

# Time- and pH-Dependent Copper Binding to A $\beta$ (1–16) Peptide: An Electrospray Ionization-Mass Spectrometric Approach

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**Abstract** An elevated concentration of copper ions in the brain of Alzheimer's disease patients has been reported in many studies and might be associated with an increased aggregation of  $\beta$ -amyloid (A $\beta$ ) peptides. In the present work, the interaction with copper ions of a model  $\beta$ -amyloid peptide, A $\beta$ (1–16), was investigated by electrospray ionization-mass spectrometry (ESI–MS) at two pH values, 7.4 and 6.6, as well as at various peptide: copper ion ratios in the first minutes after components mixing and time intervals. Our results indicated that copper ions specifically bound to A $\beta$ (1–16) peptide in solution and that the complex formation increased with time. Once formed in solution, Cu<sup>2+</sup>-A $\beta$ (1–16) complexes could easily be detected in the gas phase by ESI–MS. The pH shift from 7.4 to 6.6 only slightly influenced the Cu<sup>2+</sup> binding to A $\beta$ (1–16). No oligomerization of A $\beta$ (1–16) peptide was noticed in the first minutes of copper-peptide interaction.

**Keywords** ESI–MS ·  $\beta$ -Amyloid peptide · Copper-peptide complex · pH · Alzheimer's disease

## Introduction

There is increasing evidence that heavy metal ions are involved in the pathogenesis of neurodegenerative diseases, such as Alzheimer's disease (AD) (Zecca et al. 2004; Bush 2003; Barnham et al. 2004; Gaggelli et al. 2006; Gradinaru et al. 2011). The aggregation of  $\beta$ -amyloid peptides (A $\beta$ ) and subsequent neurotoxicity may be triggered by abnormal interactions with neocortical metal ions, especially copper, zinc, iron and aluminum (Bush 2003, 2013). Recent data suggest that the intracellular pool of A $\beta$  is regulated by the activity of endothelin-converting enzymes at the sites of production. (Pacheco-Quinto and Eckman 2013) However, the deregulation of copper seems to be intimately involved in the pathogenesis of AD (Eskici and Axelsen 2012; Damante et al. 2009). Copper is known to accelerate the aggregation of A $\beta$ (1–40) and A $\beta$ (1–42) peptides, the major components of A $\beta$  deposits (Bush and Tanzi 2008). Nevertheless, short and easy-to-handle peptides have been used to study the structures and mechanisms that are also relevant for the native and longer peptides involved in neurodegenerative diseases (Alies et al. 2013; Ali et al. 2006). Moreover, some previous ESI–MS studies have reported on the binding of Cu<sup>2+</sup> to A $\beta$ (1–16) at physiological pH and its effect on peptide conformation and oligomerisation (Ali et al. 2006). The copper binding sites are located in the N-terminal region of A $\beta$  peptides (Bush 2003; Syme et al. 2004; Minicozzi et al. 2008; Smith et al. 2006). Furthermore, the mode of copper binding to peptides seems to be highly pH dependent (Schlosser et al. 2007; Drochioiu et al. 2009; Murariu et al. 2007, 2010a). The most common methods used to investigate the interaction of metal ions with amyloidogenic peptides have already been reviewed (Faller et al. 2012; Grasso 2011). Yet, it is less known if copper-peptide

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complexes are formed in the gas phase as non-specific adducts or in solution.

We explore here the hypothesis that under in-solution conditions (pH, time of reaction, metal and peptide concentrations) various species of copper-peptide complexes may result, which are mostly conserved in the gas phase and can be detected by ESI-MS. Thus, the present work aims at investigating the formation of complexes between A $\beta$ (1–16) and copper ions in solution, both at pH 6.6 and pH 7.4.

## Experimental

### Materials

All reagents were of analytical grade or highest available purity. The solutions were prepared using MilliQ grade water (18 M $\Omega$  cm). The A $\beta$ (1–16) peptide (H-DAE-FRHDSGYEVHHQK-OH) was purchased from Bachem AG (Bubendorf, Switzerland).

### ESI-Ion Trap Mass Spectrometry

Electrospray ionization mass spectrometric analyses were carried out on a Bruker Daltonics Esquire 3000plus ion trap mass spectrometer (Bremen, Germany). Spectra were acquired in the 50–2,000 *m/z* range. Binding of metal ions to A $\beta$ (1–16) peptide was investigated by ESI-ion trap MS using 5 mM ammonium acetate, pH 7.4 or 6.6 as a solvent. Peptide concentration was 10  $\mu$ M and the peptide:copper ion ratios were 1:1, 1:2 and 1:10. A $\beta$ (1–16) and CuSO<sub>4</sub> were first dissolved in 5 mM ammonium acetate and mixed prior to MS analysis. Copper binding to A $\beta$ (1–16) peptide was followed over time, in the first minutes after mixing, while recording the mass spectra (no preincubation of the samples was performed). The experiments were carried out either under nearly physiological conditions (5 mM salt concentration, pH 7.4) or at pH 6.6, representing the pH value determined in the case of inflammatory processes.

### Circular Dichroism (CD) Spectroscopy

CD spectra were recorded on a Jasco J-715 spectropolarimeter at room temperature, in quartz cells of 0.05 cm path length, under constant nitrogen flush. The A $\beta$ (1–16) peptide was dissolved in 5 mM ammonium acetate, at a concentration of 0.2 mM, while the molar ratio Cu<sup>2+</sup>:A $\beta$ (1–16) was 2:1. The spectra were recorded after preincubating the samples for 1 and 7 min, respectively; the spectra were averages of six scans in the wavelength range 180–260 nm. Molar ellipticity was expressed as deg cm<sup>2</sup> dmol<sup>–1</sup>.

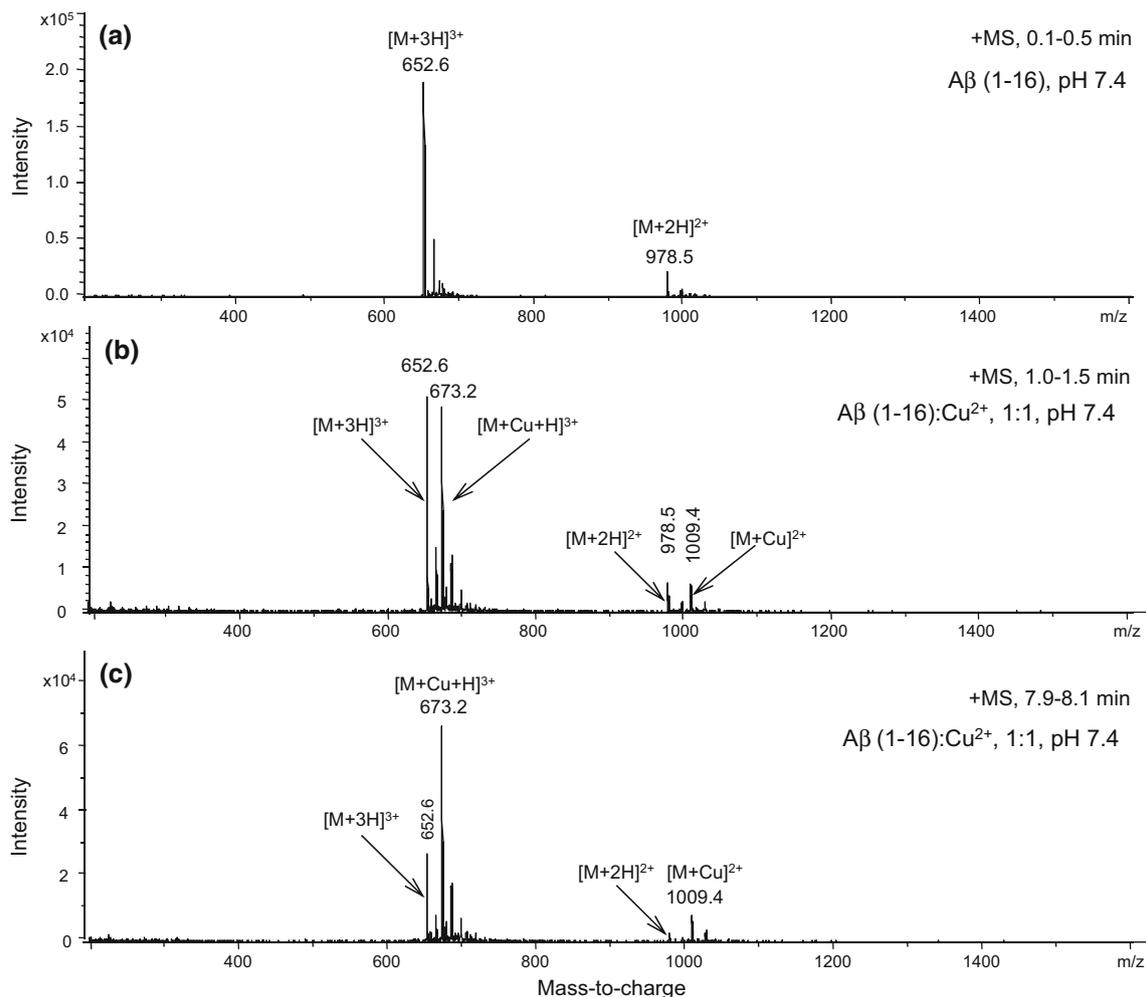
## Results

### Copper Binding to A $\beta$ (1–16) Peptide

Using ESI-MS, we found that the binding of Cu<sup>2+</sup> to A $\beta$ (1–16) peptide was significantly influenced by the molar ratio of peptide:copper ion and the time of peptide–metal ion contact, being only slightly influenced by the pH. However, more copper was bound to A $\beta$ (1–16) at pH 6.6 than at pH 7.4, although the complex formation was faster at pH 7.4. A $\beta$ (1–16) peptide, at a concentration of 10  $\mu$ M in 5 mM ammonium acetate, pH 7.4, displayed a peak in the ESI-mass spectrum at *m/z* 652.6 and another one at *m/z* 978.5 that were assigned to [M+3H]<sup>3+</sup> and [M+2H]<sup>2+</sup> ions (Fig. 1a). Under the same experimental conditions and upon adding Cu<sup>2+</sup>, the intensity of the peptide peak decreased to 26.8 % in the first 0.1–0.5 min after mixing the peptide with the copper ions. The average mass spectrum between 7.3 and 7.6 min showed a further decrease in the intensity of the peptide peak (15.3 %), whereas that of [M+Cu+H]<sup>3+</sup> ion was found to be 220.7 % higher than that of the peptide (Fig. 1c). Copper ions demonstrated a high affinity toward A $\beta$ (1–16), since [M+Cu+H]<sup>3+</sup> ion appeared immediately after mixing the two components and its intensity was high enough (96.1 % of peptide intensity). The formation of copper–peptide complex was also observed at *m/z* 1009.4, the peak being assigned to [M+Cu]<sup>2+</sup> ion.

A $\beta$ (1–16) peptide bound only one Cu<sup>2+</sup> at both pH 7.4 and 6.6, at a molar ratio of 1:1 peptide:Cu<sup>2+</sup>. At pH 6.6, for the time interval 0.2–0.7 min from mixing the components (Fig. 2a), the [M+Cu+H]<sup>3+</sup>: [M+3H]<sup>3+</sup> intensity ratio was found to be lower than that at pH 7.4 (57.1 vs 96.1 %). However, while the intensity of the peak corresponding to [M+Cu+H]<sup>3+</sup> ion was rather the same (6·10<sup>4</sup> at pH 6.6 vs 4.9·10<sup>4</sup> at pH 7.4), that of the peptide ion was much higher (10.5·10<sup>4</sup> at pH 6.6 vs 5.1·10<sup>4</sup> at pH 7.4), most probably due to the higher proton concentration of the solution and better ionization of the peptide under slightly acidic conditions. Over time (7.9–8.1 min, pH 6.6), the intensity of [M+Cu+H]<sup>3+</sup> ion doubled, while that of the peptide ion decreased three times (Fig. 2b). When compared with the results obtained at pH 7.4, both the [M+Cu+H]<sup>3+</sup> intensity and the [M+Cu+H]<sup>3+</sup>: [M+3H]<sup>3+</sup> intensity ratio were found to be higher (12.0·10<sup>4</sup> vs 6.4·10<sup>4</sup>; 3.43 vs 2.66).

When copper:A $\beta$ (1–16) ratio increased up to 10, the intensity of the peaks corresponding to the triply charged ions, [M+Cu+H]<sup>3+</sup>, suddenly increased as well; no trace of peptide ion was found at pH 7.4, while a low intensity peak was observed at *m/z* 652.7, being assigned to [M+3H]<sup>3+</sup> molecular ion. At pH 6.6 and about 0.5 min reaction time, the [M+3H]<sup>3+</sup>: [M+Cu+H]<sup>3+</sup>: [M+2Cu–H]<sup>3+</sup> intensity ratio was 1:8.9:2.8. In only 8 mins,



**Fig. 1** ESI-ion trap mass spectra of A $\beta$ (1–16) peptide (10  $\mu$ M peptide concentration), which show time-dependent formation of complexes with copper ions at pH 7.4 in 5 mM ammonium acetate solution

[M+3H]<sup>3+</sup> ion completely disappeared, [M+Cu+H]<sup>3+</sup> decreased dramatically, while [M+2Cu–H]<sup>3+</sup> became the main peak in the mass spectrum (Fig. 2d). Both at pH 7.4 and pH 6.6, the [M+2Cu–H]<sup>3+</sup> ion peak had the same intensity. Interestingly, [M+Cu+H]<sup>3+</sup> ion displayed at m/z 673.3 and pH 7.4 a peak as intense as that characteristic for the [M+3Cu–3H]<sup>3+</sup> ion at pH 6.6. Hence, more copper was bound to A $\beta$ (1–16) at pH 6.6 than at pH 7.4, although the complex formation was faster at pH 7.4.

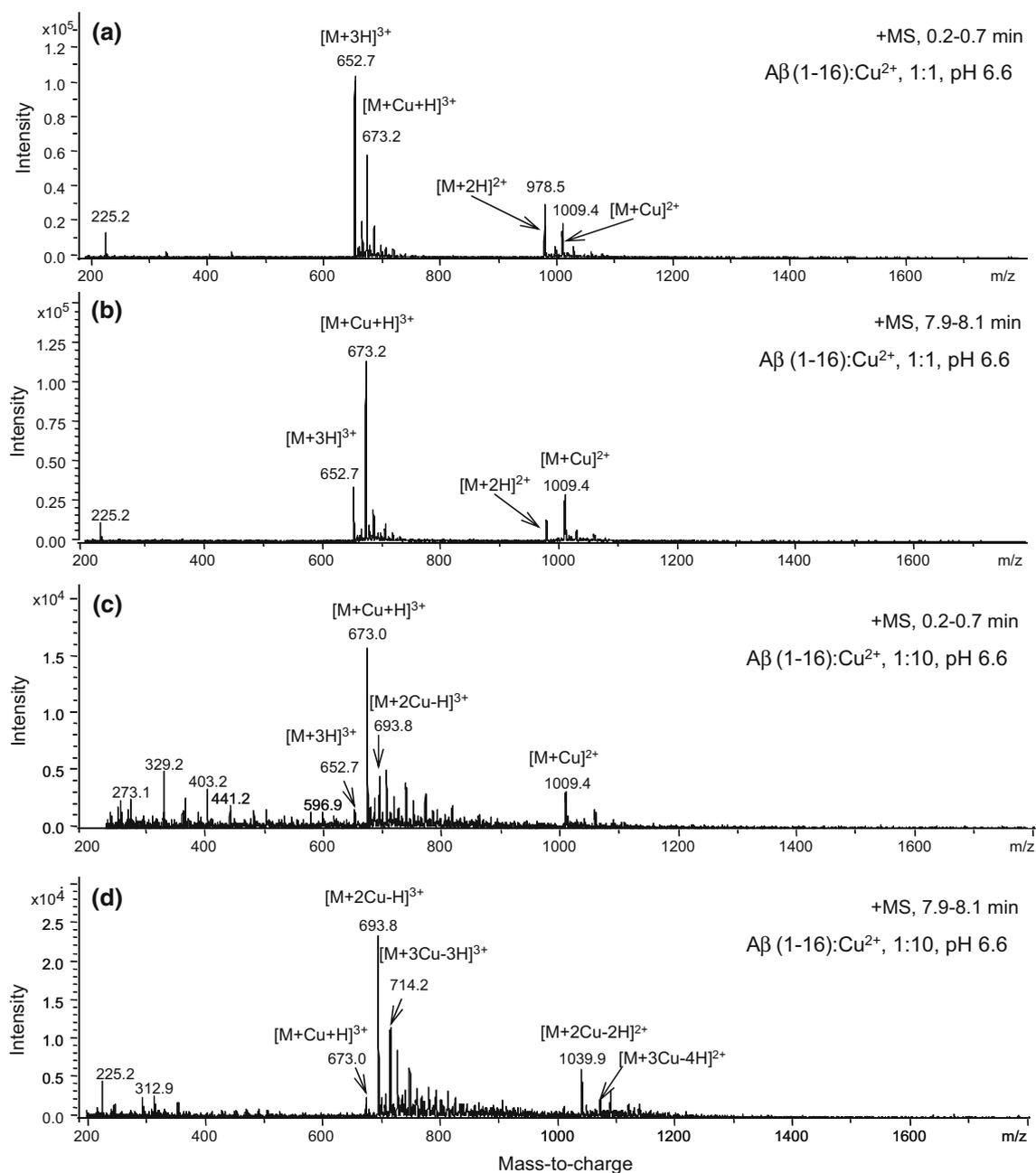
#### Copper Binding Stoichiometry

ESI–MS showed binding stoichiometries of up to 1:3 (pH 7.4) and 1:4 (pH 6.6) for the peptide–Cu<sup>2+</sup> complexes, when Cu<sup>2+</sup>:peptide molar ratio was 10:1 (Fig. 3). We assumed that in spite of the elevation of both peptide and copper ion concentrations during the ionization process than in the incubation buffer, the ratio between the copper ion and A $\beta$ (1–16) would be fixed even after evaporation of

the solvent as shown by other studies (Ali et al. 2006; Murariu 2013). Moreover, since there is an increase in the copper content of Cu<sup>2+</sup>–A $\beta$ (1–16) complexes from fractions with higher copper to peptide ratios, this would suggest that the metal was attached to the peptide prior to ESI–MS analysis. We demonstrate here that A $\beta$ (1–16) is able to bind up to three Cu<sup>2+</sup> ions at pH 7.4, and up to four Cu<sup>2+</sup> ions when the pH value decreased to 6.6.

#### CD Spectroscopy

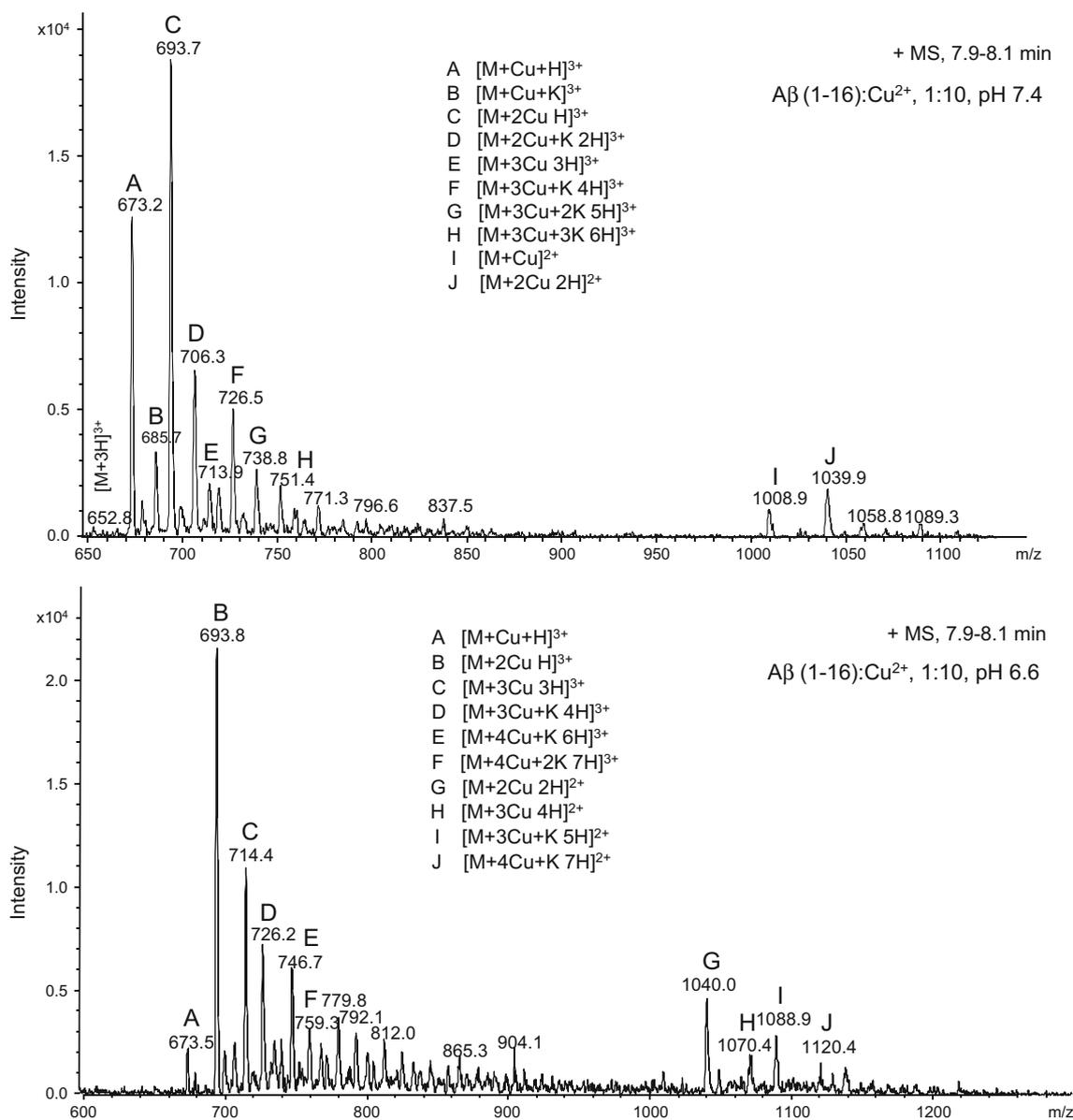
The CD spectra presented in Fig. 4 indicated that the binding of copper ions to A $\beta$ (1–16) could significantly influence the peptide conformation. The far UV CD spectrum of an aqueous solution of A $\beta$ (1–16) (0.2 mM concentration) was characterized by two negative maxima at 191 and 198 nm, and other two positive ones at 183 and 220 nm, indicating predominant random coil conformation. In the first minute of copper interaction with A $\beta$ (1–16), when a relatively small



**Fig. 2** ESI-ion trap mass spectra of A $\beta$ (1–16) peptide (10  $\mu$ M peptide concentration in 5 mM ammonium acetate solution, pH 6.6) in the presence of copper ions (1:1 and 1:10 peptide:copper molar ratios)

proportion of metal ions was bound to the peptide, a slight conformational change of the peptide was observed. The spectral features were comparable to those of the peptide in the absence of copper ions; however, the bands at 183 and 191 nm had increased intensity, while the negative maximum at 198 nm shifted to 196.5 nm. Moreover, the positive band at 200 nm decreased to negative values and shifted to 203 nm. Over time (7–8 min), more copper bound to A $\beta$ (1–16), resulting in a completely different CD spectrum

of the peptide, in which only its ellipticity in the range from 200 to 260 nm was similar to that for the spectrum recorded at about 1 min from mixing. Two negative maxima at 199.5 nm (69.1 % of the negative ellipticity of peptide at 198 nm) and 194.5 nm (73.7 %) were the main changes in the spectrum, while the positive maximum at 183 nm shifted to 186 nm and had decreased ellipticity. The CD spectrum after 7 min may indicate the presence of a mixture of conformers.



**Fig. 3** ESI-ion trap mass spectra of A $\beta$ (1–16) peptide in the presence of highly concentrated copper ion solutions, at two pH values (10  $\mu$ M peptide concentration in 5 mM ammonium acetate; pH 7.4 and 6.6,

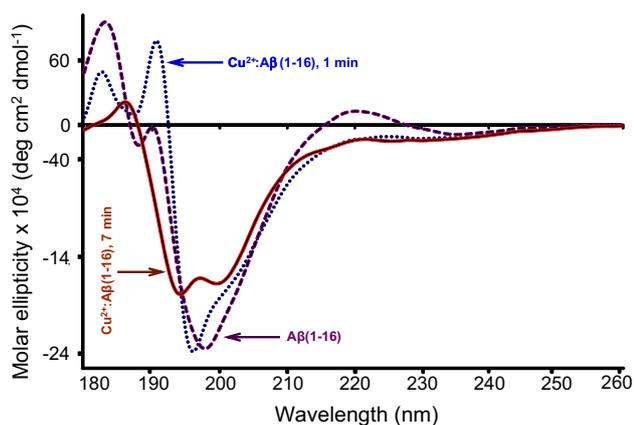
respectively; [Cu<sup>2+</sup>]:[A $\beta$ (1–16)] 1:10). The spectra show the formation of Cu<sup>2+</sup>-A $\beta$ (1–16) complexes in which the monomer peptide binds up to three or even four copper ions

These data clearly indicate that copper ions bind to A $\beta$ (1–16) over time in solution and not in the gas phase. Nevertheless, the possibility of forming non specific adducts in the gas phase was not excluded.

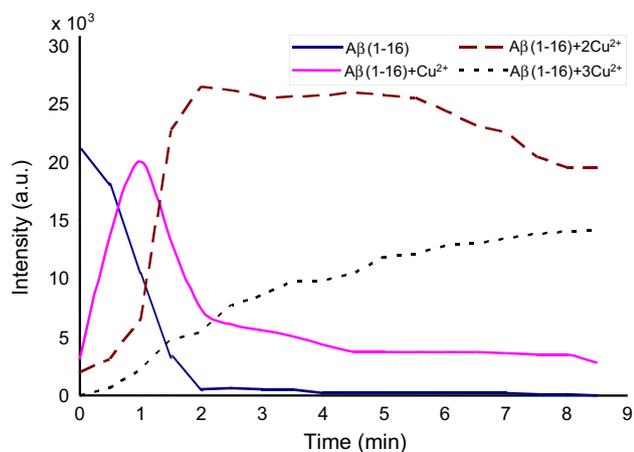
#### Time- and pH Dependences of Copper Binding

The time-dependent formation of species of A $\beta$ (1–16):Cu<sup>2+</sup> complex was followed by mass spectrometry at pH 6.6 and 1:10 peptide:copper molar ratio (Fig. 5), and at pH 7.4 and 1:1 peptide:copper molar ratio

(Fig. 6). At high copper:peptide molar ratio, the free peptide almost disappeared in the first 2 min, while the intensity of 1:1 Cu<sup>2+</sup>:A $\beta$ (1–16) species increased dramatically in the first minute after mixing to decrease quickly in the second one. Once formed, the 1:1 Cu<sup>2+</sup>:A $\beta$ (1–16) complex did bind the second copper ion to form the 2:1 Cu<sup>2+</sup>:A $\beta$ (1–16) complex, whose intensity also increased for the time interval from minute 1 to 2, and remained almost constant up to minute 6. The 3:1 Cu<sup>2+</sup>:A $\beta$ (1–16) complex showed an increasing intensity for 1.5 min after mixing the solutions, followed by a rather unchanged



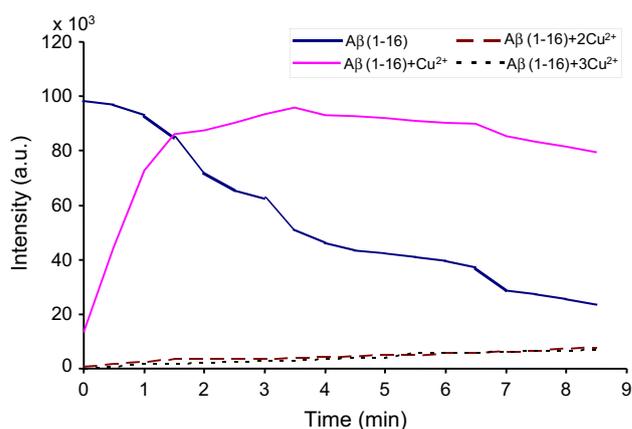
**Fig. 4** Circular dichroism spectra of A $\beta$ (1–16) and its copper complexes formed in the first minutes after mixing the two solutions



**Fig. 5** Time dependent copper binding to A $\beta$ (1–16) at pH 6.6 and 1:10 peptide:copper molar ratio

intensity of MS peak for the time interval of 0.5 min, between minute 1.5 and 2, the time when the intensity of 2:1 Cu<sup>2+</sup>:A $\beta$ (1–16) complex increased based on copper binding to 1:1 Cu<sup>2+</sup>:A $\beta$ (1–16) complex (Fig. 5). After minute 6, an increase in the intensity of 3:1 Cu<sup>2+</sup>:A $\beta$ (1–16) complex on the expense of 2:1 Cu<sup>2+</sup>:A $\beta$ (1–16) complex intensity was observed, while the 3:1 Cu<sup>2+</sup>:A $\beta$ (1–16) complex formation for 2–4.5 min interval was dependent on 1:1 Cu<sup>2+</sup>:A $\beta$ (1–16) complex. Taken together, the sum of the intensities of complexes with one, two, and three copper ions increased quickly in the first 2 min and remained almost constant for the next minutes of experiments.

At 1:1 copper:A $\beta$ (1–16) peptide molar ratio, the intensity of free peptide peak decreased slowly and continuously over the experimental time (Fig. 6). The 1:1 Cu<sup>2+</sup>:A $\beta$ (1–16) complex formed quickly in the first 2 min from



**Fig. 6** Time dependent copper binding to A $\beta$ (1–16) at pH 7.4 and 1:1 peptide:copper molar ratio

mixing the two solutions, but the process continued up to minute 4, when a maximum of intensity was attained. Both intensities of 2:1 Cu<sup>2+</sup>:A $\beta$ (1–16) and 3:1 Cu<sup>2+</sup>:A $\beta$ (1–16) complexes increased slowly.

## Discussion

Our results revealed the time-dependent formation of peptide-metal ion complexes in solution, namely A $\beta$ (1–16):Cu<sup>2+</sup> complexes that could be detected by ESI-MS in the gas phase. The complex formation was mainly dependent on the metal concentration and less on pH; however, it seemed to be faster at higher pH, whereas the lower pH stimulated the appearance of complexes containing more than two copper ions.

In contrast to other studies demonstrating the formation of oligomeric species of A $\beta$ (1–16) peptide in the presence of Cu<sup>2+</sup> ions, (Bush 2003; Alies et al. 2013; Ali et al. 2006) we noticed no oligomerization in the first minutes of copper-peptide interaction during the ESI-MS analysis. Working under different experimental conditions (preincubated samples, pH 5.5, higher concentrations of copper ions and peptide), copper was found to accelerate the aggregation of A $\beta$ (1–16) (Ali et al. 2006). However, such complexes did not appear under the experimental conditions described here, probably due to the short time of copper-peptide interaction.

One can expect to find more copper ions bound to A $\beta$  monomer at pH 7.4 than at pH 6.6, since higher pH may favor copper binding regardless of peptide sequence (Drochioiu et al. 2006). Nevertheless, more copper was found to be bound at lower pH, suggesting that brain pH changes could also play a role in various neurodegenerative pathologies.

These results are in agreement with other findings related to pH-dependent metal binding to peptides

(Murariu et al. 2009, 2010b, 2013; Drochioiu et al. 2013). Our data could be highly important to understand metal binding to peptides *in vivo*. For example, if transient concentrations of metals may appear in the body, peptides and proteins do not immediately bind such metals. The binding process takes minutes as we showed in this work and depends on the metal and peptide concentrations, as well as on pH variations. These findings may explain why short stress conditions followed by a period of rest do not damage the cell, while long acting, but low intensity stressors may result in neurodegeneration.

### Concluding Remarks

Taken together, our results indicate that copper ions specifically bind to A $\beta$ (1–16) peptide in solution and that this process is time-dependent. Changing the pH value from 7.4 to 6.6, only slightly influences the Cu<sup>2+</sup> binding to the peptide. Nevertheless, more copper ions bind to A $\beta$ (1–16) at pH 6.6 than at pH 7.4, although the complex formation is faster at pH 7.4. These results could be useful to understand the long lasting, pH-dependent interaction of copper ions with  $\beta$ -amyloid peptides involved in the pathogenesis of AD.

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**Conflict of interest** Marilena Manea, Gitta Schlosser, and Manuela Murariu declare that they have no conflict of interest.

**Human and Animal Rights and Informed Consent** This article does not contain any studies with human or animal subjects performed by any of the authors.

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