, hyper- or hypo-phosphorylation can be observed at the phosphorylation sites, causing physiological imbalances [1,24,66]. In studies of AD pathology, phosphorylation is most often

linked to tau [1,24,66].

It is a consensus that the incredibly stable, insoluble NFTs are primarily made up of phosphorylated tau [67]. Furthermore, phosphorylated tau can undergo additional modifications (that either promote or inhibit further aggregation) that account for the complexity and uncertainty surrounding the roles of the protein in the onset and progression of AD [68,69]. Lysine residues are the primary amino acid important in tau aggregation and toxicity [70]. The ubiquitin proteasome system is thought to be the primary means of ubiquitylated tau degradation [66,71,72]. Therefore, impaired function of the ubiquitin proteasome system is thought to be a principal aspect of PHF tau build-up. Ubiquitylation sites have been observed and identified on tau isolated from AD brains and were shown to be localized to the microtuble-binding region which further suggests that a lack of adequate de-ubiquitylation, positively correlates degradation resistant tau aggregation [66,73]. A 2017 article by Thomas and Yang, outlines the ways in which LC-MS/MS can be utilized to clarify the different types of lysine residue modifications and their roles in AD neuropathology. The methodology in this paper will be discussed in more detail shortly.

In addition to phosphorylation, tau has also been shown to undergo acetylation [14,15,74]. Acetylation occurs when the acetyl-coenzyme A transfers an acetyl group to the ε-amino group of a lysine residue of a target protein [73]. This PTM is reversible and catalyzed by histone acetyl transferases (HATs) and histone de-acetylases (HDACs). Like phosphorylation, an imbalance in the two enzymes (HAT/HDAC) equilibrium can cause a disruption in homeostasis of the central nervous system [73]. Acetylated tau has been detected to exclusively occur in the brains of those with various tauoapthies [73]. Lys174, for instance, was characterized as an acetylation site during early stages of AD using ESI MS/MS of cell lysates from AD brains [14]. Both phosphorylation and acetylation can reduce tau affinity for microtubules and thus facilitates tau aggregation [14]. Additionally, it has been documented that tau acetylation can cause hyperphosphorylated tau aggregation due to lowered protein degradation [74]. Acetylation most commonly occurs on the lysine and arginine amino groups and exhibits a characteristic 42 Da mass shift [33].

While phosphorylation is one of the most common modifications linked to AD, it can be one of the most difficult to analyze by mass spectrometry. Phosphopeptides are typically present in low quantities compared to other peptides in a biological sample and are therefore particularly susceptible to ion suppression [35]. It has been reported that the more phospho-groups a peptide has, the lower the sensitivity for this PTM [75,76]. Although, this phenomenon has been challenged. In a recent work, the correlations of the extent of phosphorylation and signal intensity were investigated by MALDI and ESI. Mass spectra of three peptide standards containing zero to three phosphotyrosines were collected and analyzed and the spectra originating from ESI and MALDI each showed a decrease in signal intensity with increasing phosphorylation [35]. Due to the labile nature of phosphoryl groups, it is not uncommon for modified peptides to lose sites of phosphorylation during the ionization process. According to Parker et al., this can partially be accounted for when using MALDI as the mode of analysis. Some organic matrices (such as 2,5- dihydroxybenzoic acid – DHB) transfer less energy to the analyte and are therefore more suitable for phosphopeptide analysis [35]. Phosphorylation is typically identified by a characteristic 80 Da shift in the mass of the modified peptide although it is common practice to first attempt to enrich the sample for the phosphopeptides of interest due to their low abundance. However, there is no standard protocol for doing this, as different phosphopeptides require different steps for enrichment. A secondary technique is to compare spectra of peptides before and after being treated with phosphatase which will result in a loss of 80 Da from those signals corresponding to phosphorylated peptides [34,77].

Quantification of PTMs may be one of the most important aspects of analyzing biological samples. As previously mentioned, protein modifications are not all negative and their regulation is important in normal biological function. Multiple reaction monitoring (MRM) is the main MS-based technology utilized to simultaneously quantify multiple modified peptides in a sample [33,34,36,73]. This, and similar mass spectrometric techniques, have been previously utilized in the determination of the abundance of the polyubiquination of tau in human AD [66] and the abundance of tau and amyloid-beta in human CSF [78–81]. Thomas and Yang [73] outline a thorough means of identifying and quantifying lysine-residue modifications in tau. Their method however, does not specify an exact means of tau isolation. Although, it is strongly suggested that, at a minimum, 20 μg of purified tau is used as starting material. It is well documented that the isolation and purification of tangles from AD brains is non-trivial [82–84]. Additionally, great care should be taken during isolation procedure because solvents utilized for preparation and storage can have negative effects on the mass spectra produced [57,85,86].

Thomas et al. (2012) aimed to utilize nanoflow LC-MS/MS to characterize modifications of tau [6]. The resulting mass spectra identified a monomethylation of lysine which had never been reported. This methylation occurred across 7 amino acids, all of which were part of the microtubule-binding region and one of which was a competing site for ubiquitylation [6]. To confirm the presence of these modified sites, post-mortem AD tissue was subject to confocal fluorescence microscopy with the addition of anti-tau and anti-methyl lysine antibodies [6]. This paper is a prime example of the ways in which mass spectrometry was able to give more insight into the characterization of the PTM. The subsequent microscopy was critical to confirmation, however.

6. α-Tubulin

While most researchers agree that hyperphosphorylated tau causes a breakdown of microtubules, evidence that this reduction correlates to an increase of PHFs is lacking. This suggests that other modification mechanisms are occurring in conjunction with the hyperphosphorylation of tau that lead to PHF build-up [28]. Zhang et al. (2015) focused on the modifications associated with tubulin dimers to investigate how they affect the stability of the microtubules [28]. It was confirmed from previous studies that the abundance of α-tubulin in the brains of those with AD is significantly decreased. All modified forms of α-tubulin (polyglutamated, tyrosinated, detyrosinated etc.) were also decreased. However, the proportion of acetylated α-tubulin to other forms of the protein was increased [28]. This was determined by immunocytochemistry (using mouse antibodies for the various modifications being investigated), double-label immunofluorescence imaging (using inverted fluorescence and inverted laser-scanning confocal fluorescence microscopy), and western blotting. While the data is convincing, particularly due to the fact that it corroborates previously published results, advancements in mass spectrometry have been shown to be a robust means of characterizing and analyzing tubulin [87–89]. Additionally, it has been shown that SRM or MRM are more informative, primarily when it comes to quantification and highly complex sample analysis, than western blot analysis alone, although they may still be used in conjunction [90,91].

7. PTMs associated with oxidative stress

Oxidative stress can compromise many biological molecules through redox reactions [92,93]. Additionally, various forms of oxidative stress can trigger PTMs by oxidation of amino acid side chains [8,94]. Oxidative stress encompasses any imbalance in processes that cause the formation of harmful free radical species such as O_2^-, HO·, and H_2O_2 [1]. Although, in the normal state, the function and removal of these species is strictly regulated, in disease states such as with AD, oxidative stress can lead to the unintended modifications of proteins resulting in the misfolding of these proteins and eventual neuronal death [8,92,95,96].

The role of oxidative stress in neuronal disease and aging first

became apparent in the early 1990s. A branch of the oxidative stress theory centralizes on the aggregation of amyloid-beta through dityrosine cross-links produced by Cu-mediated redox chemistry [97]. There is significant prior work that suggests that Cu(II) binds to histidine and tyrosine amino acid residues on the amyloid-beta backbone [57,98–100]. In the presence of an oxidant (H_2O_2), it is thought that the Cu(II) mediates tyrosine cross-linking that potentially aids in the aggregation of amyloid-beta peptides and strengthens the insolubility of the structure [97], while yielding reactive oxygen species (ROS) polymerization that cause oxidative cell death [39]. However, a recent mass spectrometry-based investigation into the dityrosine cross-links produced by reactive oxidation of amyloid-beta peptides was accomplished with the omission of the transition metal [101]. Currently, there is equivocal evidence that the metal species (Cu, Zn etc.) play a significant role in the aggregation of amyloid-beta or other proteins in human brains. Furthermore, damage by free radicals has been implicated in AD pathology due to the elevated source from microglia [102,103], amyloid-beta [104,105], elevated levels of neuronal iron [106–108], and general imbalance in the homeostasis of metal ions [92]. Silva et al. published a comprehensive table of common oxidative modifications and the common mass shifts associated to them [33]. Like some of the other modifications discussed, oxidative modifications are low in abundance and unstable. Additionally, it is possible to observe oxidation of analytes caused by MS analysis [33]. A positive aspect of MS analysis over other forms analysis, comes from the ability to account for modifications in database searches and use common mass shifts for identification with nonspecific modification analysis. We have already demonstrated the usefulness of MALDI-TOF MS for the analysis of metal-bound amyloid-beta [57] and are able to use the methods developed to analyze other oxidative modifications directly from complex human samples, such as isolated senile plaques, blood and CSF.

A branch of oxidative stress modulated PTMs stems from lipid and sugar oxidation adducts. Increased lipid peroxidation has been documented to occur in AD brains and within CSF [109–113]. 4-hydroxynonenal (HNE) is one of the most abundant aldehyde by-products of lipid peroxidation [109–111]. This area of PTM research in AD is one that may benefit the most from the application of mass spectrometry. Research attempting to solve the roles of HNE, in particular, in AD pathology has been primarily accomplished through the use of antibodies and while informative, little information regarding abundance or localization is available by those means. Smith et al. (2007) published a comprehensive review on the roles of oxidation products in AD pathology, with minimal cited work being attributed to mass spectrometry analysis [8].

Due to the irreversible nature of AD, targeting risk factors or early stages of the disease is of extreme importance. While samples from preclinical (PCAD) and mildly cognitively impaired (MCI) brains are more difficult to procure, analysis of oxidative modifications in these earlier stages of the disease must be considered [114,115]. A major problem with PCAD is that the potential for future disease progression is unknown. MCI is more closely related to AD and optimal studies would consider all 3 stages simultaneously, however this is often unrealistic. PCAD and MCI brains have been analyzed by both mass spectrometric methods and immunochemical methods. A 2010 article by Aluise et al. describes oxidative stress, amyloid-beta expression and proteomic profile differentiation between inferior parietal lobule (IPL) samples from pre-clinical AD and control brains [116]. PCAD brains show high levels of normal hallmarks of AD such as senile plaques and NFTs, however no resultant symptoms such as memory problems are apparent. The group determined that there was a significant increase in expression of soluble monomeric amyloid-beta42 which is thought to be non-toxic and, in some cases, neuroprotective, in PCAD brains compared to control brains. However, there was no significant increase in insoluble oligomeric amyloid-beta42. Similarly, oxidative stress assays (protein carbonyl, 3-nitrotyrosine (3-NT) and protein-bound HNE) were utilized to conclude that there was no significant difference in oxidative stress measurements between PCAD and control brains. Additionally, differences in protein expression between PCAD and control brains were analyzed via mass spectrometry [116]. While the oxidative stress assays showed no significant differences, utilizing mass spectrometry for this portion of the study would have been beneficial. Although informative, immunochemical assays can be non-selective, expensive, and non-quantitative and thus a more complete picture of these modifications must be gathered through the mass spectrometry methods described herein. Oxidative modification studies of MCI brains are rarer and are needed to bridge the gap between PCAD and AD to better elucidate the mechanism of oxidative stress as the disease progresses.

While PCAD is not entirely representative of the disease end state, MCI more closely relates to progressed AD. Hence, a means of analyzing modifications present in MCI is imperative. Garcia-Blanco adds that even though there are some clinical biomarkers utilized to differentiate between AD, MCI, and healthy controls, biomarkers are needed to distinguish between non-AD MCI and MCI due to AD [31]. MRI and PET have had some success in determining accumulations of amyloid-beta and tau in the brain, however, these techniques are expensive and lack specificity. Therefore, biomarkers for MCI are needed to better understand the progression to AD at a molecular level. In a comprehensive review by Swomley and Butterfield [30], the importance of redox proteomics in the analysis of AD and MCI is discussed. It is noted that a primary theory in oxidative stress and AD is that the commonly seen mitochondrial leak of superoxide radicals that contribute to normal aging is exaggerated with mitochondrial damage in AD, pushing radical formation. Swomley and Butterfield developed protocols for gel-based, and non-gel based proteomic analysis of proteins with oxidative modifications from MCI and AD brains. Utilizing various mass spectrometry techniques, modifications indicative of glycation, HNE, nitration, phosphorylation, and carbonylation were observed in MCI analysis and suggested that important metabolic pathways are affected early in the onset and progression of AD [30].

8. Other PTMs in blood and CSF

As AD is a neurodegenerative disease, brain samples yield the most information regarding the mechanisms of the disease state. However, often time, brain samples are unavailable, particularly in the case of individuals with PCAD or individuals who have not begun showing symptoms. Therefore, another aspect of AD research that is currently lacking, is the development of methods for analyzing CSF, blood, and other peripheral fluids for potential biomarkers. A blood-based biomarker panel for the identification of PCAD would be invaluable and investigating varying levels of different blood proteins in different stages of AD by mass spectrometry is the first step to accomplishing this [117]. In their review article, Blanco et al. discuss the increased levels of oxidized proteins in MCI fluids (blood, plasma, CSF etc.) but comment on the low statistical significance of much of the data on the subject [30]. The authors document the various studies which have been completed with peripheral fluids from patients (PCAD, MCI, and advanced AD). Most studies have utilized blood and plasma and although most data have been shown to be not statistically relevant, there has been consistency in increased levels of oxidized proteins within the fluids with disease progression and lipid peroxidation has also been shown to increase with severity, suggesting that this may be an early AD biomarker. More advanced, and selective analytical techniques, such as mass spectrometry, should therefore be utilized to shed more light on the subject.

Transthyretin (TTR), an important transport protein for thyroxine and retinol, is a primary protein that has been investigated as a biomarker for AD within CSF and blood [118,119]. Biroccio et al. described the ability of TTR to bind to amyloid-beta and to have certain neuroprotective properties [118]. PTMs associated with the protein were investigated by nano-LC MS/MS and MALDI-TOF MS and it was

found that oxidized forms of TTR were actually less abundant in CSF from AD patients as opposed to from healthy patients. Popov et al. described PTMs of transthyretin in blood by bottom-up and top-down proteomics [119]. 11 different modifications of the protein were identified. Several of these modifications, such as glycosylation, are mutations of genes which are thought to lead to various diseases and affect aggregation and neurotoxicity of amyloid-beta in AD [119]. Mass spectrometry surveys such as this are imperative in the better understanding of the changes that occur in CSF and blood proteins with disease progression.

9. Future directions

While great progress has been made in understanding the roles of PTMs in Alzheimer's disease, particularly with the application of mass spectrometry as a go-to analytical technique, much is still unknown about the relationship between the structure and function of the modifications. The next step in determining the function of the modifications is to be able to determine their localization, and variation between tissue types (healthy and diseased). Preliminary work involving synthetic peptides and animal models are crucial for a basic understanding of disease etiology and determination of research focus, however, the development of techniques to easily analyze human tissue is imperative for clinical applications. Imaging mass spectrometry (IMS), mentioned briefly earlier, is a prime example of where mass spectrometry can take PTM research. There are few reports on the application of this technique to the analysis of PTMs [120,121]. A way in which we have been utilizing MALDI IMS involves the comparison of control and diseased (or damaged) serial tissue sections to investigate the identity of molecular targets specific to the damage type in an attempt to make better sense of the biological mechanisms occurring and potential means of therapy or recovery [122–124]. Hundreds of molecules from a single tissue section can be simultaneously analyzed for their molecular and spatial information [122–125]. The primary advantages of MALDI imaging stem from the combination of pathological and histological approaches of tissue analysis and mass spectrometric approaches of complex biological systems analysis [124,125]. MALDI imaging is a label-free approach that does not rely on antibodies for species confirmation [125]. Often times, antibodies are not selective for only one analyte in a complex mixture or antibodies do not exist for an analyte of interest, making some antibody-based confirmation experiments difficult [122]. Additionally, if the complex sample involves unknown components, site-specific histology yields little information about the identities. The ability to resolve molecular localization without the need for labels makes MALDI IMS superior to common histological approaches. New molecular-based approaches of sample analysis are imperative to PTM and AD research in general if therapies are on the horizon.

Transparency document

The Transparency document associated with this article can be found, in online version.

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