Stoichiometry of Heavy Metal Binding to Peptides Involved in Alzheimer's Disease: Mass Spectrometric Evidence

23

Monica Jureschi, Ancuta Veronica Lupaescu, Laura Ion, Brînduşa Alina Petre, and Gabi Drochioiu

Abstract

Mass spectrometry is a powerful analytical technique becoming increasingly important in different biomedical research area. Mass spectrometric based methods were developed and applied to detect and identify multiple metal ion complexes of peptides and proteins with high sensitivity and high mass accuracy. Aggregation of amyloid-β (Aβ) peptides is one of the main pathological features of Alzheimer's disease (AD), and some metal ions seem to play a key role in AD pathogenesis. Consequently, mass spectrometry was used to investigate heavy metal binding to AD-related peptides. Therefore, the purpose of this chapter is to review the methodology and application of identifying coordination chemistry and binding properties of several metal ion-binding sites to synthetic β-amyloid (Aβ) and anti-amyloid model peptides. The selective metal–amyloid-β peptide interaction studies using (a) Matrix-assisted laser desorption/ionization mass spectrometry (MALDI); (b) Electrospray ionization mass spectrometry (ESI-MS), and (c) Tandem mass spectrometry (MS/MS^n) will be reported.

Keywords

Amyloid-β peptide · Mass spectrometry · Metal binding · Neuroprotective peptide

Monica Jureschi and Ancuta Veronica Lupaescu contributed equally to this work.

M. Jureschi · A. V. Lupaescu · L. Ion · G. Drochioiu (✉)
Faculty of Chemistry, Alexandru Ioan Cuza University of Iasi, Iaşi, Romania
e-mail: gabidr@uaic.ro

B. A. Petre (✉)
Faculty of Chemistry, Alexandru Ioan Cuza University of Iasi, Iaşi, Romania

Center for Fundamental Research and Experimental Development in Translation Medicine – TRANSCEND, Regional Institute of Oncology, Iaşi, Romania
e-mail: brindusa.petre@uaic.ro

Abbreviations

AD	Alzheimer's disease
ADNF	Activity-dependent neurotrophic factor
ADNP	Activity-dependent neurotrophic protein
AFM	Atomic force microscopy
APP	Amyloid precursor protein
Aβ	Beta-amyloid peptide
CHCA	α-Cyano-4-hydroxycinnamic acid
CID	Collision-induced dissociation
DHB	Dihydroxybenzoic acid
ECD	Electron capture dissociation
ESI	Electrospray ionization
ETD	Electron transfer dissociation
EXAFS	Extended X-ray absorption fine structure spectroscopy
FTIR	Fourier-transform infrared spectroscopy
LC	Liquid chromatography
MALDI	Matrix-assisted laser desorption/ionization
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NFTs	Neurofibrillary tangles
NMR	Nuclear magnetic resonance
ROS	Reactive oxygen species
SA	Sinapinic acid
SFM	Scanning force microscopy
THAP	Trihydroxyacetophenone,
TNFα	Tumor necrosis factor α
ToF	Time of flight

23.1 Introduction

Mass spectrometry (MS) is an important analytical tool, which can detect and identify multiple metal ion complexes of peptides and proteins with high sensitivity and high mass accuracy [1, 2]. Electrospray ionization (ESI) and

© Springer Nature Switzerland AG 2019

A. G. Woods, C. C. Darie (eds.), *Advancements of Mass Spectrometry in Biomedical Research*,
Advances in Experimental Medicine and Biology 1140, https://doi.org/10.1007/978-3-030-15950-4_23

matrix-assisted laser desorption/ionization (MALDI) are both 'soft' ionization techniques that produce ions of low energy. Matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI-ToF-MS) is widely used in a variety of measurements because of its speed, ease of use, high sensitivity, and wide detectable mass range [3, 4]. For example, the complexes of peptides or proteins with chelated metal ions can be detected efficiently and rapidly by MALDI-ToF-MS [5]. On the other hand, the electrospray ionization–mass spectrometry (ESI–MS) is used in a wide variety of fields to examine the formation, stoichiometry and speciation of complexes involving metals and organic ligands [6]. In a recent review, the ESI ionization process is presented as an ideal and versatile method for studying noncovalent interactions between peptides and metals [7].

Neurodegenerative diseases are associated with neuronal death that generally occurs in the specific brain area, such as cerebral cortex and hippocampus [8]. In the last decades, major advances have been made to better understand the pathogenesis of neurodegenerative diseases. Currently, AD is an incurable neurodegenerative disorder and the world's most common dementing illness that affects over 150 million patients around the world, and its incidence is expected to increase dramatically [9]. Neuropathophysiologically, AD is characterized by the presence of intracellular neurofibrillary tangles (NFTs) containing Tau protein as major component, and extracellular senile plaques that are predominantly localized in areas with neuronal loss [10]. The senile plaques, also called neuritic, dendritic or amyloid plaques consist primarily of several Aβ peptides sequences [11]. Amyloid fibrils have been shown to be associated with only a limited number of diseases, including Alzheimer's disease, Parkinson's disease, Prion disease and about twenty-seven other diseases of varying severity. Besides accumulation of Aβ peptides and Tau protein, the brain of patients with AD contains increased inflammatory markers such as protein complement factors, acute-phase protein or proinflammatory cytokines [12]. Thus, the AD pathogenesis seems to be restricted not only to the neuronal compartment, but includes strong interactions with immunological systems in the brain [13]. External factors, including systemic inflammation and obesity, may interfere with immunological processes in the brains and thus promote AD progression [14].

23.1.1 Aβ Peptide Associated with AD

The amyloid-β (Aβ) peptide was discovered for the first time by *Glenner* and *Wong* in 1984 as the major component of the amyloid deposits [15, 16]. Aβ peptide, contains 39–43 amino acid residues in its sequence and is an unspecific cleavage product resulted from APP (amyloid precursor protein). Proteolytic processing of APP is mediated by three different secretases, α-secretase, β-secretase and γ-secretase [17]. Beta-secretase defines the N-terminal of Aβ and cleaves the APP throw the "amyloidogenic pathway". Alternatively, APP cleavage can choose the "non-amyloidogenic pathway" generated by alpha-secretase that prevents Aβ formation. The gama-secretase complex mediates the C-terminal truncation of Aβ while its composition defines the length of the peptide: from 39 to 43 amino acids. Several different types of Aβ can be produced, the predominant species being: Aβ(1–38), Aβ(1–40) and Aβ(1–42) comprising 38, 40 and 42 amino acid residues, respectively [18]. Once Aβ is cleaved enzymatically from APP, it becomes a soluble monomeric peptide in an aqueous medium and is removed from the brain in healthy individuals. In pathological cases, Aβ peptide forms aggregates and becomes neurotoxic [19]. It has been shown that Aβ forms a variety of quaternary structures including amyloid fibrils as well as a class of intermediary structures called oligomers [20]. Hence, the abnormal accumulation of neurotoxic forms of Aβ in the brain is a result of an altered proteolytic processing of APP. The causes of the modifications are still unclear, but may be related to the increase in oxidative stress, impaired energy metabolism and disturbed cellular ion homeostasis [21]. When Aβ is excessively produced in the cerebral tissue, the aggregation of amyloid monomers is favored, resulting oligomers, protofibrils, fibrils and ultimately senile deposits [22]. The ability to form insoluble deposits is commonly affected by temperature, pH, the presence of metal ions and Aβ peptide concentration [23]. The Aβ peptide has been shown to be implicated in the toxicity of primary and clonal neuronal cell lines in vitro, including the aggregation of β-amyloid necessary for cytotoxic activity and generation of chemical radicals. There is good evidence for their accumulation in AD brains, where the toxic mechanism involves the production of reactive oxygen species (ROS) [24]. Amyloid oligomers have emerged as toxic species of Aβ and they might explain the lack of correlation between amyloid plaques and memory impairment or cellular dysfunction [25].

23.1.2 Metal Involvement in AD

Metals are found all over the organism and their specific functions make them indispensable for a multitude of biological processes. Even with normal ageing, metals ions such $Fe^{2+/3+}$, Zn^{2+} and $Cu^{+/2+}$ accumulate in the brain, and therefore, this organ is abundant in antioxidants to control and prevent the detrimental formation of reactive oxygen species (ROS) generated *via* Fenton chemistry [26]. Metals have been associated for the first time to AD, half a century ago, when Goodman et al. identified large iron deposits in AD brains using Perls' Prussian blue staining [27]. Since then, studies related to the distribution or their implication of

metals such as iron, copper, zinc and aluminum in Alzheimer's pathology have been conducted [28, 29]. It has been reported that metal ions have an important role in the aggregation process of Aβ peptide and the generation of reactive oxygen species [30]. In fact, high concentrations of metal(s) identified in the brain are associated to normal aging and a number of diseases liked to neurodegeneration [31]. In the synapse signaling, during neurotransmission, important amounts of Zn^{2+} and Cu^{2+} ions are released and could favor the Aβ precipitation [32]. Transitional metals such as copper and iron accelerate the peptide aggregation at acidic pH [33]. High ratios of Fe^{3+} produced species that are completely different from those of the other investigated metal ions, such as copper, nickel and zinc ions [34]. Moreover, the conformational modifications generated by the metal ions have a great impact over the plaque formation [35–37]. Thus, studies related to Aβ interaction with different metal ions can offer more information about the metal-protein/peptide relationship and the pH influence [38–40]. For example, conformational changes that involve the loss of water molecules diminish the capacity of Aβ(1–40) peptide to bind metal ions [38]. The association of Cu^{2+}, Zn^{2+} and Fe^{3+} ions with Aβ could explain the recently reported enrichment of these metal ions in amyloid plaques in AD brains [33]. However, the disruption way of metal ion homeostasis, which affects the disease, is still obscure. Nevertheless, metal ions are increasingly recognized to play an important role in molecular processes underlying AD [41], and the only recent advances in analytical technology allow detailed investigation of metalloproteins. Investigation of individual metal-peptide complexes may yield new mechanistic details about the role of metal ions in AD. However, in the literature are reported different mechanisms by which metal ions can aggravate Alzheimer's disease. Metal ions and the oxidative stress hypothesis focus on understanding how metal dyshomeostasis could favor AD progression [8]. In addition, it is possible a synergic action of more heavy metal ions that interact both with Aβ peptides and Tau protein [42].

Other features displayed by the disease are related to oxidative stress, mitochondrial dysfunction, excitotoxicity and metal ions accumulation [10]. The presence of metals in amyloid plaques has been attributed to a connection between the formation of amyloid fibrils and metal ions [43]. In addition, the neurotoxicity of Aβ oligomers involves the production of reactive oxygen species (ROS) and modifications in cellular calcium homeostasis [21]. Free radicals are a major cause in the progression of traumatic brain injury [44]. Following interaction of Aβ oligomers with Fe^{3+} or Cu^{2+} ions, the reduction of metal ions is facilitated along with reactive oxygen species (ROS) and hydrogen peroxide (H_2O_2) production [45]. Redox couples, like Cu(I)/Cu(II) and Fe(II)/Fe(III), stimulate the activation of molecular oxygen and generate destructive hydroxyl radicals ($OH^{\bullet}$) through a Fenton-type reaction [46]. Besides, metal dyshomeostasis increases the cytotoxicity of ROS by decreasing the value of glutathione, a potent cellular antioxidant [47]. Such findings led the researchers to the hypothesis that antioxidants could play an important role in preventing AD progression [45].

One of the existing hypotheses that attempt to explain the occurrence of neurodegenerative diseases focuses on abnormal accumulations of metals such as Cu^{2+}, Zn^{2+}, Fe^{3+}, Al^{3+} at the neuronal level, which lead to the formation of toxic peptide aggregates [48]. As shown in Fig. 23.1 this process is not solitary in AD pathology and is related to the inflammatory process that occurs due to the generation of physiological uncontrolled reactive oxygen species. Recent evidence suggests that precipitation of Aβ peptide and the toxicity in AD are induced by abnormal interactions with more neocortical metal ions, especially Zn^{2+}, Cu^{2+}, and $Fe^{3+}$and not a single metal ion [49]. The authors used atomic force microscopy (AFM), Thioflavin T-induced fluorescence (ThT), and FTIR spectroscopy to investigate the co-incubation of Aβ(1–42) peptide with Cu^{2+}, Zn^{2+}, and Fe^{3+} and reported significantly altered morphology of the resulted aggregates.

23.1.3 Neuroprotective Peptides

Neurotrophic proteins and neuropeptides play an important role in immunity and neuroprotection and can be recognized as important factors in drug development [50]. Major neurodegenerative disorders, including Alzheimer's disease, are characterized by elevated tissue iron, and miscompartmentalization of copper and zinc, which accumulate in amyloid [51]. Increasingly sophisticated medicinal chemistry approaches to correct such metal abnormalities without causing systemic disturbance of these essential minerals are being tested.

Activity-dependent neurotrophic factor and protein (ADNF and ADNP) are proteins that protect the neurons from dying and are essential for brain function. A small peptide fragment of ADNP that provides neuroprotection at very low concentrations is the eight amino acid neuroprotective peptide NAP, with the following amino acid sequence: NAPVSIPQ [52, 53]. NAP was shown to prevent the trauma-induced accumulation of TNFα (tumor necrosis factor α), a proinflammatory cytokine, and to inhibit the Aβ fibrils formation [54]. Besides, results showing the formation of NAP–Cu complexes provided new information about the mechanism of interaction with Aβ aggregates [55]. Thus, this peptide can be considered a veritable drug candidate against Aβ-induced neurotoxicity. The active core of ADNF, named ADNF-9 or SAL (SALLRSIPA), exhibits structural and functional similarities with NAP. Its capacity to preserve against cognitive impairment was highlighted on different animal models where it increase the neuronal survival

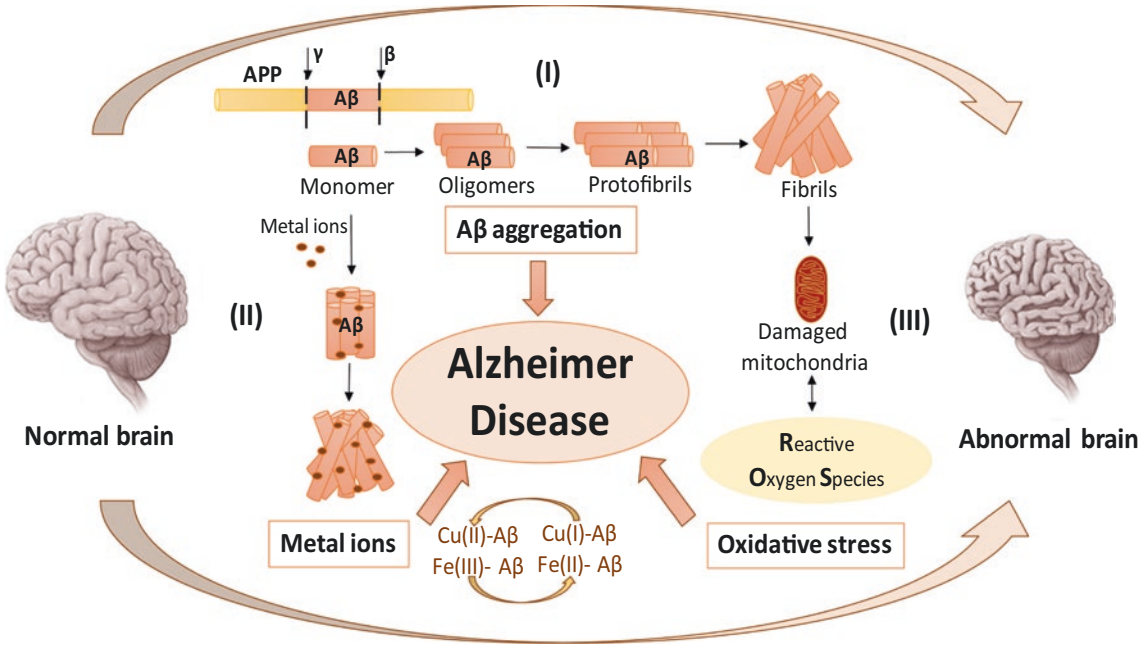

Fig. 23.1 Overview of possible hypotheses related to Alzheimer's disease (AD): (I) the hypothesis of amyloid peptide aggregation, (II) the metal ions hypothesis, and (III) the hypothesis of oxidative stress

following exposure to neurotoxins [56]. Research related to natural products and their effect on AD development, revealed that methanolic extract could also be used as neuropharmacological agents against cognitive impairment [57, 58]. Thus, studies investigating the effect of methanolic extract from *Lactuca capensis* leaves on AD rat model revealed benefic effects on cognitive performance, acetylcholinesterase activity and brain-derived neurotrophic factor [57]. Another study investigating the lavender essential oils conducted on scopolamine-induced dementia rat model showed potent neuroprotective effects by increasing the activities of antioxidant enzymes [58].

23.2 Mass Spectrometry and AD

An important issue for the improvement of Aβ-targeted AD therapies is given by the possibility to detect and classify Aβ levels *in vitro* and *in vivo* studies [59]. Mass spectrometry (MS) is a useful tool in characterizing Aβ *ex vivo*, because it allows post-acquisition analysis of sample [60]. Several new Alzheimer-related protein and peptide panels have been designed using the combination of mass spectrometry and targeted approaches [61]. For example, Aβ isoforms from different brain regions were analyzed using immunoprecipitation in combination with matrix-assisted laser desorption ionization time-of-flight (MALDI-ToF MS) or Nano flow liquid chromatography (LC), electrospray (ESI), high resolution tandem mass spectrometry (MS/MS) to give better accuracy of peptide mass [62]. MALDI ToF MS technique can also be used to highlight the Aβ population from complex samples. According to the "metal hypothesis" of AD, metals such as copper, iron, zinc and aluminum bind to the amyloid peptide and promote the Aβ misfolding and plaque aggregation [63]. An altered distribution of neuronal metal ions may induce alternative Aβ aggregation pathways [64]. Changes induced by metal ions on the structure of Aβ peptide are detected by MS using the electrospray ionization (ESI) technique. This method allows the detection of Aβ-metal interactions in order to determine the stoichiometry, specificity, and fragmentation mechanisms of complexes [39]. Thus, MS investigation has an important role in understanding the biomolecular mechanisms involved in aggregates formation and proved to be crucial in the development of drug therapies. The high complexity of biomolecules and their biological pathways implication have caused difficulties for studying proteins by some techniques, including sometimes mass spectrometry as well [7]. In such cases, peptides, smaller and less complex biomolecules, or their fragments and mutants have been found to be suitable models to mimic the interactions of entire proteins. One technique in particular, ESI-MS being a soft ionization technique that ionizes molecules in mild native condition has proven to be fruitful to study the peptide–metal interaction. The speed, sensitivity, and selectivity of MS, along with the information that can be interpreted from mass spectra, have driven this technique to the forefront for understanding the nature of peptide–metal complexes. The stoichiometry of peptide–metal complexes or even mixtures of complexes were identified by MS [38]. The specific amino acids residues to which

the metal cations are bound and the degree of association in these complexes can also be determined from ESI-MS spectra.

Neurodegenerative disorders, including AD, share similar metabolic processes such as protein aggregation and oxidative stress, both of which are associated with the involvement of heavy metal ions [65]. Chelation therapy could provide a valuable therapeutic approach to AD, since metals, particularly iron, are pharmacological targets for the rational design of new therapeutic agents. However, one-dimensional treatments demonstrated that AD is a complex disease, which requires new approaches for preventing and treating. Undoubtedly, drugs for targeting specific sites of the neurodegenerative cascade are in development. Some are focus on blocking the β- or γ-secretases, enzymes responsible for the amyloidogenic process of APP [66]. Metal chelators to target metal-induced Aβ aggregates have attracted more and more attention for AD therapy [67, 68]. Metal-involved strategies are capable to regulate metal homeostasis and prevent the Aβ aggregation. It is well known that by chelating Aβ-bound metal ions, the neurotoxicity and ROS generation are visibly diminished [69, 70]. For example, clioquinol, a metal protein-attenuating compound (MPAC) and similar compounds or derivatives have been shown to improve the cognition of AD patients [71] due to their capacity of disrupting the interaction between metals and the Aβ peptide in the brain. This chapter reviews the literature of recent years on the binding of metals to peptides involved in Alzheimer's disease, by using different mass spectrometric approaches. The need for more standard reporting of quality assurance data is also highlighted, to improve the application of mass spectrometry to metal–peptide complexes further. Therefore, our study emphasizes the use of mass spectrometry in the determination of metal binding to amyloid-β and anti-amyloid-β peptides linked to AD, but also the relationship of heavy metal ions with the amyloid precursor protein (APP) and Tau protein. In this regard, recent approaches for the use of (1) MALDI-ToF MS, (2) ESI-MS, and (3) tandem mass spectrometry, MS/MS in the field of metal–peptide investigation are described.

23.2.1 MALDI-ToF Mass Spectrometric Approach of Peptides

Matrix Assisted Laser Decomposition/Ionization mass spectrometry (MALDI-MS) is an analytical technique that ensures the rapid determination of the molecular weight of proteins and other biomacromolecules [72]. The MALDI ionization method in combination with a ToF (time-of-flight) mass analyzer has proved to be a particularly convenient analytical tool for these analyses. One of the particular facts is the dominance of singly charged ions and the ability to ionize a mixture of peptides at once, which makes this technique to be a popular choice for rapid analysis of protein digestion [73]. Sample preparation including proteins, peptides, either individual components or mixtures, is relatively simple. The procedure requires co-crystallizing the sample of interest with an excess of UV-absorbing organic compound named matrix [74].

The MALDI process generally involves depositing a diluted solution of analyte and a highly concentrated matrix solution on a target plate, either separately or together as a mixture of the two solutions and allowing them to dry. Combining the sample with the matrix is relatively simple by adding the matrix to the sample on the MALDI target plate in a ratio of 1:1, which will lead to high-quality spectra. The samples can also be prepared by mixing the sample solution and the matrix solution in a microcentrifuge tube. For example, if the sample is a mixture of peptides and requires prepurification, it is possible to obtain desalting the peptide solution before being mixed with the matrix. This procedure can be accomplished using micro C18 pipettes (e.g. ZipTip) according to the Millipore User Guide for ZipTipMC Pipettes (www.millipore.com). In this way, the sample is concentrated on this capillary column, the salts are removed by washing steps and the analyte is eluted directly for the measurement [74]. As mentioned, this method requires the ionization of the sample using a compound called matrix capable of absorbing UV light. The matrix is usually a small, soluble organic compound with similar features to a proton donor aromatic compound capable to incorporate and co-crystallize the analyte. More exactly, the analyte and matrix solution are co-precipitated in order to facilitate the sublimation and ionization of sample under the UV laser pulse [75]. The choice of matrix is extremely important since it is related to the quality of MALDI spectra. The most often used MALDI matrices are: α-cyano-4-hydroxycinnamic acid (CHCA) [76], 2,5-dihydroxybenzoic acid (2,5-DHB) [77], 2,4,6-trihydroxyacetophenone, (2,4,6-THAP), and sinapinic acid (SA) [3]. For a peptide with a mass >1000 Da, CHCA is preferred, whereas DHB has a lower profile for lower-grade peptides and is more tolerant to buffer salts at low peptide concentrations. The sinapinic acid is usually the matrix of choice for very large peptides and proteins. Matrices are prepared as solutions of 10 mg/ml in solvent, generally 0.1% trifluoroacetic acid in 20–50% acetonitrile (ACN) [78].

The procedure of spotting the sample and the matrix onto the plate can influence the crystal quality and further the mass analysis sensitivity. The most common method, called the dried droplet method, includes spotting first the sample and over it the matrix solution. After the solvent is evaporated, the plate containing fresh crystals can be immersed in the MALDI and irradiated with a pulsed laser beam. Usually, the analysis is made in positive ion mode but the negative mode can also be used. Negative ion MALDI data scanning

gives sometimes more pronounced peaks for the complexes of peptides with heavy metal ions [79]. The popularity of MALDI-MS for peptide analysis is due to the availability of modern commercial instruments, which are easier to operate and provide greater performance at a lower cost. The search for more useful matrix compounds is an active area for MALDI-MS research since the birth of the technique in 1988, this being an essential part of this mass spectrometric method. Despite increased knowledge of the MALDI ionization process, matrix selection and optimization of the preparation protocol are still empirical procedures.

23.2.1.1 Examples of Peptide-Metal Interaction Studied by MALDI Mass Spectrometry

Metal ions are capable to promote amyloid β-peptide aggregation into insoluble fibrils following noncovalent interaction. However, they can bind to peptides by two distinct ways: the inter-molecular mode engages "Aβ–metal–Aβ" bridges formations, while the intra-molecular one is based on the atoms involved in the metal coordination site [80]. Studies involving chemical modification and mutation provide information regarding the metal binding site of Aβ. Histidine (His^{6}, His^{13}, His^{14}) aspartate (Asp^{1}, Asp^{7}) and glutamate residues (Glu^{3}, Glu^{11}) are considered the main binding site for Zn^{2+} and Cu^{2+} ions. Other possible coordinating residues for copper are: tyrosine (Try^{10}), aspartate (Asp^{23}), glutamate (Glu^{22}), methionine (Met^{35}), deprotonated amides of the peptide backbone and carbonyl groups. Regarding $Fe^{3+/2+}$ and Al^{3+} binding sites, there is still under investigation where the preferential regions are situated [81].

The *in vitro* MALDI MS studies are performed by incubating the peptide samples with metal ions in different metal to peptide ratio and at various pH values. Usually, for complexing experiments are used salts containing ions such as Fe^{3+}, Fe^{2+}, Cu^{2+}, Cu^{+}, Ni^{2+}, Zn^{2+}, Al^{3+} known to have an important influence on disease progression. In the case of amyloid peptides, the obtained results help us in understanding the propagation mechanism that leads to senile deposits [82]. As for neuroprotective peptides, this kind of experiments is used for evaluating the chelating properties of studied peptides. The Aβ peptide sequences and neuroprotective peptides were synthesized by solid phase peptide synthesis (SPPS) using Fmoc and side-chain protection chemistry (t-butyl, trityl) obtaining appropriate crude peptides after final deprotection using predominantly acidic condition for cleavages. All peptides were lyophilized and further subjected to final purification by semipreparative reversed phase—high performance liquid chromatography (RP-HPLC) and characterised by MALDI ToF mass spectrometry [82, 38].

In our studies we have used synthetic Aβ peptides to analyze the interaction of partial Aβ(9–16) peptide fragment with copper metal ions. In order to identify their binding capacities, Aβ(9–16) peptide was incubated with copper (II) ions under 350 rpm for 24 h at room temperature. The samples were loaded onto a 384-polished steel MALDI target plate using the dried-droplet method: the sample and the matrix solution were mixed together on the target and allowed to dry in the ambient air. The matrix contained a saturated solution of HCCA (a-cyano-4-hydroxycinnamic acid) in acetonitrile: 0.1% TFA (2:1). The obtained spectra are shown in Fig. 23.2, where beside the signal from m/z = 996.852 corresponding to the pure peptide other peaks were attributed to the sodium (m/z = 1018.890) and potassium (m/z = 1034.821) adducts. However, the most intense peak from m/z = 1058.825 was assigned to Aβ-Cu(I) complex ($[M+Cu(I)]^{+}$). The high affinity of Aβ(9–16) peptide for metal ions is provided by the tyrosine (Tyr^{10}) and the two histidine (His^{13}, His^{14}) residues found in its structure. Thus, the MALDI-ToF mass spectra of the Aβ(9–16) model peptides in the presence of metal ions confirmed the existence of metal-peptide interactions at peptide: metal ration of 1:10 (mol/mol). However, the laser intensity was less than 30% in order to avoid the peptide-metal complex dissociation under UV laser irradiation. The direct detection of noncovalent complex in MALDI is often more problematic than in electrospray ionization mostly due to labile dissociation of nonstable complexes during the desorption/ionization process [83, 84]. Moreover, the MALDI matrix choice was shown to have a major contribution for successful studies of noncovalent protein or protein–metal ions interactions [85, 86].

Binding interaction of metals with neuroprotective peptides that are known to exhibit antiaggregation activity in AD patient were also investigated. For example, NAP peptide, an octapeptide known to protect against Aβ peptide fibrillogenesis, and its histidine mutant (NAPH) were incubated with copper(II) ions for 24 h under slow mixing at 350 rpm and room temperature. The samples were then directly loaded onto a 384-polished steel MALDI target plate using the same dried-droplet method. The matrix contained a saturated solution of HCCA (a-cyano-4-hydroxycinnamic acid) in acetonitrile: 0.1% TFA (2:1). In Fig. 23.3 are shown the spectra of NAP peptide (a) and NAPH peptide (b) in presence of copper ions. In both cases the strongest peak was assigned to $[M-17]^{+}$ ion (at m/z = 809.508 for NAP and m/z = 859.583 for NAPH) which is formed due to the rule "N-terminal Q → Q[−17.027]" applied for N-terminal glutamines [87]. The peptide-Cu(I) ion complex showed a signal at m/z = 887.424 for NAP peptide (a) and another one at m/z = 937.495 for NAPH peptide (b). Thus, the MS spectra of the synthesized peptides in the presence of metal ions confirmed the existence of metal-peptide interactions favored by the presence of serine and histidine residues. Furthermore, the observed signals showed a high affinity of NAP peptides toward Cu^{+} ion which may interfere with copper oxido-

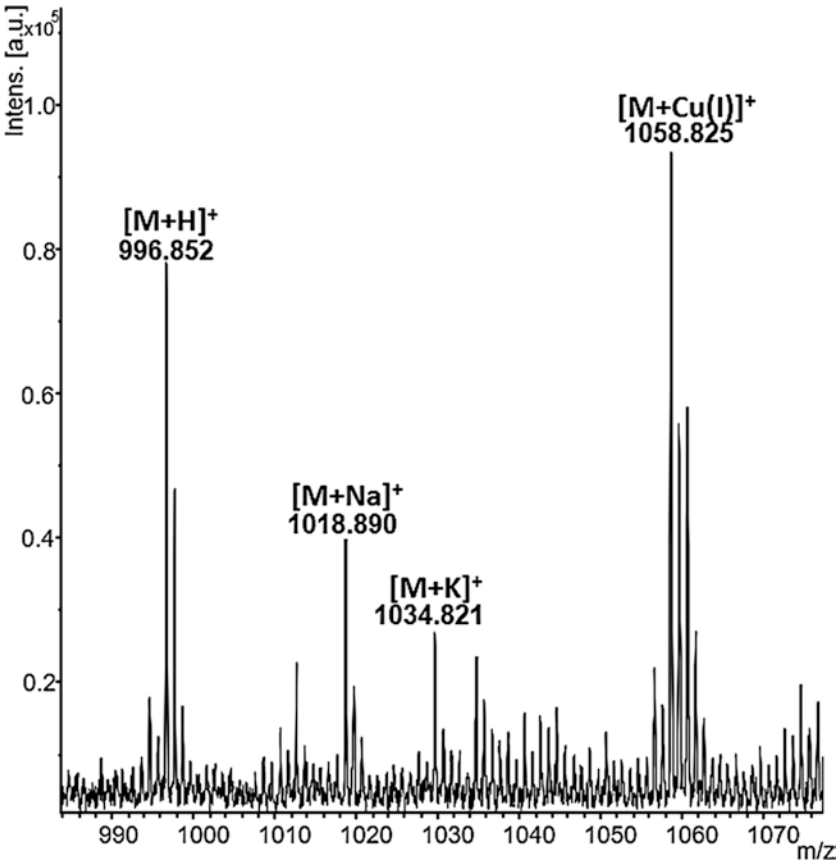

Fig. 23.2 MALDI-ToF mass spectra of native peptide Aβ(9–16) (GYEVHHQK), in the presence of copper ions. The synthesized peptide (2 mM) were subjected to copper metal ions at a peptide metal ratio of 1:10. The sample were prepared in deionized water, at pH 7

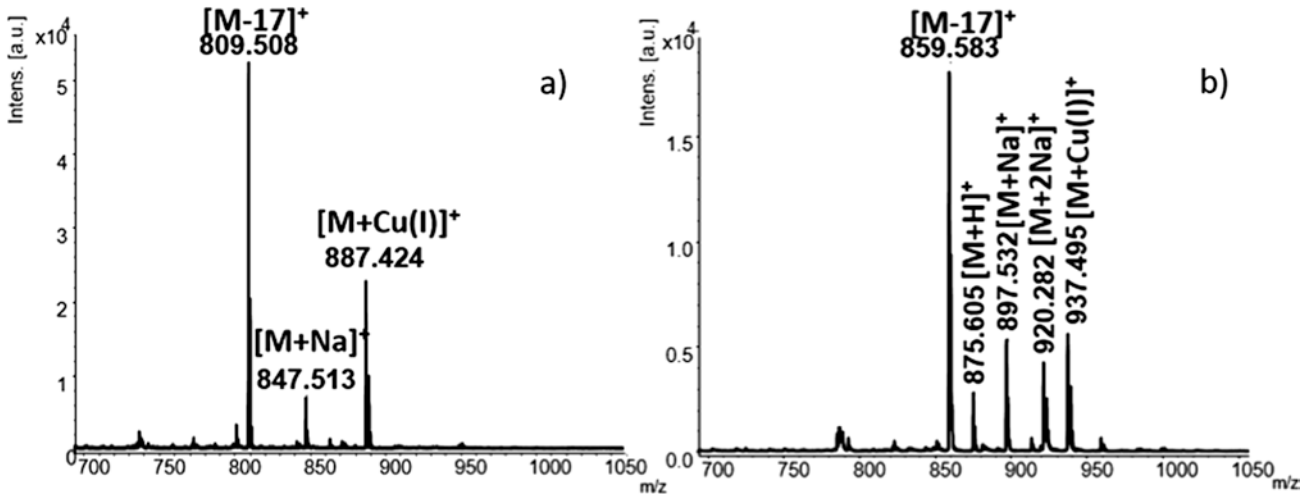

Fig. 23.3 MALDI-ToF mass spectra of (**a**) native peptide NAP (NAPVSIPQ) and (**b**) its mutant, NAPH (NAPV**H**IPQ), in the presence of copper ions. The synthesized peptide (2 mM) were subjected to copper metal ions at a peptide metal ratio of 1:10. The sample were prepared in deionized water, at pH 7

reduction reaction catalyzed by Aβ, and not Cu^{2+} as expected. Other signals found in the spectrum were attributed to the $[M+H]^+$ ion and its sodium adducts ($[M+Na]^+$).

Similar studies were performed using NAP cysteine mutant (NAPV**C**IPQ) in the presence of iron(III) ions [88] and copper(II) ions [89]. In both cases the MALDI MS spectra showed beside the peaks assigned to both pure peptides and their adducts with sodium and potassium ions, signals corresponding to an peptide-iron(II) or peptide-copper(I) complex. In addition, the MS spectra proved the formation of Fe^{2+}–peptide complexes/Cu^{+}-peptide complexes even if the experiment was made using Fe^{3+} ions/Cu^{2+} ions.

23.2.2 ESI-MS Evidence for Metal Binding to Aβ Peptide

Electrospray ionization mass spectrometry (ESI–MS) has been used to study protein-ligand interactions driven by non-covalent forces. However, its ability to gently shift the analyte from solution to gas phase, made it suitable for metal-organic ligand complex analysis [90]. This technique can provide information regarding the influence of pH and concentration on peptide-metal complexes. Stoichiometry of the complex can be easily obtained from the resulting mass spectra due to direct analysis in the MS. Modification on metal oxidation state and stoichiometry of complexes can also be detected. However, changes in solution chemistry was shown to affect the relative ion intensity of species [6]. The ionization process involves the use of electrical energy and generates singly charged species that result in the mass spectra with m/z peaks. According to the optimum detection conditions required by the compound, the technique can be easily switched from positive to negative ion mode. Moreover, its flexibility regarding the sample medium, make this technique approachable for a wide range of solvents and allows pH variability [91]. Thus, by ESI-MS ionic species can be analyzed with increased sensitivity.

Several fundamental issues regarding the amyloid fibril accumulation pathway have been elucidated using MS approaches [15]. The stoichiometry, specificity and fragmentation mechanisms of peptide–metal ion complexes can be studied in detail by ESI-MS [92]. Also, iron ions binding to Aβ(1–40) peptide was demonstrated through ESI-MS measurements performed on a Bruker Daltonics Esquire 3000 Plus (Bremen, Germany) ion trap mass spectrometer [39]. Thus, ESI–MS is able to provide direct information on changes in speciation with pH and metal: ligand ratio, identify metal ion charge directly and allow insight into competitive interactions in ternary systems [6]. CD spectroscopy, Thioflavine-T (ThT) induced fluorescence and scanning force microscopy (SFM) measurements were used to prove the MS results. ESI-MS is also complementary to other biophysical methods, such as nuclear magnetic resonance (NMR) and analytical ultracentrifugation [64].

Multiple heavy metal ions bound to Aβ peptides studied by ESI-MS showed a complex pattern of metal–metal competition for Aβ(1–40) binding sites, which depend essentially on the involved metal ions, their concentration, and pH changes. Moreover, it was previously reported that metal ions bind specifically to Aβ(1–40) peptide and change dramatically its conformation [93]. However, mass spectrometry measurement offers advantages in speed and sensitivity [94]. Nevertheless, the quantitative ESI–MS data should be addressed in detail, with reference to differences in the ion intensities of species, signal suppression and quantifying species distributions [6].

Tandem mass (MS/MS or MS^n) spectrometry offers information about the structure and the primary sequence involved in the non-covalent bindings. This method requires the isolation of the interest peak (precursor ions) followed by specific fragmentation of peptide back-bond depending on the fragmentation method used. The resulted product ions are indicated by a, b, or c if the charge is retained on the N-terminus and by x, y or z if the charge is maintained on the C-terminus [95, 96]. In tandem MS product ions are generated by different processes such as collision-induced dissociation (CID) [97, 98], electron capture and transfer methods (ECD—electron capture dissociation, and ETD—electron transfer dissociation) [99, 100], photodissociation [101], and others [102, 103]. The resulting ions are then separated and detected in a second stage of mass spectrometry (MS^2), when an ion in MS^2 is selected for a further fragmentation an MS^3 mass spectrum is generated and the process may continue taking into account the sample availability and capabilities of the MS instrument. In case of CID fragmentation of a metal–peptide complex, the **b** type and **y** type ions achieved after fragmentation will provide identification of the metal-binding site [62]. Tandem mass spectrometry (MS/MS) can be used also to identify specific modification such as tryptophan and methionine oxidation products in the protein sequence [68, 104] of phosphorylation sites [105, 106]. In this case, following the exposure to oxidative factors, the protein or peptide must be digested with trypsin before analyzing the sample by tandem MS.

23.2.2.1 Examples of Peptide-Metal Interaction Studied by ESI and Tandem MS^n Mass Spectrometry

Lu et al. evaluated the metal binding properties of Aβ(1–16) peptide fragment using soluble anodes for generating metal ions capable to interact with peptides and proteins [107]. Unlike the use of metal salt solutions, this method only produces metal ions without counter ions capable of charge neutralization. For example, a copper anode connected to an ESI ionization source is able to produce both types of copper ions (Cu^+ and Cu^{2+}). These ions can be further scavenged by biomolecules to form complexes. In the case of Aβ(1–16) peptide, following the interaction with copper ions generated by this approach, doubly and triply charged peptide-metal complexes were observed in ESI mass spectra after 10 min of electrospraying time [107].

As shown in Fig. 23.4, the metal complex bound with one copper ion $Cu^+(A\beta\ 1–16)^+$ was observed at m/z = 672.9 and m/z = 1009.4, respectively, while the metal complex bound with two copper ions $Cu^{2+}(A\beta\ 1–16)^+$ was observed at m/z = 694.0 and m/z = 1040.3. By increasing the spray time, the number of copper ions bound to Aβ(1–16) increased up to six copper ions [107]. Observing both Cu(I) and Cu(II) ions produced in solution by using this approach the isotopic

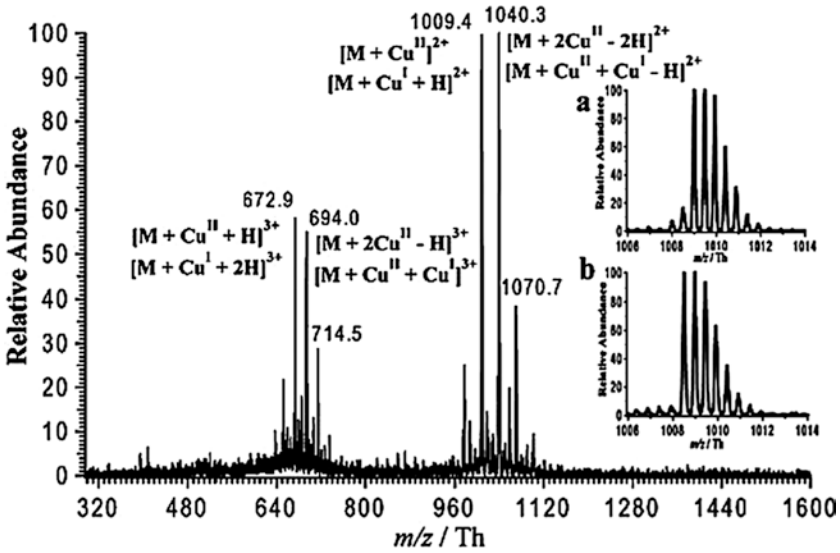

Fig. 23.4 ESI MS spectrum of Aβ 1-16 peptide in the presence of copper ions generated on-line using a sacrificial copper electrode. The insert (**a**) shows the isotopic distribution of copper-Aβ16 complexes generated from copper electrode in the presence of ascorbic acid and the insert (**b**) shows the isotopic distribution of copper(II)-peptide complex obtained from the addition of Cu(II) salt as reference. Reprinted from Metallomics (2010), Vol. 2, pp. 474–479, with permission from Copyright Clearance center

distribution of each observed peak in mass spectra was used to identify the peptide oxidation states of copper ions and their concentrations as previously described [108]. Theoretically, the isotopic distribution of the mixture of Cu(I)- and Cu(II)-Aβ peptide complexes should be the sum of the isotopic distribution of each Cu(I)- and Cu(II)-Aβ complexes. In this case, by calculating the isotopic peaks of mixture it was found that 55% ± 10% of the complexes contain Cu(I). To prove this result, ascorbic acid solution, as scavenger, was added in the Aβ-metal solution before spraying. As shown in Fig. 23.4a, the isotopic distribution of the doubly charged copper complex at m/z = 1009.4 obtained by using a copper electrode in the presence of ascorbic acid was different from that of a Cu(II) complex. Moreover, the peak at m/z = 1009.4 obtained by analyzing a solution of Aβ(1–16) mixed with Cu(II) salt showed an isotopic distribution corresponding to Cu(II) complex (Fig. 23.4b), confirming that a great amount of Cu(I) complex was generated in solution and not in the gas phase. Using a soluble copper electrode in the presence of ascorbic acid and applying the same isotopic peaks distribution calculation method it was confirmed that 86% ± 4% of Cu(I)-Aβ(1–16) complex was obtained and its stability was set for about half an hour [107].

In order to determine exactly the binding site between copper(I) ion and Aβ(1–16) peptide, Lu et al. used collision-induced dissociation (CID) tandem MS to generate fragments of Cu(I)-Aβ(1–16) complex. As shown in Fig. 23.5, the CID spectrum of $[M+Cu^{I}+H]^{2+}$ at 30% of collision energy displayed many product ions [107].

The most fragment ions obtained are conventional b-ions and complexes of b-ions and y-ions bound to Cu^{+}. Analyzing all these fragments, the binding site of Cu^{+} to Aβ(1–16) have been deducted sequentially at His^{13} and His^{14}, common residues that are known to bind metals. These results indicated that Cu(I) is coordinated to Aβ(1–16) peptide by two imidazole groups of His^{13} and His^{14} respectively (see insert in Fig. 23.5), in agreement with structural studies of Cu(I)-Aβ(1–16) complex by extended X-ray absorption fine structure spectroscopy (EXAFS) [109, 110]. These mass spectrometric data confirmed for the first time in literature that both His^{13} and His^{14} are binding sites of Aβ(1–16) peptide and Cu(I) complex.

The following example described in this chapter refers to the reaction of platinum phenanthroline ($PtCl_2$(phen)) with metal bound Aβ(1–16) peptide. It was previously reported that chelation therapy using platinoid complexes inhibit the aggregation process of Aβ by non-covalent interactions [111, 112] and is a novel approach to combat Aβ neurotoxicity [113]. Ma et al. analyzed the interactions between $PtCl_2$(phen) and metal-bound Aβ peptide ([Cu^{2+}-Aβ1–16]) complex by ESI-MS and tandem mass spectrometry (MS/MS).

The mass spectra showed several m/z peaks corresponding to platinated adducts from the reaction of $PtCl_2$(phen) with Aβ1–16 peptide in the presence of Cu^{2+} salt at pH 5.0 (Fig. 23.6). The free peptide Aβ1–16 peptide and the [Cu(II)-Aβ1–16] complex were detected as triple charged ions at m/z = 652.30 and 673.24, respectively. The mono-platinated adduct [Pt(phen)+Aβ1–16] (at 589.92 m/z,(4+); 776.96 m/z, (3+)) and bi-platinated adduct [2 Pt(phen)+Aβ1–16] (at 676.46 m/z, (4+); 901.30, m/z (3+)) were observed. Moreover, the ions peaks at m/z = 797.26 (3+) and 691.94 (4+) are corresponding to the complexes [Pt(phen)+Aβ1–16+Cu(II)] and [2Pt(phen)+Aβ1–16+Cu(II)], respectively providing explicit evidence of the formation of Pt-Cu bimetallic complexes. By the reaction of [Cu(II)–Aβ1–16] with $PtCl_2$(phen) at higher pH (pH 7.4) only mono-platination adduct [Pt(phen)+Aβ1–16] was observed on the mass spectra [114], bis-platination adduct was suppressed by Cu^{2+} coordination under neutral conditions. To further analyze

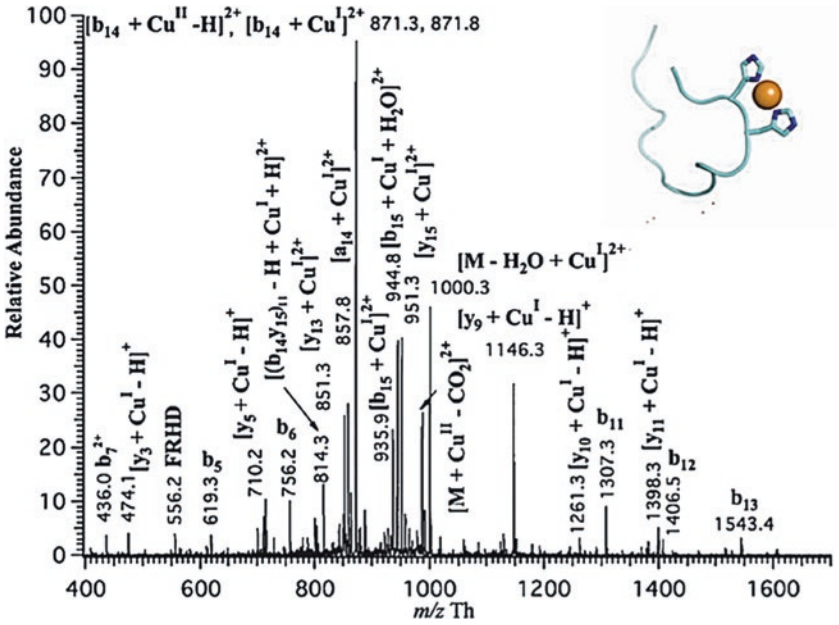

Fig. 23.5 The MS/MS spectrum of *m/z* 1009.4 parent ion corresponding to the copper-Aβ16 complex obtained with a Cu electrode at 30% of collision energy and possible mode of Cu^{+} coordinating to Aβ16 schematically drawn by software Pymol[26]. Reprinted from Metallomics (2010), Vol. 2, pp. 474–479, with permission from Copyright Clearance center

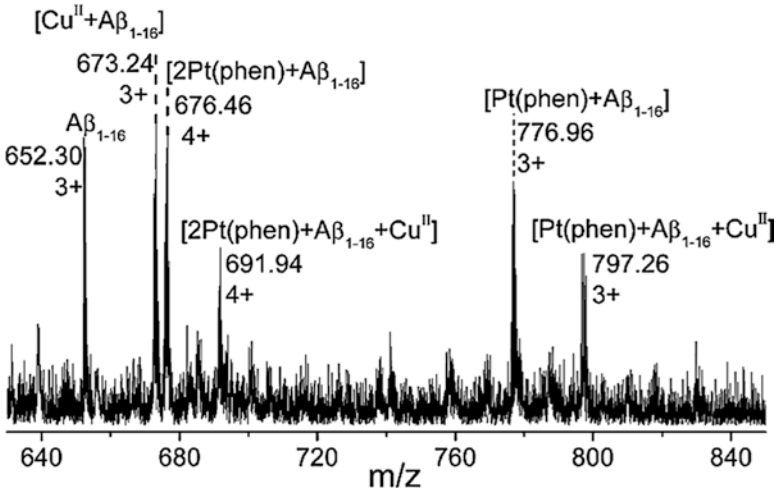

Fig. 23.6 ESI MS specta of Aβ 1-16 peptide with $PtCl_2$(phen) in the presence of copper ions in DMSO–H_2O (v/v = 1/2) at 298 K, pH 5.0 after 6 hours. Reprinted from Metallomics (2013), Vol. 5, pp., 879–887, with permission from Copyright Clearance center

the reaction of $PtCl_2$(phen) to [CuII-Aβ1–16] complex, the ternary complex [Pt(phen)+Aβ1–16+Cu(II)] was investigated by tandem MS/MS.

The triple charged ion at m/z = 797.26 was selected as parent ion and subjected to fragmentation in the collision cell of the MS. The CID mass spectra results are shown in Fig. 23.7, including b_n/y_n of Pt-free fragments, b_n*/y_n* of platinated or Cu^{2+} loaded fragments and b_n**/y_n** of [Pt(phen)+Cu]-loaded fragments, all annotated in the legend. The most abundant triple charged ion at m/z = 782.56 could be generated by the loss of acylamino or carboxyl group (44.10 Da) from the parent ion. The coordination of two metal ions stabilizes the Aβ1–16 fragmentation in the MS/MS spectra. In the b/y ions series of ternary complex, the dimetallic fragments y_4**–y_5**, $y_{12}**$ and $y_{14}**$–$y_{15}**$ were observed (Fig. 23.7). The smallest y_4** fragment suggests that the sequence of His^{13}-Lys^{16} is involved in the coordination of both $[Pt(phen)]^{2+}$ and Cu^{2+}. The y_4* and y_5* ions, carrying $[Pt(phen)]^{2+}$ unit, provide evidence of platinated sites on His^{13}-Lys^{16} peptide sequence. The smallest $b_{13}**$ indicates that the Asp^{1}-His^{13} peptide sequence contains the bimetallic coordination and the presence of b_{10}–b_{12} and y_2 metal-free ions, confirmed that His^{13} is the coordination site for Cu^{2+}. These results showed that $PtCl_2$(phen) binds to the metal coordination sites in Aβ(1–16) peptide, and the platination may alters the binding capacity of Cu(II). The phenanthroline ligand remains coordinated to the platinum and the ternary complex [Pt(phen)+Aβ+Cu(II)] was detected in the ESI mass spectra.

In previous studies Drochioiu et al. studied the interaction and stoichiometry of synthetic amyloid-β(1–40) peptide toward simple and paired metal ions investigated by electrospray ion trap mass spectrometry (ESI-MS) and showed that

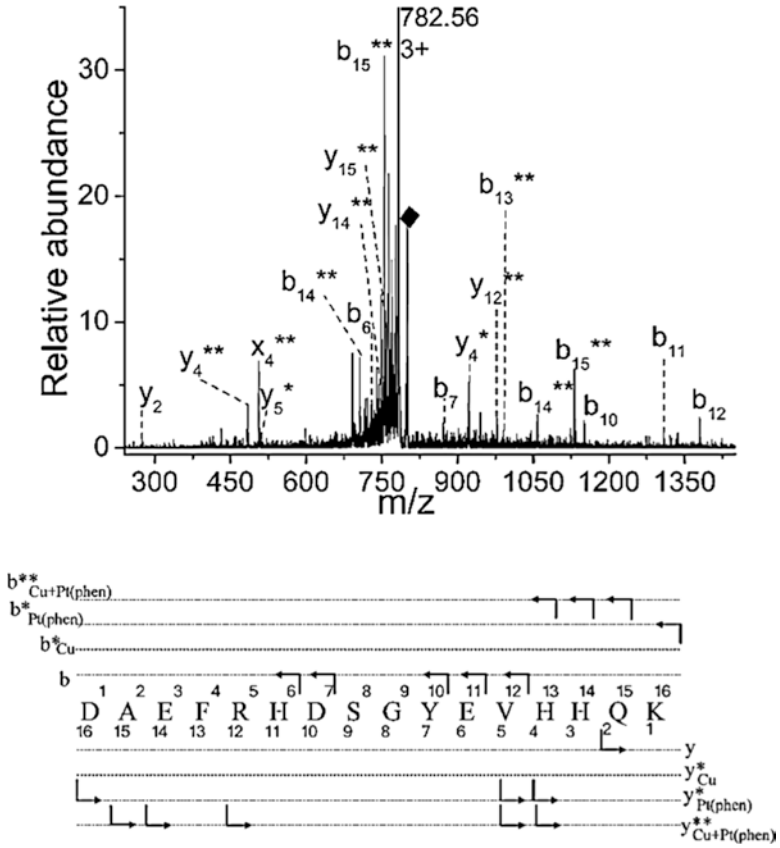

Fig. 23.7 The MS/MS spectrum of *m/z* 797.26 parent ion corresponding to the [Pt(phen)+Aβ$_{1-16}$+Cu^{II}] complex and the fragmentation scheme of the complex based on MS/MS spectrum (bottom). Reprinted from Metallomics (2013), Vol. 5, pp., 879–887, with permission from Copyright Clearance center

pH-dependent metal binding may induce conformational changes on the amyloid peptide [38, 39]. Thus, at a higher pH value, complexes containing more than one metal ion bound to the amyloid peptide were identified in the ESI-MS spectra. In addition, metal ions proved to enhance Aβ oligomerization. Metal–metal interactions during the binding process can be studied by ESI-MS as well. In this case, samples containing two different metal ions are allowed to compete for binding to Aβ peptide. For instance, following copper–silver interaction to Aβ, in the mass spectra was observed an increase in bound copper while the silver-complex showed less noncovalent affinity. In addition, new signals assigned to Cu+Ag-peptide complex ([M+Cu+Ag+3H]$^{6+}$ ion) were identified. Thus, preferable binding of amyloid peptides to metal ions was confirmed. However, it is required to take into account the presence of free amino and carboxylate group beside the two histidine residues from the amyloid peptide structure, in order to explain the production of such a large number of complexes [38].

23.3 Conclusion

The gentleness of the ESI or MALDI processes allows intact metal-peptide/protein complexes to be directly detected with high sensitivity and high resolution by mass spectrometry. Therefore, we highlighted here the feasibility of MS techniques to investigate both Aβ peptides involved in Alzheimer's disease and their complexes with heavy metal ions. The pH dependent stability of peptide-metal ion complexes can be followed easily by MS. Evidence from the literature suggests that the MS data for these weakly bound systems reflect, to some extent, the nature of the interaction found in the liquid phase, trying to mimic body fluids. There is a complex pattern of metal–metal competition for Aβ(1–16 or 1–40) binding sites, which depend essentially on the involved metal ions, their concentration, and pH changes as revealed by mass spectrometric studies and not only.

In conclusion, with the help of ESI MS technique the competition between different metal ions toward amyloid peptide can be analysed. Also, the interaction between metal-amyloid complexes and different chelation compound can be also investigated. Regarding the MALDI method, once the right matrix for the analysis, the desired noncovalent interaction can be detected easily. Both MS approaches are able to perform MS/MS studies that allow us to obtain more information about the specific active sites of the studied probes.

Acknowledgements Funding from the Romanian Government (UEFISCDI Bucharest, PN-III-P4-ID-PCE-2016-0376, Contract 56/2017) is gratefully acknowledged by the authors.

References

1. Ong, S. E., & Mann, M. (2005). Mass spectrometry-based proteomics turns quantitative. *Nature Chemical Biology, 1*(5), 252–262.
2. Aebersold, R., & Mann, M. (2003). Mass spectrometry-based proteomics. *Nature, 422*(6928), 198–207.
3. Pan, C., Xu, S., Zhou, H., Fu, Y., Ye, M., & Zou, H. (2007). Recent developments in methods and technology for analysis of biological samples by MALDI-TOF-MS. *Analytical and Bioanalytical Chemistry, 387*(1), 193–204.
4. Mann, M., & Talbo, G. (1996). Developments in matrix-assisted laser desorption/ionization peptide mass spectrometry. *Current Opinion in Biotechnology, 7*(1), 11–19.
5. Salih, B., Masselon, C., & Zenobi, R. (1998). Matrix-assisted laser desorption/ionization mass spectrometry of noncovalent protein–transition metal ion complexes. *Journal of Mass Spectrometry, 33*(10), 994–1002.
6. Keith-Roach, M. J. (2010). A review of recent trends in electrospray ionisation–mass spectrometry for the analysis of metal–organic ligand complexes. *Analytica Chimica Acta, 678*(2), 140–148.
7. Carlton Jr., D. D., & Schug, K. A. (2011). A review on the interrogation of peptide–metal interactions using electrospray ionization-mass spectrometry. *Analytica Chimica Acta, 686*(1–2), 19–39.
8. Kepp, K. P. (2012). Bioinorganic chemistry of Alzheimer's disease. *Chemical Reviews, 112*(10), 5193–5239.
9. Jellinger, K. A. (2010). Basic mechanisms of neurodegeneration: A critical update. *Journal of Cellular and Molecular Medicine, 14*, 457–487.
10. Kepp, K. P. (2017). Alzheimer's disease: How metal ions define β-amyloid function. *Coordination Chemistry Reviews, 351*(15), 127–159.
11. Iwatsubo, T., Saido, T. C., Mann, D. M., Lee, V. M., & Trojanowski, J. Q. (1996). Full-length amyloid-beta (1–42(43)) and amino-terminally modified and truncated amyloid-beta 42(43) deposit in diffuse plaques. *The American Journal of Pathology, 149*(6), 1823–1830.
12. Akiyama, H. (2000). Inflammation and Alzheimer's disease. *Neurobiology of Aging, 21*(3), 383–421.
13. Heneka, M. T., Carson, M. J., Khoury, J. E., Landreth, G. E., Brosseron, F., Feinstein, D. L., et al. (2015). Neuroinflammation in Alzheimer's disease. *Lancet Neurology, 14*(4), 388–405.
14. Singhrao, S. K., Harding, A., Simmons, T., Robinson, S., Kesavalu, L., & Crean, S. (2014). Oral inflammation, tooth loss, risk factors, and association with progression of Alzheimer's disease. *Journal of Alzheimer's Disease, 42*(3), 723–737.
15. Calvano, C. D., Monopoli, A., Cataldi, T. R., & Palmisano, F. (2018). MALDI matrices for low molecular weight compounds: An endless story? *Analytical and Bioanalytical Chemistry, 410*, 4015–4038.
16. Glenner, G. G., & Wong, C. W. (1984). Alzheimer's disease: Initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochemical and Biophysical Research Communications, 120*(3), 885–890.
17. Wolfe, M. S. (2012). Processive proteolysis by γ-secretase and the mechanism of Alzheimer's disease. *Biological Chemistry, 393*(9), 899–905.
18. Heneka, M. T., Golenbock, D. T., & Latz, E. (2015). Innate immunity in Alzheimer's disease. *Nature Immunology, 16*(3), 229–236.
19. Selkoe, D. J. (2006). Toward a comprehensive theory for Alzheimer's disease hypothesis: Alzheimer's Disease Is Caused by the Cerebral Accumulation and Cytotoxicity of Amyloid β-Protein. *Annals of the New York Academy of Sciences, 924*(1), 17–25.
20. Petkova, A. T., Ishii, Y., Balbach, J. J., Antzutkin, O. N., Leapman, R. D., Delaglio, F., et al. (2002). A structural model for Alzheimer's – Amyloid fibrils based on experimental constraints from solid state NMR. *Proceedings of the National Academy of Sciences of the United States of America, 99*(26), 16742–16747.
21. Mattson, M. P. (2004). Pathways towards and away from Alzheimer's disease. *Nature, 430*(7000), 631–639.
22. Knowles, T. P. J., Vendruscolo, M., & Dobson, C. M. (2014). The amyloid state and its association with protein misfolding diseases. *Nature Reviews. Molecular Cell Biology, 15*(6), 384–396.
23. Pike, C., Burdick, D., Walencewicz, A., Glabe, C., & Cotman, C. (1993). Neurodegeneration induced by beta-amyloid peptides in vitro: The role of peptide assembly state. *The Journal of Neuroscience, 13*(4), 1676–1687.
24. Davis, J. B. (1996). Oxidative mechanisms in β-amyloid cytotoxicity. *Neurodegeneration, 5*, 441–444.
25. Kayed, R., & Lasagna-Reeves, C. A. (2013). Molecular mechanisms of amyloid oligomers toxicity. *Journal of Alzheimer's Disease, 33*(1), S67–S78.
26. Smith, D. G., Cappai, R., & Barnham, K. J. (2007). The redox chemistry of the Alzheimer's disease amyloid-β peptide. *Biochimica et Biophysica Acta-Biomembranes, 1768*(8), 1976–1990.
27. Goodman, L. (1953). Alzheimer's disease: A clinico-pathologic analysis of twenty-three cases with a theory on pathogenesis. *The Journal of Nervous and Mental Disease, 118*, 97–130.
28. Lau, T. L., Gehman, J. D., Wade, J. D., Masters, C. L., Barnham, K. J., & Separovic, F. (2007). Clioquinol modulation of Aβ(1–42) interaction with phospholipid bilayers and metals. *Biochimica et Biophysica Acta-Biomembranes, 1768*, 3135–3144.
29. White, A. R., Barnham, K. J., Huang, X., Voltakis, I., Beyreuther, K., Masters, C. L., et al. (2004). Iron inhibits neurotoxicity induced by trace copper and biological reductants. *Journal of Biological Inorganic Chemistry, 9*, 269–280.
30. Adlard, P. A., & Bush, A. I. (2006). Metals and Alzheimer's disease. *Journal of Alzheimer's Disease, 10*, 145–163.
31. Lahiri, D. K., Chen, D. M., Lahiri, P., Bondy, S., & Greig, N. H. (2005). Amyloid, cholinesterase, melatonin, and metals and their roles in aging and neurodegenerative diseases. *Annals of the New York Academy of Sciences, 1056*, 430–449.
32. Lee, J. Y., Cole, T. B., Palmiter, R. D., Suh, S. W., & Koh, J. Y. (2002). Contribution by synaptic zinc to the gender-disparate plaque formation in human Swedish mutant APP transgenic mice. *Proceedings of the National Academy of Sciences of the United States of America, 99*(11), 7705–7710.
33. Atwood, C. S., Moir, R. D., Huang, X., Scarpa, R. K., Bacarra, N. M. E., Romano, D. M., et al. (1998). Dramatic aggregation of Alzheimer Aβ by Cu(II) is induced by conditions representing physiological acidosis. *The Journal of Biological Chemistry, 273*, 12817–12826.
34. Schlosser, G., Stefanescu, R., Przybylski, M., Murariu, M., Hudecz, F., & Drochioiu, G. (2007). Copper-induced oligomerization of peptides: A model study. *European Journal of Mass Spectrometry, 13*(5), 331–337.
35. Drago, D., Bettella, M., Bolognin, S., Cendron, L., Scancar, J., Milacic, R., et al. (2008). Potential pathogenic role of β-amyloid1–42–aluminum complex in Alzheimer's disease. *The International Journal of Biochemistry & Cell Biology, 40*(4), 731–746.
36. Chen, Y. R., Huang, H. B., Chyan, C. L., Shiao, M. S., Lin, T. H., & Chen, Y. C. (2006). The effect of Aß conformation on the metal affinity and aggregation mechanism studied by circular dichroism spectroscopy. *Journal of Biochemistry, 139*, 733–740.
37. Gaggelli, E., Grzonka, Z., Kozlowski, H., Migliorini, C., Molteni, E., Valensin, D., et al. (2008). Structural features of the Cu(II) complex with the rat Aβ(1–28) fragment. *Chemical Communications, 3*, 341–343.

38. Drochioiu, G., Manea, M., Dragusanu, M., Murariu, M., Dragan, E. S., Petre, B. A., et al. (2009). Interaction of β-amyloid(1–40) peptide with pairs of metal ions: An electrospray ion trap mass spectrometric model study. *Biophysical Chemistry, 144*(1–2), 9–20.
39. Drochioiu, G. (2009). An electrospray ionization mass spectrometric study of iron binding to amyloid-β peptides. *European Journal of Mass Spectrometry, 15*(5), 651–659.
40. Zirah, S., Stefanescu, R., Manea, M., Tian, X., Cecal, R., Kozin, S. A., et al. (2004). Zinc binding agonist effect on the recognition of the β-amyloid (4–10) epitope by anti-β-amyloid antibodies. *Biochemical and Biophysical Research Communications, 321*(2), 324–328.
41. Hare, D. J., Rembach, A., & Roberts, B. R. (2016). The emerging role of metalloproteomics in Alzheimer's disease research. *Systems Biology of Alzheimer's Disease, 1303*, 379–389.
42. Di Natale, G., Bellia, F., Sciacca, M. F., Campagna, T., & Pappalardo, G. (2018). Tau-peptide fragments and their copper (II) complexes: Effects on amyloid-β aggregation. *Inorganica Chimica Acta, 472*, 82–92.
43. Faller, P., & Hureau, C. (2009). Bioinorganic chemistry of copper and zinc ions coordinated to amyloid-β peptide. *Dalton Transactions, 7*, 1080–1094.
44. Beit-Yannai, E., Kohen, R., Horowitz, M., Trembovler, V., & Shohami, E. (1997). Changes of biological reducing activity in rat brain following closed head injury: A cyclic voltammetry study in normal and heat-acclimated rats. *Journal of Cerebral Blood Flow and Metabolism, 17*(3), 273–279.
45. Huang, W. J., Zhang, X., & Chen, W. W. (2016). Role of oxidative stress in Alzheimer's disease. *Biomedical Reports, 4*(5), 519–522.
46. Dikalov, S. I., Vitek, M. P., & Mason, R. P. (2004). Cupric–amyloid β peptide complex stimulates oxidation of ascorbate and generation of hydroxyl radical. *Free Radical Biology & Medicine, 36*(3), 340–347.
47. Kozlowski, H., Luczkowski, M., Remelli, M., & Valensin, D. (2012). Copper, zinc and iron in neurodegenerative diseases (Alzheimer's, Parkinson's and prion diseases). *Coordination Chemistry Reviews, 256*(19–20), 2129–2141.
48. Kong, X., Zhao, Z., Lei, X., Zhang, B., Dai, D., & Jiang, L. (2015). Interaction of metal ions with the his13-his14 sequence relevant to Alzheimer's disease. *The Journal of Physical Chemistry. A, 119*(14), 3528–3534.
49. Ryu, J., Girigoswami, K., Ha, C., Ku, S. H., & Park, C. B. (2008). Influence of multiple metal ions on β-amyloid aggregation and dissociation on a solid surface. *The Biochemist, 47*(19), 5328–5335.
50. Gozes, I. (2001). Neuroprotective peptide drug delivery and development: Potential new therapeutics. *Trends in Neurosciences, 24*(12), 700–705.
51. Barnham, K. J., & Bush, A. I. (2008). Metals in Alzheimer's and Parkinson's diseases. *Current Opinion in Chemical Biology, 12*(2), 222–228.
52. Gozes, I. (2011). NAP (davunetide) provides functional and structural neuroprotection. *Current Pharmaceutical Design, 17*(10), 1040–1044.
53. Gozes, I., Morimoto, B. H., Tiong, J., Fox, A., Sutherland, K., Dangoor, D., et al. (2006). NAP: research and development of a peptide derived from activity-dependent neuroprotective protein (ADNP). *CNS Drug Reviews, 11*(4), 353–368.
54. Akiyama, H., Barger, S., Barnum, S., Bradt, B., Bauer, J., Cole, G. M., et al. (2000). Inflammation and Alzheimer's disease. *Neurobiology of Aging, 21*(3), 383–421.
55. Ashur-Fabian, O., Segal-Ruder, Y., Skutelsky, E., Brenneman, D. E., Steingart, R. A., Giladi, E., et al. (2003). The neuroprotective peptide NAP inhibits the aggregation of the beta-amyloid peptide. *Peptides, 24*(9), 1413–1423.
56. Gozes, I., Sragovich, S., Schirer, Y., & Idan-Feldman, A. (2016). D-SAL and NAP: Two peptides sharing a SIP domain. *Journal of Molecular Neuroscience, 59*(2), 220–231.
57. Postu, P. A., Noumedem, J. A. K., Cioanca, O., Hancianu, M., Mihasan, M., Ciorpac, M., et al. (2017). Lactuca capensis reverses memory deficits in Aβ1-42-induced an animal model of Alzheimer's disease. *Journal of Cellular and Molecular Medicine, 22*(1), 111–122.
58. Hancianu, M., Cioanca, O., Mihasan, M., & Hritcu, L. (2013). Neuroprotective effects of inhaled lavender oil on scopolamine-induced dementia via anti-oxidative activities in rats. *Phytomedicine, 20*(5), 446–452.
59. Ford, M. J., Cantone, J. L., Polson, C., Toyn, J. H., Meredith, J. E., & Drexler, D. M. (2008). Qualitative and quantitative characterization of the amyloid β peptide (Aβ) population in biological matrices using an immunoprecipitation–LC/MS assay. *Journal of Neuroscience Methods, 168*(2), 465–474.
60. Grasso, G. (2010). The use of mass spectrometry to study amyloid-β peptides. *Mass Spectrometry Reviews, 30*(3), 347–365.
61. Zellner, M., Veitinger, M., & Umlauf, E. (2009). The role of proteomics in dementia and Alzheimer's disease. *Acta Neuropathologica, 118*(1), 181–195.
62. Portelius, E., Bogdanovic, N., Gustavsson, M. K., Volkmann, I., Brinkmalm, G., Zetterberg, H., et al. (2010). Mass spectrometric characterization of brain amyloid beta isoform signatures in familial and sporadic Alzheimer's disease. *Acta Neuropathologica, 120*(2), 185–193.
63. Zatta, P., Drago, D., Bolognin, S., & Sensi, S. L. (2009). Alzheimer's disease, metal ions and metal homeostatic therapy. *Trends in Pharmacological Sciences, 30*(7), 346–355.
64. Attanasio, F., Convertino, M., Magno, A., Caflisch, A., Corazza, A., Haridas, H., et al. (2013). Carnosine inhibits Aβ42 aggregation by perturbing the H-bond network in and around the central hydrophobic cluster. *Chembiochem, 14*(5), 583–592.
65. Hider, R. C., Ma, Y., Molina-Holgado, F., Gaeta, A., & Roy, S. (2008). Iron chelation as a potential therapy for neurodegenerative disease. *Biochemical Society Transactions, 36*(6), 1304–1308.
66. Roberds, S. L., Anderson, J., Basi, G., Bienkowski, M. J., Branstetter, D. G., Chen, K. S., et al. (2001). BACE knockout mice are healthy despite lacking the primary beta-secretase activity in brain: Implications for Alzheimer's disease therapeutics. *Human Molecular Genetics, 10*, 1317–1324.
67. Cuajungco, M. P., Frederickson, C. J., & Bush, A. I. (2005). Amyloid-beta metal interaction and metal chelation. *Sub-Cellular Biochemistry, 38*, 235–254.
68. Hegde, M. L., Bharathi, P., Suram, A., Venugopal, C., Jagannathan, R., Poddar, P., et al. (2009). Challenges associated with metal chelation therapy in Alzheimer's disease. *Journal of Alzheimer's Disease, 17*(3), 457–468.
69. Tahmasebinia, F., & Emadi, S. (2017). Effect of metal chelators on the aggregation of beta-amyloid peptides in the presence of copper and iron. *Biometals, 30*(2), 285–293.
70. Choi, J. S., Braymer, J. J., Nanga, R. P., Ramamoorthy, A., & Lim, M. H. (2010). Design of small molecules that target metal-Abeta species and regulate metal-induced Abeta aggregation and neurotoxicity. *Proceedings of the National Academy of Sciences of the United States of America, 107*(51), 21990–21995.
71. Ritchie, C. W., Bush, A. I., Mackinnon, A., Macfarlane, S., Mastwyk, M., MacGregor, L., et al. (2003). Metal-protein attenuation with iodochlorhydroxyquin (clioquinol) targeting Abeta amyloid deposition and toxicity in Alzheimer disease: A pilot phase 2 clinical trial. *Archives of Neurology, 60*(12), 1685–1691.
72. Chang, S. Y., Zheng, N. Y., Chen, C. S., Chen, C. D., Chen, Y. Y., & Wang, C. C. (2007). Analysis of peptides and proteins affinity-bound to iron oxide nanoparticles by MALDI MS.

Journal of the American Society for Mass Spectrometry, 18(5), 910–918.
73. Dashtiev, M., Wäfler, E., Röhlin, U., Gorshkov, M., Hillenkamp, F., & Zenobi, R. (2007). Positive and negative analyte ion yield in matrix-assisted laser desorption/ionization. *International Journal of Mass Spectrometry, 268*(2–3), 122–130.
74. Sandoval, W. (2014). Matrix-assisted laser desorption/ionization time-of-flight mass analysis of peptides. *Current Protocols in Protein Science, 16*(2), 1–16.2.11.
75. Fitzgerald, M. C., Parr, G. R., & Smith, L. M. (1993). Basic matrixes for the matrix-assisted laser desorption/ionization mass spectrometry of proteins and oligonucleotides. *Analytical Chemistry, 65*(22), 3204–3211.
76. Beavis, R. C., Chaudhary, T., & Chait, B. T. (1992). α-Cyano-4-hydroxycinnamic acid as a matrix for matrixassisted laser desorption mass spectromtry. *Organic Mass Spectrometry, 27*(2), 156–158.
77. Strupat, K., Karas, M., & Hillenkamp, F. (1991). 2,5-Dihydroxybenzoic acid: A new matrix for laser desorption—ionization mass spectrometry. *International Journal of Mass Spectrometry and Ion Processes, 111*, 89–102.
78. Smolira, A., & Wessely-Szponder, J. (2014). Importance of the matrix and the matrix/sample ratio in MALDI-TOF-MS analysis of cathelicidins obtained from porcine neutrophils. *Biotechnology and Applied Biochemistry, 175*(4), 2050–2065.
79. Lin, Z., & Cai, Z. (2018). Negative ion laser desorption/ionization time-of-flight mass spectrometric analysis of small molecules by using nanostructured substrate as matrices. *Mass Spectrometry Reviews, 37*(5), 681–696.
80. Stellato, F., Menestrina, G., Serra, M. D., Potrich, C., Tomazzolli, R., Meyer-Klaucke, W., et al. (2006). Metal binding in amyloid β-peptides shows intra- and inter-peptide coordination modes. *European Biophysics Journal, 35*(4), 340–351.
81. Miller, Y., Ma, B., & Nussinov, R. (2012). Metal binding sites in amyloid oligomers: Complexes and mechanisms. *Coordination Chemistry Reviews, 256*(19–20), 2245–2252.
82. Petre, B.-A., Youhnovski, N., Lukkari, J., Weber, R., & Przybylski, M. (2005). Structural characterisation of tyrosine-nitrated peptides by ultraviolet and infrared matrix-assisted laser desorption/ionisation fourier transform ion cyclotron resonance mass spectrometry. *European Journal of Mass Spectrometry, 11*(5), 513–518.
83. Loo, J. A. (2000). Electrospray ionization mass spectrometry: A technology for studying noncovalent macromolecular complexes. *International Journal of Mass Spectrometry, 200*(1–3), 175–186.
84. Mädler, S., Erba, E. B., & Zenobi, R. (2012). *MALDI-ToF mass spectrometry for studying noncovalent complexes of biomolecules. Applications of MALDI-TOF spectroscopy* (pp. 1–36).
85. Glocker, M. O., Bauer, S. H. J., Kast, J., Volz, J., & Przybylski, M. (1996). Characterization of specific noncovalent protein complexes by UV matrix-assisted laser desorption ionization mass spectrometry. *Journal of Mass Spectrometry, 31*(11), 1221–1227.
86. Lehmann, E., Zenobi, R., & Vetter, S. (1999). Matrix-assisted laser desorption/ionization mass spectra reflect solution-phase zinc finger peptide complexation. *Journal of the American Society for Mass Spectrometry, 10*(1), 27–34.
87. Chen, X., Drogaris, P., & Bern, M. (2010). Identification of tandem mass spectra of mixtures of isomeric peptides. *Journal of Proteome Research, 9*(6), 3270–3279.
88. Ciobanu, C. I., Stefanescu, R., Niculaua, M., Teslaru, T., Gradinaru, R., & Drochioiu, G. (2016). Mass spectrometric evidence for iron binding to the neuroprotective peptide NAP and its Cys5 mutant. *European Journal of Mass Spectrometry, 22*(2), 97–104.
89. Lupaescu, A. V., Jureschi, M., Ciobanu, C. I., Ion, L., Zbancioc, G., Petre, B. A., et al. (2018). FTIR and MS evidence for heavy metal binding to anti-amyloidal NAP-like peptides. *International Journal of Peptide Research and Therapeutics, 25*, 303–309.
90. Di Marco, V. B., & Bombi, G. G. (2006). Electrospray mass spectrometry (ESI-MS) in the study of metal-ligand solution equilibria. *Mass Spectrometry Reviews, 25*(3), 347–379.
91. Ho, C. S., Lam, C. W. K., Chan, M. H. M., Cheung, R. C. K., Law, L. K., Lit, L. C. W., et al. (2003). Electrospray ionisation mass spectrometry: Principles and clinical applications. *Clinical Biochemist Reviews, 24*(1), 3–12.
92. Zirah, S., Rebuffat, S., Kozin, S. A., Debey, P., Fournier, F., Lesagec, D., et al. (2003). Zinc binding properties of the amyloid fragment Aβ(1–16) studied by electrospray-ionization mass spectrometry. *International Journal of Mass Spectrometry, 228*(2–3), 999–1016.
93. Murariu, M., Dragan, E. S., & Drochioiu, G. (2007). Synthesis and mass spectrometric characterization of a metal-affinity decapeptide: Copper-induced conformational changes. *Biomacromolecules, 8*(12), 3836–3841.
94. Loo, J. A. (1997). Studying noncovalent protein complexes by electrospray ionization mass spectrometry. *Mass Spectrometry Reviews, 16*(1), 1–23.
95. McNaught, A. D., & McNaught, A. D. (1997). *Compendium of chemical terminology* (Vol. 1669, 2nd ed.). Oxford: Blackwell Science.
96. Roepstorff, P., & Fohlman, J. (1984). Proposal for a common nomenclature for sequence ions in mass spectra of peptides. *Biomedical Mass Spectrometry, 11*(11), 601.
97. Wells, J. M., & McLuckey, S. A. (2005). Collision-induced dissociation (CID) of peptides and proteins. *Methods in Enzymology, 402*, 148–185.
98. Sleno, L., & Volmer, D. A. (2004). Ion activation methods for tandem mass spectrometry. *Journal of Mass Spectrometry, 39*(10), 1091–1112.
99. Cooper, H. J., Håkansson, K., & Marshall, A. G. (2005). The role of electron capture dissociation in biomolecular analysis. *Mass Spectrometry Reviews, 24*(2), 201–222.
100. Syka, J. E., Coon, J. J., Schroeder, M. J., Shabanowitz, J., & Hunt, D. F. (2004). Peptide and protein sequence analysis by electron transfer dissociation mass spectrometry. *Proceedings of the National Academy of Sciences of the United States of America, 101*(26), 9528–9533.
101. Morgan, J. W., Hettick, J. M., & Russell, D. H. (2005). Peptide sequencing by MALDI 193-nm photodissociation TOF MS. *Methods in Enzymology, 402*, 186–209.
102. Budnik, B. A., Haselmann, K. F., & Zubarev, R. A. (2001). Electron detachment dissociation of peptide di-anions: An electron–hole recombination phenomenon. *Chemical Physics Letters, 342*(3–4), 299–302.
103. Coon, J. J., Shabanowitz, J., Hunt, D. F., & Syka, J. E. (2005). Electron transfer dissociation of peptide anions. *Journal of the American Society for Mass Spectrometry, 16*(6), 880–882.
104. Rebrin, I., Bregere, C., Gallaher, T. K., & Sohal, R. S. (2008). Detection and characterization of peroxynitrite-induced modifications of tyrosine, tryptophan, and methionine residues by tandem mass spectrometry. *Methods in Enzymology, 441*, 283–294.
105. Savitski, M. M., Lemeer, S., Boesche, M., Lang, M., Mathieson, T., Bantscheff, M., et al. (2010). Confident phosphorylation site localization using the Mascot Delta Score. *Molecular & Cellular Proteomics, 10*(2), M110.003830.
106. Glover, M. S., Dilger, J. M., Acton, M. D., Arnold, R. J., Radivojac, P., & Clemmer, D. E. (2016). Examining the influence of phosphorylation on peptide ion structure by ion mobility spectrometry-mass spectrometry. *Journal of the American Society for Mass Spectrometry, 27*(5), 786–794.
107. Lu, Y., Prudent, M., Qiao, L., Mendez Manuel, A., & Girault Hubert, H. (2010). Copper(I) and copper(II) binding to b-amyloid

16 (Ab16) studied by electrospray ionization mass spectrometry. *Metallomics, 2*(7), 474–479.
108. Prudent, M., & Girault, H. H. (2008). On-line electrogeneration of copper-peptide complexes in microspray mass spectrometry. *Journal of the American Society for Mass Spectrometry, 19*(4), 560–568.
109. Himes, R. A., Park, G. Y., Siluvai, G. S., Blackburn, N. J., & Karlin, K. D. (2008). Structural studies of copper(I) complexes of amyloid-β peptide fragments: Formation of two-coordinate bis(histidine) complexes. *Angewandte Chemie International Edition In English, 47*(47), 9084–9087.
110. Shearer, J., & Szalai, V. A. (2008). The amyloid-β peptide of Alzheimer's disease binds cuiin a linear bis-his coordination environment: Insight into a possible neuroprotective mechanism for the amyloid-β peptide. *Journal of the American Chemical Society, 130*(52), 17826–17835.
111. Barnham, K. J., Kenche, V. B., Ciccotosto, G. D., Smith, D. P., Tew, D. J., Liu, X., et al. (2008). Platinum-based inhibitors of amyloid-β as therapeutic agents for Alzheimer's disease. *Proceedings of the National Academy of Sciences of the United States of America, 105*(19), 6813–6818.
112. Kumar, A., Moody, L., Olaivar, J. F., Lewis, N. A., Khade, R. L., Holder, A. A., et al. (2010). Inhibition of Aβ42 peptide aggregation by a binuclear ruthenium(II)−platinum(II) complex: Potential for multimetal organometallics as anti-amyloid agents. *ACS Chemical Neuroscience, 1*(10), 691–701.
113. Wang, X., Wang, X., Zhang, C., Jiao, Y., & Guo, Z. (2012). Inhibitory action of macrocyclic platiniferous chelators on metal-induced Aβ aggregation. *Chemical Science, 3*(4), 1304.
114. Ma, G., Wang, E., Wei, H., Wei, K., Zhu, P., & Liu, Y. (2013). PtCl2(phen) disrupts the metal ions binding to amyloid-β peptide. *Metallomics, 5*(7), 879–887.