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# Peptidic scaffolds to reduce the interaction of Cu(II) ions with $\beta$ -amyloid protein

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**KEYWORDS:** beta-amyloid aggregation, MPAC, copper, peptide

*In memory of Prof. Juan M. Salas and his contributions to Bioinorganic Chemistry*

**ABSTRACT.** Competitive Cu(II)-binding studies have been carried out between five decapeptides (both acyclic and cyclic), namely **C-Asp**, **C-Asn**, **O-Asp**, **ODP<sub>ro</sub>-Asp** and **O-Asn**, and the A $\beta$ (1-16) and A $\beta$ (1-40) fragments. Conformational constraints in such peptidic scaffolds affect their copper-binding affinity, which can be tuned. In the present study, the ability of these peptides to compete with A $\beta$  has been assessed *in vitro*, with the objective to examine whether such soft chelating agents may be used to lessen the deleterious interaction of Cu(II) with A $\beta$ . Fluorescence spectroscopy, electron paramagnetic resonance and mass spectrometry data show that the more constrained peptide, *i.e.* cyclic **C-Asp**, which displays a Cu(II)-binding affinity comparable to that of A $\beta$ , is the only potential metal-protein attenuating compound (MPAC) candidate. *In vitro* aggregation studies with A $\beta$ (1-40) reveal that **C-Asp** can hamper the formation of copper-stabilized oligomeric A $\beta$  species, through capturing the metal ion prior to its interaction with monomeric A $\beta$ . The present study shows that (cyclic) peptides, pre-organized for Cu(II) binding, may be applied for the development of potential copper-A $\beta$  attenuating compounds.

## **INTRODUCTION**

Alzheimer's disease (AD) represents the most common form of dementia. The number of people suffering from AD is currently estimated to 47 million;<sup>1</sup> this number is expected to triplicate within the next three decades. Currently, only five drugs have been approved by the FDA; however, they only alleviate the symptoms.<sup>2</sup>

One of the pathogenic processes of AD is the accumulation of Amyloid- $\beta$  (A $\beta$ ) in the brain, generating A $\beta$  aggregates and triggering oxidative stress. Although other hallmarks have been identified for this disease, A $\beta$  has probably been the most widely chosen therapeutic target to develop potential anti-AD drugs. A number of therapies aimed at disrupting the amyloid cascade – *i.e.* a set of events involved in the production and aggregation of A $\beta$  – have reached phases II

and III of clinical trials but eventually failed.<sup>3</sup> This suggests that the strategy of inhibiting A $\beta$  production and its aggregation is too simplistic, and that other biological target(s) should also be considered.

Some studies have pointed to the abnormal accumulation of some transition-metal ions, mainly Cu(I/II), Zn(II), and Fe(II/III), in amyloid plaques and in some areas of AD brains, contributing to metal homeostasis dysregulation.<sup>4</sup> Metal misbalance has also been detected in other neurodegenerative pathologies such as Parkinson's disease.<sup>5</sup> Some evidence indicates that traces of copper ions may be implicated in pathogenic aggregation pathways and neurotoxicity of A $\beta$ , accelerating the progression of AD. A $\beta$  can bind with high affinity copper ions and subsequently generate toxic amyloid aggregates in the form of soluble oligomers, which are considered as the most toxic aggregates.<sup>6</sup> In addition, the higher concentration of this redox-active metal ion in A $\beta$  plaques and in neuropil regions of the brain contributes to the oxidative stress observed in AD.

Given the damaging effects of the Cu-A $\beta$  species, shifting the equilibrium of the A $\beta$ -copper complex toward less-toxic aggregated states, while lowering extracellular copper levels and restoring brain metallostasis, could be an alternative therapeutic strategy for treating AD.<sup>7</sup> One of the approaches being explored in such direction is the use of metal-protein attenuating compounds (MPACs). MPACs differ from traditional chelators since, instead of binding strongly and removing the metal ion (in this case copper) from the affected tissue, they correct abnormal metal interactions and have subtle effects on metal homeostasis, inhibiting the interaction with the pathogenic protein, *viz.* A $\beta$ . This promotes the resuspension and clearance of A $\beta$  and inhibits redox reactions that generate neurotoxic dihydrogen peroxide. Consequently, MPACs might provide a viable therapeutic strategy for slowing down or preventing the progression of AD.<sup>8</sup> For instance, two MPACs, namely clioquinol (PBT1) and 5,7-dichloro-2-[(dimethylamino)methyl]quinolin-8-

ol (PBT2), reduce the metal-induced A $\beta$  deposition in brain mice and improve cognitive performance in AD patients.<sup>9</sup> Although the use of PBT1 has been limited by side effects issues, PBT2 is undergoing phase II clinical trials. The use of MPACs remains a highly promising anti-AD alternative.

The exploration of small peptides as MPACs is relatively recent in the field of anti-AD therapeutics.<sup>10</sup> Peptides are biocompatible and less likely to be immunogenic. Importantly, the enormous advances achieved in solid-phase synthesis and chromatographic techniques, together with the possibility for automatization, accelerate the synthesis and facilitate almost unlimited derivatization alternatives, which allow a fine tuning of the metal-binding properties by changing the amino acid sequence. Namely, histidine-containing oligopeptides have shown a strong potential to act as high-affinity and selective copper chelators that are able to displace Cu(II) ions from A $\beta$ , without disturbing its native distribution, so as of other metals.<sup>10-11</sup> The strategic location of histidine residues and geometric restrictions in the main chain also promote a high selectivity towards Cu(II) ions against other metal ions (Ca(II), Zn(II) and Mg(II)) that are more abundant *in vivo* and exist freely in the synaptic cleft and neuronal cytoplasm.<sup>12</sup>

Certain sequences such as that corresponding to the albumin binding mode (ATCUN motif) - *viz.* H-Xaa-Xaa-His including H-His-Xaa-His - are particularly effective in redox-silencing Cu(II) through the impediment of its reduction by endogenous reducing agents and the subsequent production of ROS.<sup>13</sup> Both the high binding affinity towards Cu(II) and the stabilization of its oxidation state have been attributed to the involvement of two deprotonated amide nitrogen atoms from the peptide backbone; such deprotonation is favored at medium to high pH values, as it is the case for physiological conditions. The imidazole ring of the His3 residue completes the formation

of three fused five- and six-membered chelating rings, leading to a very stable square-planar complex.

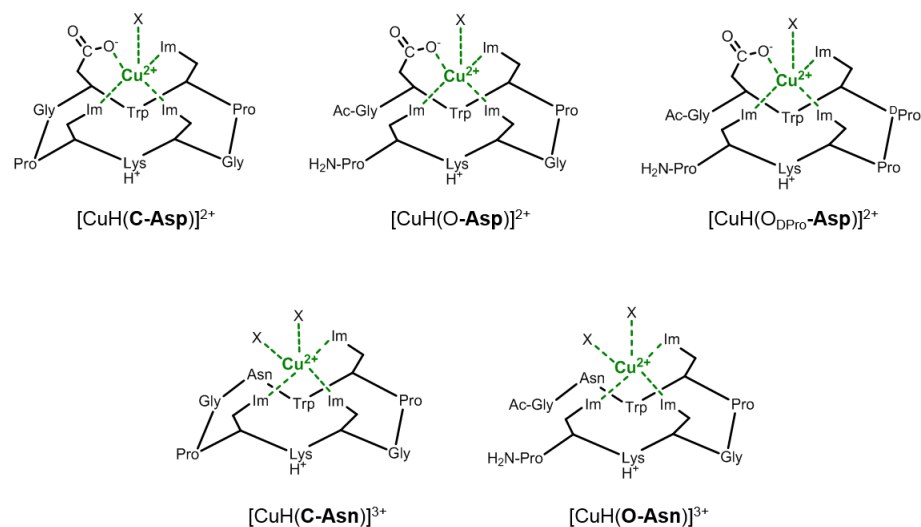
However, a too high affinity and selectivity towards copper ions may affect its native distribution and critically alter the normal functioning of the cells. Inspired by native copper proteins, which accommodate their scaffold to attain a large variety of biological functions,<sup>14</sup> a family of His-containing decapeptides having different degrees of conformational constrain and structural pre-organization were previously designed (Table 1).<sup>15</sup> The study of their copper coordination properties revealed that they all bind the metal ion, giving rise to similar Cu(II) species at close to neutral pH value ( $[\text{CuH}(\text{C-Asp})]^{2+}$ ,  $[\text{CuH}(\text{O-Asp})]^{2+}$ ,  $[\text{CuH}(\text{ODPro-Asp})]^{2+}$ ,  $[\text{CuH}(\text{C-Asn})]^{3+}$ , and  $[\text{CuH}(\text{O-Asn})]^{3+}$ ). The metal ion is coordinated by the side-chain functionalities of the His and the Asp residues, if present in the sequence, in a square-planar or square-pyramidal geometry (Fig. 1). The replacement of the Asp residue by an Asn decreased the Cu(II) affinity of these decapeptides, suggesting that the amide functionality is weakly or not coordinated to metal centre. Interestingly, the different intrinsic nature of their peptidic scaffolds, namely cyclic *vs* linear and ProGly *vs* <sup>D</sup>ProPro (a stronger  $\beta$ -turn inducer unit), and the replacement of Asp by Asn, had an important impact on their formation constants, their pH-dependent stability, their Cu<sup>2+</sup>-exchange rates, as well as on their redox potentials (ascribed to the Cu<sup>2+</sup>/Cu<sup>+</sup> process).<sup>15</sup>

On the other hand, these peptides are expected to be resistant to *in vivo* degradation since they are either cyclic or protected at their terminal positions. Additionally, one of them contains a D-amino acid. This aspect encourages their study for potential therapeutic uses.

**Table 1.** Amino acid sequences of the designed decapeptides.

Peptide	Sequence (X <sub>1</sub> -----X <sub>10</sub> )
<b>C-Asp</b>	Cyclo(GDWHPGHKHP)
<b>O-Asp</b>	Ac-GDWHPGHKHG-NH <sub>2</sub>
<b>O<sub>DPro</sub>-Asp</b>	Ac-GDWH <sup>D</sup> PPHKHG-NH <sub>2</sub> [a]
<b>C-Asn</b>	Cyclo(GQWHPGHKHP)
<b>O-Asn</b>	Ac-GQWHPGHKHG-NH <sub>2</sub>

[a] <sup>D</sup>P refers to D-Proline



**Figure 1.** Schematic representation of the proposed structures of the Cu(II) complexes [CuH(C-Asp)]<sup>2+</sup>, [CuH(O-Asp)]<sup>2+</sup>, [CuH(O<sub>DPro</sub>-Asp)]<sup>2+</sup>, [CuH(C-Asn)]<sup>3+</sup>, and [CuH(O-Asn)]<sup>3+</sup>. For the sake of clarity, only the side chains of Asp and His residues are shown. Im = imidazole, and X = H<sub>2</sub>O or counterion.

Considering the Cu(II) coordination properties of these five decapeptides (**C-Asp**, **C-Asn**, **O-Asp**, **ODPro-Asp** and **O-Asn**) and their Cu(II) binding affinities, their ability to attenuate the interaction of Cu(II) ions with A $\beta$  has been examined in the present study by fluorescence spectroscopy, EPR studies and mass spectrometry. The ultimate aim is to explore the potential use of pre-organized peptide structures<sup>16</sup> to develop biocompatible and effective copper-protein attenuating compounds that would inhibit/reverse A $\beta$  aggregation and help to restore metallostasis in AD.

## RESULTS AND DISCUSSION

### Cu(II) coordination properties of the decapeptides.

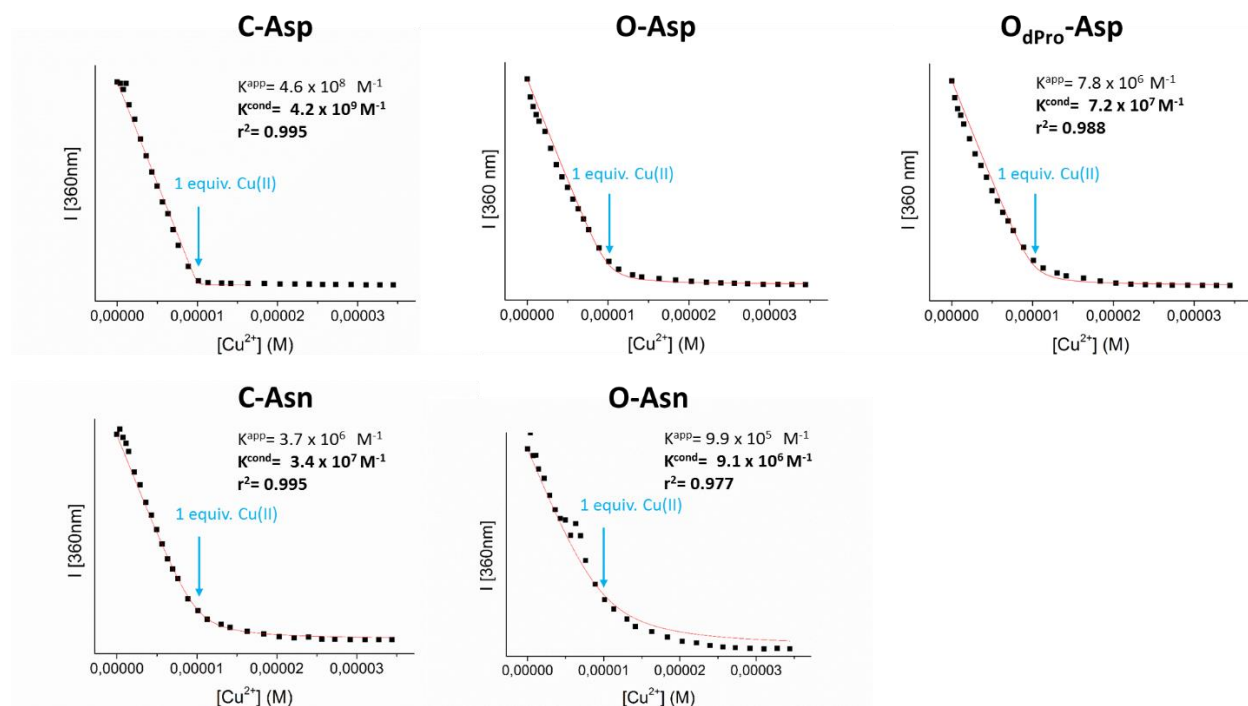
The protonation constants of the decapeptides and the stability constants of their Cu(II) complexes were previously determined by potentiometric titrations.<sup>15</sup> These data allowed us to generate the corresponding speciation diagrams (Fig. S1, Table S1) at 1:1 Cu/peptide ratio and at 10  $\mu$ M (concentration relevant to the experiments described in this study). The data indicate that at pH 7.4, only the **C-Asp** peptide forms a single, major species (*i.e.*  $[\text{CuH}(\text{C-Asp})]^{2+} = [\text{CuHL}]^{2+}$  94.7%). For the rest of the decapeptides, various species at different concentrations are observed at this pH (Table S1). For the sake of comparison and to get a better insight into the affinity of the decapeptides for Cu(II) at pH 7.4, the conditional binding constants ( $K^{\text{cond}}$ , defined as  $\Sigma[\text{Cu}_m\text{H}_{i+j}\text{L}_i]/\Sigma[\text{H}_i\text{L}] \Sigma[\text{H}_j\text{Cu}]$ ) were calculated.<sup>17</sup> At a molar Cu/peptide ratio of 1:1 (10  $\mu$ M), the conditional affinity to Cu(II) ions at pH 7.4 and 25  $^{\circ}$ C follows the trend ( $\log K^{\text{cond}}$ ) **C-Asp** (10.4) > **O-Asp** (9.1)  $\approx$  **ODPro-Asp** (9.1)  $\approx$  **C-Asn** (9.0) > **O-Asn** (8.5). The conditional affinity increases when using a molar Cu/peptide ratio of 2:1 for all peptides, and the trend is **C-Asp** > **C-Asn** > **ODPro-Asp** > **O-Asp** > **O-Asn** .



Since the ability of these peptides to compete for Cu(II) against A $\beta$  will be assessed through the emission of the tryptophan residue (see next section), we found crucial to study the fluorescence-quenching effect of Cu(II) ions for each decapeptide and use this parameter to evaluate their Cu(II) binding affinities ( $K^{\text{cond}}$ ), and compare the values with those obtained by the potentiometric experiments under similar (physiological) conditions and peptide concentration, *i.e.* pH 7.4, 25 °C, 10  $\mu$ M peptide (Fig. 2). A significant quenching of the fluorescence of tryptophan was observed upon the addition of increasing amounts of Cu(II), until up to 1 equivalent of metal per decapeptide (Fig. 2). These results are consistent with the formation of 1:1 Cu/peptide species. The intensity data were fitted to Eq. (1),<sup>18</sup> which has been previously reported for 1:1 Cu-biomolecule systems and used in other studies for metal-biomolecule interactions:<sup>19</sup>

$$I = I_0 + \frac{I_{lim} - I_0}{2[L]} \left( [L] + [Cu^{2+}] + \frac{1}{K^{app}} - \sqrt{\left( [L] + [Cu^{2+}] + \frac{1}{K^{app}} \right)^2 - 4[L][Cu^{2+}]} \right) \quad (1)$$

where  $I_0$  and  $I_{lim}$  denote the fluorescence intensities of respectively free and fully bound ligand (peptide in this case);  $K^{app}$  is the apparent binding constant;  $L$  stands for peptide.



**Figure 2.** Fitting of the fluorescence titration data to Eq. (1) and the resulting apparent and conditional affinity constants obtained for the five tested peptides. [Peptide] = 10  $\mu$ M in HEPES 10 mM, pH 7.4.

The contribution of the buffer as a weak metal-binding competitor (HEPES can form a 1:1 complex with Cu(II), favored by its excess, 10 mM) was corrected by using Eq. (2) to obtain the conditional binding constant ( $K^{cond}$ ), which is the affinity constant at zero-buffer concentration:<sup>20</sup>

$$\log K^{cond} = \log K^{app} + \log \left( 1 + \beta_{Cu-buffer} \times \frac{[buffer]}{1 + 10^{-pH + pK_a}} \right) \quad (2)$$

where  $\beta_{Cu-buffer}$  is the stability constant of the Cu(II)-HEPES complex ( $\log \beta_{Cu-buffer} = 3.22$ ), and  $K_a$  is the protonation constant of HEPES buffer ( $pK_a = 7.41$ ).<sup>20</sup>

The obtained  $K^{cond}$  values (Fig. 2) follow the trend ( $\log K^{cond}$ ) **C-Asp** (9.6) > **O-Asp** (7.9)  $\approx$  **O<sub>dPro</sub>-Asp** (7.9) > **C-Asn** (7.5) > **O-Asn** (7.0), which is fairly consistent with the trend previously obtained by potentiometry and highlight the higher Cu(II) affinity of the **C-Asp** peptide.

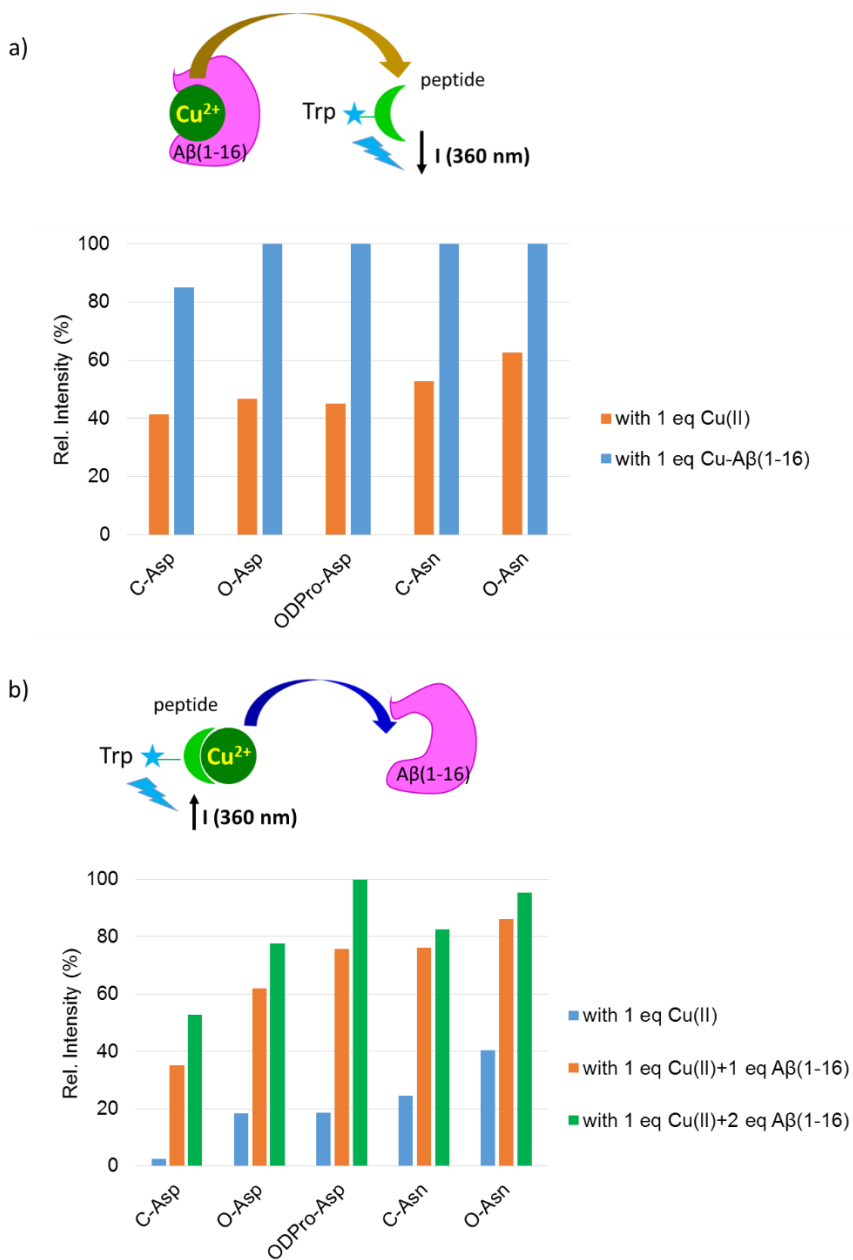
It must be stressed here that to obtain more accurate  $K^{\text{cond}}$  values through fluorescence quenching, binding experiments in the presence of a competitor with similar copper affinity (to that of the compound investigated) are required.

### **Cu(II)-binding competition against A $\beta$ (1-16)**

Next, the capability of the peptides to sequestrate Cu(II) ions and therefore prevent their interaction with A $\beta$  fragments was assessed. The non-aggregating A $\beta$ (1-16) fragment was selected for this study to avoid aggregation-derived interferences in the spectroscopic measurements; this fragment contains the whole metal-binding region of the aggregation-prone A $\beta$  fragments that are responsible for the formation of senile plaques, such as A $\beta$ (1-40) and A $\beta$ (1-42).<sup>21</sup> It should be noted, however, that the affinity of A $\beta$ (1-16) towards copper is about 2.5-fold lower than that of A $\beta$ (1-40).<sup>22</sup> Nevertheless, A $\beta$ (1-16) is widely used to evaluate the binding affinity of potential metal-protein attenuating compounds (MPACs).<sup>11, 23</sup>

The tryptophan fluorescence of all the peptides is quenched (to a different extent) upon addition of 1 equivalent of Cu(II) salt (Fig. 3a). Interestingly, the addition of Cu-A $\beta$ (1-16) to C-**Asp** produced the partial quenching of the emission of the free C-**Asp** peptide. The other peptides displayed the same emission intensity before and after adding 1 equivalent of Cu-A $\beta$ (1-16). This suggests that C-**Asp** either rapidly coordinates A $\beta$ -bound Cu producing a ternary species, or the peptide is capable of removing Cu(II) from the Cu-A $\beta$ (1-16) complex. After incubation for 4 hours at 37 °C (Fig. S3), the copper-induced quenching increased for all peptides, even when treated with Cu-A $\beta$ (1-16). This observation illustrates the importance of kinetics regarding these interactions and reveals a dynamic copper exchange between the decapeptides and A $\beta$ (1-16), whose study is beyond the scope of this work.

In order to determine to which extent the addition of higher amounts of decapeptide favors the displacement of Cu(II) ions from A $\beta$ (1-16), analogous tests were performed with 2 equivalents of peptide with respect to the preformed Cu-A $\beta$ (1-16) complex (Fig. S4). Although most of the fluorescence emission results from the excess of free peptide, some effect can be noticed with **O-Asp** (apart from **C-Asp**), for which the overall relative emission is lower than that obtained with only one equivalent of peptide. Reversely, the emission of Cu(II)-peptide complexes obtained after addition of 1 and 2 equivalents of A $\beta$ (1-16) was then registered. As observed in Fig. 3b, all peptides displayed partial recovery of their intrinsic emission upon addition of 1 equivalent of A $\beta$ (1-16). When the amount of A $\beta$ (1-16) was increased up to 2 equivalents, a total or nearly total recovery of the emission of **O<sub>DPro</sub>-Asp** and **O-Asn** was achieved, respectively, suggesting a complete displacement of the metal ion by A $\beta$ (1-16). Up to 80 % of emission recovery was obtained for **C-Asn** and **O-Asp**, and only 53 % for **C-Asp**. These data are in agreement with the  $K^{\text{cond}}$  values (see above), except for **O<sub>DPro</sub>-Asp**. Control measurements (data not shown) showed that A $\beta$ (1-16) itself does not affect the emission of the peptides.



**Figure 3.** a) Effect on the emission intensity of each peptide (relative to the emission of the free peptide) reflecting a) the displacement of Aβ(1-16)-bound Cu(II) ions upon incubation with equimolar amounts of peptide, and b) the displacement of peptide-bound Cu(II) ions upon addition of increasing amounts of Aβ(1-16). [Peptide] = [CuCl<sub>2</sub>] = [Aβ(1-16)] = 10 μM.

Fluorescence data depicted in the graphs have been calculated from averaged intensity values of three independent measurements.

Based on the results obtained so far, two scenarios may be proposed, that are: (i) the formation of a stable ternary A $\beta$ -Cu-peptide complex, and (ii) the occurrence of an equilibrium between Cu-A $\beta$  and Cu-peptide binary species.

To assess whether stable A $\beta$ -Cu-peptide ternary complexes can be formed, EPR spectra of the five different systems were recorded at 77 K, using 100  $\mu$ M solutions of 1:1.1 Cu/peptide in the presence of an excess of A $\beta$ (1-16) (Fig. S2, S5 and Table S2). Upon addition of increasing amounts of A $\beta$ (1-16) (up to 4 equivalents) to the Cu-**C-Asp** complex, the corresponding spectra are indicative of the presence of a mixture of Cu-**C-Asp** and Cu-A $\beta$ (1-16) species. For the less efficient copper chelator **O-Asp**, the EPR data are similar to that of Cu-A $\beta$ (1-16); however, the poor resolution and great similarity between the hyperfine structures do not allow to discard the presence of Cu-**O-Asp**, or even of the formation of Cu-A $\beta$ -**O-Asp** species. Analogous features were observed for **ODPro-Asp** and **C-Asn**. In the case of **O-Asn**, a solution of the Cu-peptide complex in the presence of 2 equivalents of A $\beta$ (1-16) produced a typical spectrum for Cu-A $\beta$ , suggesting that this species is (at least) the most abundant in the mixture.

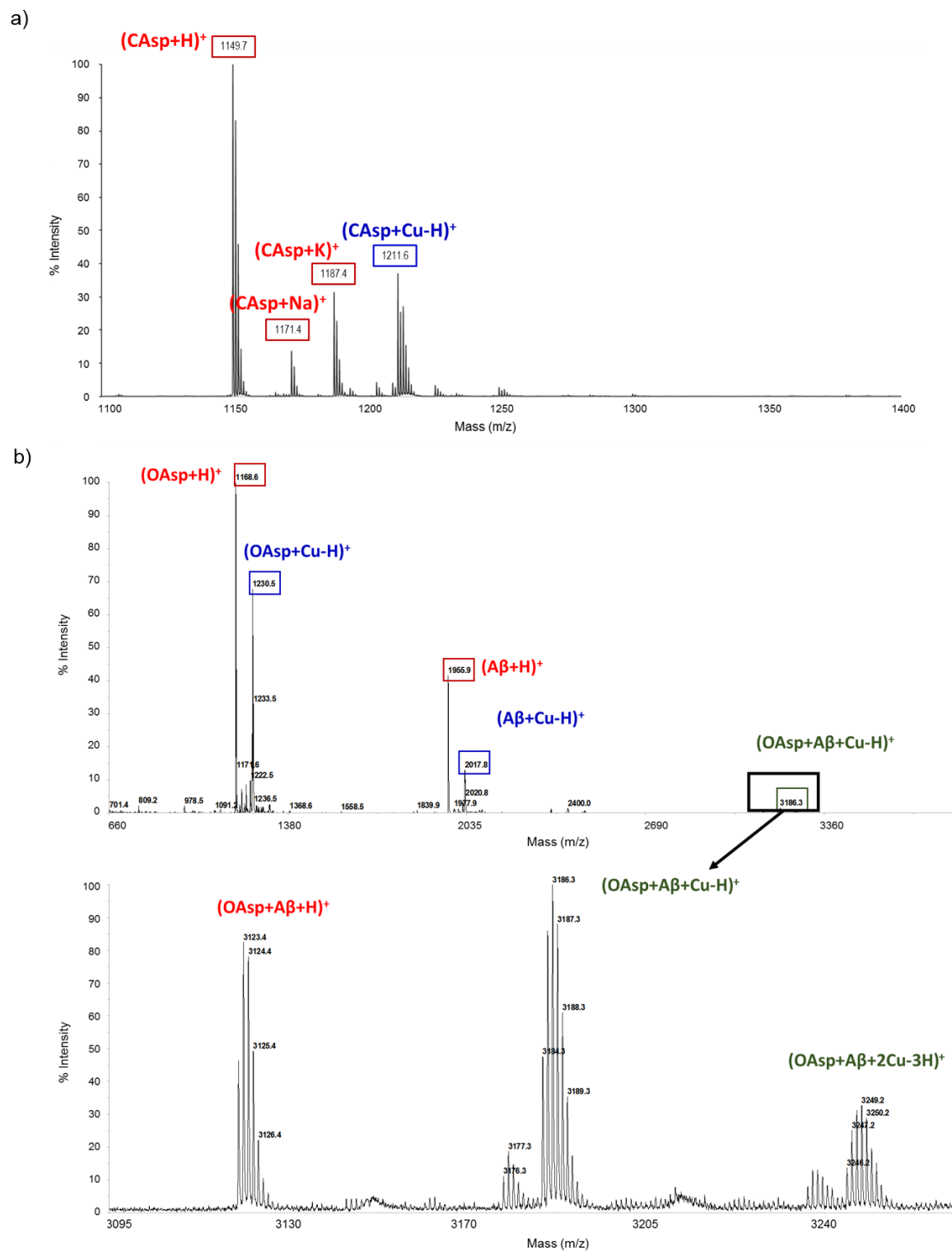
Since the EPR studies did not allow to detect ternary species, MALDI-TOF experiments were subsequently carried out using Cu-peptide-A $\beta$ (1-16) mixtures of different molar ratios (1:1:2-4), and the same conditions as those used in EPR, mixing the three components before starting incubation.

The mass spectra of **C-Asp** and **O-Asn**, obtained from 1:1:4 and 1:1:2 Cu-peptide-A $\beta$ (1-16) solutions, respectively, did not show peaks corresponding to ternary species (Figs. 4a and S6).

The major peaks correspond to the  $[\text{pept}+\text{H}]^+$  and  $[\text{pept}+\text{Cu}-\text{H}]^+$  species. The presence of  $[\text{A}\beta+\text{Cu}-\text{H}]^+$  could also be detected for **O-Asn**.

The incubation of **OD<sub>Pro</sub>-Asp**, **O-Asp** and **C-Asn** with one equivalent of Cu(II) and an excess of A $\beta$ (1-16) generated mass spectra where the species  $[\text{pept}+\text{Cu}-\text{H}]^+$  and  $[\text{A}\beta+\text{Cu}-\text{H}]^+$  could be seen (as for **C-Asp** and **O-Asn**), but a smaller signal ascribed to  $[\text{pept}+\text{A}\beta+\text{Cu}-\text{H}]^+$  species could be detected as well (Figures 4b, S7 and S8). In contrast, when Cu(II) ions were incubated with one equivalent of A $\beta$ (1-16), prior to the addition of one equivalent of peptide,  $m/z$  peaks due to ternary species were not noticed. Thus, these data suggest that the formation of stable ternary Cu-peptide-A $\beta$  complexes with **OD<sub>Pro</sub>-Asp**, **O-Asp** and **C-Asn** is not taking place.

It should be noted that Cu(II)-containing species could not be detected by ESI-MS, under the experimental conditions applied for the MALDI-TOF studies.





The mass-spectrometry results illustrate whether relatively stable ternary species, that are intermediates in the Cu(II) exchange between the chelating peptides and A $\beta$ (1-16), are formed. In the case of the strongest and the weakest metal chelators, **C-Asp** and **O-Asn** respectively, the exchange appears to occur fast enough (towards the peptide or A $\beta$ , respectively) to impede the detection of ternary peptide-Cu-A $\beta$  species. For the other decapeptides, which show a Cu(II)-binding affinity in between those of **C-Asp** and **O-Asn**, the exchange intermediate could be detected.

In summary, it has been demonstrated that **C-Asp** is the strongest copper-chelating peptide and the only one capable of competing effectively with A $\beta$ (1-16). Within a solution containing one equivalent of A $\beta$ (1-16) and **C-Asp** at pH 7.4, an equilibrium is established, which is consistent with  $\log K^{\text{cond}}$  (10.4) of the peptide that is similar to that of A $\beta$ (1-16).<sup>22</sup> **O-Asn** ( $\log K^{\text{cond}} = 8.5$ ) is the weakest chelator and it is not able to displace Cu(II) ions bound to A $\beta$ (1-16), nor to compete with A $\beta$  to bind it. For both **O-Asn** and **C-Asp**, the potential “ternary intermediates” are not stable enough to be detected.

**O<sub>DPro</sub>-Asp**, **O-Asp** and **C-Asn**, whose  $\log K^{\text{cond}}$  is 9.1, can compete with A $\beta$ (1-16) for binding Cu(II) ions, but only if they are added before or simultaneously to A $\beta$ (1-16); equimolar amounts of them cannot remove Cu(II) ions bound to A $\beta$ (1-16).

From the series of decapeptides investigated, **C-Asp** appears to be the best potential candidate as MPAC; therefore it was selected for further studies *in vitro* (*vide infra*) with aggregating A $\beta$ (1-40).

## **Effect of C-Asp on the aggregation kinetics of A $\beta$ (1-40) in the absence and presence of Cu(II) ions**

Thioflavin T (ThT) is a dye that recognizes selectively amyloid fibrils but not monomers, oligomers and other prefibrillar species. This fluorescent dye is therefore widely used to monitor the aggregation of amyloid-prone proteins. Thus, ThT was selected to analyze the effectiveness of **C-Asp** to chelate Cu(II) and to hinder the effects of Cu(II) ions on A $\beta$ (1-40) aggregation.

As observed in the kinetic experiments using ThT, the aggregation of free A $\beta$ (1-40) exhibits a characteristic sigmoidal profile, illustrating a process in which a rate-limiting nucleation stage is followed by a comparatively fast fibril growth step.<sup>24</sup> Cu(II) is known to stabilize oligomeric states of the amyloid protein, which are not detected by ThT, leading to flat kinetic profiles.<sup>21</sup> We have first determined, by ThT fluorescence, the minimal amount of copper that is necessary to inhibit completely the formation of fibrillary A $\beta$ (1-40) aggregates. As depicted in Fig. 5a, ThT emission measurements after incubating Cu:A $\beta$  mixtures at different molar ratios (from 0 to 1) reveal a gradual decrease of fibrillation upon addition of copper and reaches a minimum at a Cu:A $\beta$  molar ratio of 0.5. At higher molar ratio values, the intensity remains similarly low, which indicates that fibrils are no longer formed. Remarkably, as evidenced in Fig. 5b, copper seems to be able to inhibit the fibrillation only when it interacts with the monomeric form of the protein; once small aggregation nuclei start forming, the subsequent addition of Cu(II) ions does not prevent such aggregates to fibrillate; this effect is evidenced in Fig. 5c with the observed decrease of the fibrillation degree. The inhibition of the fibrillation of monomers but not of small aggregation nuclei is in agreement with the gradual prolongation of the nucleation period (lag time) upon increasing the metal concentration until a completely flat curve is obtained, which characterizes a complete inhibition of fibrillation (Fig. S9); such lengthening of the lag time is

caused by lower concentrations of fibrillation-prone protein. Taking into account these observations, we selected a Cu:A $\beta$  ratio of 1:1 in our further studies with **C-Asp**. Upon incubating one equivalent of copper with the fully monomeric form of the protein, the formation of oligomeric species was confirmed by TEM with negative staining (Fig. S10).

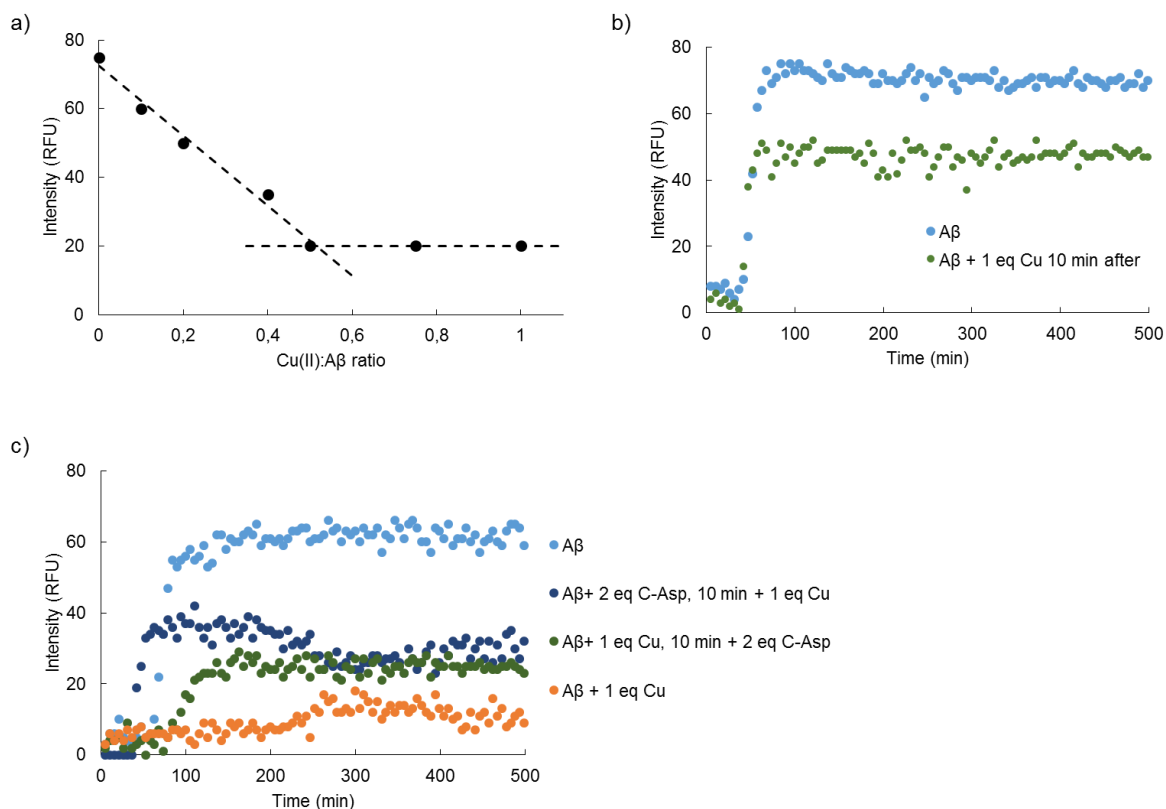
According to previous reports, metal-containing oligomers are considered to be the main toxic species.<sup>25</sup> Hence, an efficient chelating agent would in principle hamper the copper-induced stabilization of oligomers and trigger the aggregation of A $\beta$ (1-40) with the typical sigmoidal profile.

The study of the ability of **C-Asp** to sequester copper ions and affect the aggregation of A $\beta$  revealed that the aggregation profile of monomeric A $\beta$ (1-40) is not altered by the presence of this peptide (Fig. S11). Next, we assessed how the copper-chelating properties of **C-Asp** changes the aggregation of A $\beta$ (1-40) before and after the addition of the metal ion. **C-Asp** indeed acts as an effective chelator since the ThT emission decreased markedly in both cases (Figure 5c). When **C-Asp** is preincubated with the protein for 10 min prior to the addition of Cu(II), competitive binding of A $\beta$  and **C-Asp** is observed, leading to a partial inhibition (*ca.* 50%) of the fibrillation (Fig. 5c, dark blue dots). The shortening of the nucleation time compared with that of pure A $\beta$  may be due to a catalytic effect of the interaction of Cu(II) with weakly interacting A $\beta$ -**C-Asp** species (see ESI-MS results at the end of this section).

When the protein is preincubated for 10 min with copper (Fig. 5c, green dots), a fraction of A $\beta$  monomers is stabilized as non-amyloidogenic species (oligomers) by the metal ion, the rest remaining either as monomers or evolving into small prefibrillary structures. Hence, the subsequent addition of the peptidic chelator would in principle sequester the copper ions that have not interacted with A $\beta$  monomers, therefore hampering the formation of further copper-

stabilized oligomeric species. The preformed nuclei and the non-copper-interacting monomers follow the typical sigmoidal aggregation profile of amyloid. A final fibrillation of about 50% is reached, which is similar to the case described above. However, the longer nucleation time observed when copper is preincubated with A $\beta$  most likely results from a lower fibrillation-prone protein concentration, due to the prior formation of copper-induced irreversible non-amyloidogenic species.

Overall, these results suggest that pre-formed oligomers may act off-pathway of the aggregation process and that the sequestration of Cu(II) ions by **C-Asp** prevents such irreversible oligomerization.



**Figure 5.** a) ThT emission of A $\beta$ (1-40) monomers incubated for 18 h with different Cu:A $\beta$ (1-40) molar ratios. The intersection at Cu:A $\beta$  = 0.5 characterizes the minimal molar ratio at which the stabilization of oligomeric species occurs; b) ThT fluorescence curves for pure A $\beta$ (1-40) and A $\beta$ (1-40) left aggregating for 10 min before the addition of CuCl<sub>2</sub>; c) ThT emission over time illustrating the effect of **C-Asp** on the aggregation kinetics of A $\beta$ (1-40), applying different sequences of addition of the peptide and the copper salt. [A $\beta$ (1-40)] = [CuCl<sub>2</sub>] = 25  $\mu$ M; [C-Asp] = 50  $\mu$ M, PBS pH 7.4.

The effect of **C-Asp** on Cu-stabilized oligomers of A $\beta$ (1-40) was investigated further by electrospray mass spectrometry (positive mode). A set of four different experiments was applied, as described below (see also Fig. S12).

Samples of A $\beta$ (1-40) oligomers stabilized by Cu(II) ions were prepared in 10 mM ammonium formate buffer (contrarily to PBS, this buffer is compatible with mass spectrometry studies), in two microcentrifuge tubes, in the presence of ThT. Two equivalents of **C-Asp** were added to one of these tubes, and the two samples were incubated for 22 h at 37 °C (Fig. S12a). Two analogous samples were simultaneously prepared without ThT (Fig. S12b). The first two samples (with ThT) confirmed by ThT emission that fibrils were not formed (data not shown), whereas the ones not containing ThT were used for the mass spectrometry experiments (to avoid any misinterpretation(s) due to the presence of the dye). Before analysis, the tubes were centrifuged, and both the supernatants and the pellets were measured, separately (Fig. S12b).

Control experiments (data not shown) containing only A $\beta$ (1-40) monomers showed that the aggregation of A $\beta$  in 10 mM ammonium formate is much less favored than that in PBS (due to the less saline conditions), as reflected by the corresponding lag time of up to 11 h, instead of about 30 min (in PBS). However, at the end of the (longer) fibrillation process, the same amount of A $\beta$ (1-40) aggregates was produced, compared with that observed in PBS. The ESI-MS(+) spectra of the isolated and re-dissolved pellets (Fig. S13a,b), corresponding to Cu-A $\beta$ (1-40) aggregates formed with **C-Asp** present, did not show *m/z* peaks ascribed to A $\beta$  bound to copper ion(s); only the protonated (free) protein could be detected (Fig. S13b). It can be pointed out that a control sample consisting of A $\beta$ (1-40) incubated with (solely) copper(II) chloride was analyzed for comparison, which did display a peak attributable to [A $\beta$ +Cu+H]<sup>3+</sup> (Fig. S14). Traces of [C-Asp+Cu]<sup>2+</sup> could be observed in the pellets (Fig. S13a). Interestingly, protonated forms of **C-Asp** were present in both the pellets and the supernatants, but in much higher amounts in the former; this may be explained by the occurrence of some weak interactions between **C-Asp** and A $\beta$ . Cu-**C-Asp** species were found in the supernatant fractions, but no

peaks for A $\beta$  or A $\beta$ +Cu species could be detected (Fig. S13c). These results confirm that the decapeptide can displace Cu ions from A $\beta$ . It should be noted that the poorly saline conditions used here for the fibrillation are remarkably less favorable than those used above in PBS (see Figure 5). Likewise, it is possible that the Cu-induced oligomerization is less stabilized, and **C-Asp** can compete more favorably. Thus, displacement of Cu ions not only from monomeric species but also from oligomeric forms cannot be discarded under these conditions.

### **Inhibition of ROS production/release**

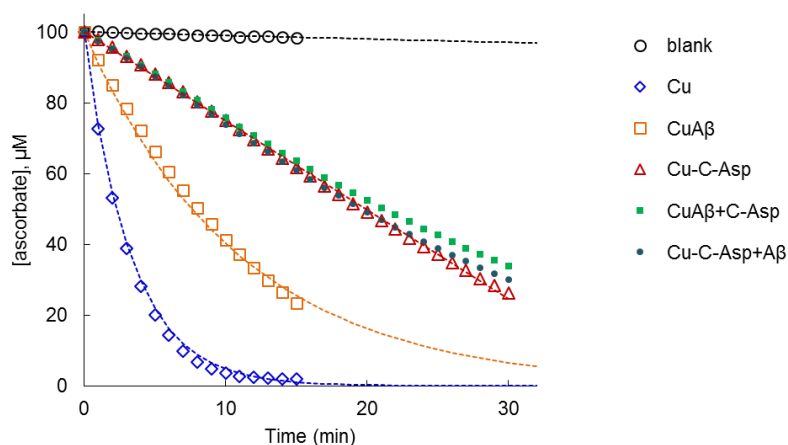
Besides their involvement in A $\beta$  aggregation, redox-active Cu(II) ions can also induce oxidative stress in the presence of biological reducing agents like ascorbate. The toxic effects of Cu(II) are specific to its interaction with A $\beta$ (1-40) and A $\beta$ (1-42), which generates ROS in the presence of dioxygen, thereby inducing oxidative stress.<sup>26</sup>

Therefore, a well-designed MPAC should not be limited to competitive Cu(II) binding with A $\beta$ , but it should also be able to prevent or, at least, to lessen copper-catalyzed oxidative stress.

Figure 6 displays the comparative results achieved regarding the generation of ROS under aerobic conditions and using ascorbate as reducing agent, in the presence of CuCl<sub>2</sub> and Cu-A $\beta$ (1-16) or Cu-**C-Asp**. The oxidation of ascorbate was monitored by spectrophotometry during a period of 30 min. The kinetic curves obtained show that Cu-A $\beta$ (1-16) consumed 77% of the initial amount of ascorbate after 15 min, while ascorbate consumption was only of 38% for Cu-**C-Asp**. Accordingly, complexation of copper by **C-Asp** gives rise to a lessening of the generation of toxic ROS (compared to A $\beta$ (1-16)), and therefore makes metal mislocation safer for the organism. The kinetics of ascorbate oxidation was also followed right after adding **C-Asp** to a Cu-A $\beta$  solution, and *vice versa*, for 30 min. As evidenced in Figure 6, almost

indistinguishable curves were obtained in both cases, their profiles resembling that resulting from ascorbate consumption in the presence of Cu-C-Asp; however, some deviations from zero-order kinetics are observed at longer times, as noticed for Cu-A $\beta$ . These data suggest that a rapid equilibrium between Cu-A $\beta$ (1-16) and the Cu-C-Asp species is established, with a clear predominance of the formation of Cu-C-Asp over Cu-A $\beta$ (1-16).

In addition to the above-described ability to lessen copper-induced oxidative stress, the soft chelating character of MPAC-like molecules as C-Asp is expected to avoid the alteration of the native metal distribution and thus the normal functioning of the brain.



**Figure 6.** ROS formation mediated by CuCl<sub>2</sub>, Cu(II)-C-Asp, and Cu(II)-A $\beta$ (1-16), determined by the consumption of ascorbate through its absorbance at 265 nm. The CuA $\beta$ +C-Asp and Cu-C-Asp+A $\beta$  samples were preincubated with the first ligand; the second ligand was added just before starting to monitor the ascorbate consumption; [CuCl<sub>2</sub>] = 5  $\mu$ M, [C-Asp] = [A $\beta$ (1-16)] = 5.5  $\mu$ M, [ascorbate] = 100  $\mu$ M, phosphate buffer 100 mM, pH 7.4. Data fitting to zero- (blank, Cu-C-Asp) and first-order kinetics (Cu, CuA $\beta$ ) is depicted by dotted lines.



## CONCLUSIONS

The exploration of small peptides as MPACs is relatively recent in the field of anti-AD therapeutics. Five His-containing decapeptides, showing different degrees of conformational constrain and structural pre-organization, have been tested for their potential ability to interfere with the deleterious interaction of A $\beta$  with Cu(II) ions, a pathogenic process that takes place in AD.

The five peptides used in the present study, *viz.* **C-Asp**, **C-Asn**, **O-Asp**, **OD<sub>Pro</sub>-Asp** and **O-Asn**, are structurally pre-organized for Cu(II) binding. The cyclic peptide **C-Asp**, displaying the highest Cu(II)-binding affinity, in the same order of magnitude as that of A $\beta$ , was the only one capable of competing with the amyloid protein. *In vitro* aggregation studies with A $\beta$ (1-40) revealed that **C-Asp** could hamper the formation of copper-stabilized A $\beta$  oligomers, facilitating the typical fibrillation pathway of monomeric A $\beta$ . **C-Asp** complexation also reduces the copper-catalyzed production of ROS. The results obtained highlight the potential of peptidic scaffolds as copper-A $\beta$  attenuating compounds and therefore, as potential anti-AD therapeutics.

## EXPERIMENTAL SECTION

**Peptide synthesis and purification.** The peptides **C-Asp**, **C-Asn**, **O-Asp**, **OD<sub>Pro</sub>-Asp**, and **O-Asn** were synthesized following procedures reported by Frago *et al.*<sup>15b, 15c</sup> CuCl<sub>2</sub>·2H<sub>2</sub>O was used as a source of Cu(II) ions in all the studies described in the present report. A $\beta$ (1-16) and A $\beta$ (1-40) were purchased as trifluoroacetate salts from Innovagen (Sweden) and Bachem (Switzerland), respectively, and used without further purification.

**Stock solutions.** Fresh solutions of the purified peptides in Milli-Q water were prepared for the different studies and the concentrations of peptide were determined by UV/Vis spectroscopy by

using the extinction coefficient of Trp ( $\epsilon_{280\text{nm}} = 5690 \text{ M}^{-1} \text{ cm}^{-1}$ ). The absorption spectra were recorded on a Varian Cary 100 Scan spectrophotometer at room temperature employing a 1 cm path length cuvette.

### **Fluorescence spectroscopy**

Fluorescence measurements were carried out using a HORIBA Jobin–Yvon iHR320 spectrofluorometer at room temperature. The photomultiplier detector voltage was set at 950 V and the instrument excitation and emission slits were both set at 5 nm. In all cases, the pH was fixed at 7.4 with 10 mM HEPES buffer, except for the EPR experiments, where 100 mM HEPES was employed instead.

*Calculation of the conditional binding constants:* The apparent affinity constants of the Cu(II) complexes with **C-Asp**, **C-Asn**, **O-Asp**, **ODPro-Asp**, and **O-Asn** were calculated by fitting the fluorescence titration data to Equation (1) (see above). Since HEPES, the buffer used for the measurements, can form a 1:1 complex with Cu(II) and can thus act as a weak competitor, its influence on the binding equilibrium was taken into account.<sup>20</sup> Therefore, the affinity constants at zero buffer concentration, that is, the conditional binding constants ( $K^{\text{cond}}$ ), were determined. For very large quantities of HEPES, with respect to the other components present in the solution, Equation (2) (see above) can be applied.

### **Mass spectrometry**

To assess the potential formation of binary and ternary Cu(II) complexes between A $\beta$ (1-16) (in excess) and the peptides, MALDI-TOF spectra were acquired on a 4800 Maldi-TOF/TOF (ABSciex), using 2,6-dihydroxyacetophenone (DHAP) as matrix. The spectra were acquired in positive-ion and negative-ion modes. The solutions were prepared in Milli-Q water with [CuCl<sub>2</sub>]

= 0.1 mM, [peptide] = 0.11 mM, [A $\beta$ (1-16)] = 0.22 or 0.44 mM. The pH was adjusted to pH 7.4 with NaOH.

For the studies of the effect of **C-Asp** on the aggregation of a 1:1 mixture of Cu-A $\beta$ (1-40) preincubated for 2 h, electrospray ionization (ESI) mass spectra were recorded on a LC/MSD-TOF spectrometer (Agilent Technologies) equipped with an electrospray ionization (ESI) source. The spectra were acquired in the positive-ion mode at 125 V. ThT-free samples obtained after 24 h incubation in 10 mM ammonium formate were centrifuged at 13000 rpm for 30 min. The supernatants and the pellets containing the aggregates were analysed separately in the same medium (see Fig. S12).

### **Electron Paramagnetic Resonance**

X-band EPR spectra of frozen solutions were recorded at 77 K on a Bruker ESP300 spectrometer. WINEPR 2.11 software (Bruker) was used to process and simulate the spectra. Aqueous 0.1 mM Cu<sup>2+</sup> solutions containing CuCl<sub>2</sub>/peptide mixtures with molar ratio of 1:1.1 or CuCl<sub>2</sub>/peptide/A $\beta$ (1-16) mixtures with molar ratios of 1:1.1:2.2 or 1:1.1:4.4 were used in 100 mM HEPES buffer (pH 7.4).

### ***In vitro* A $\beta$ (1–40) aggregation kinetic studies**

*Preparation of aggregate-free amyloid- $\beta$* : A $\beta$ (1–40) (5 mg) was solubilized in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP; 1 mL) under vigorous stirring at room temperature for 1 h. The resulting solution was sonicated for 30 min and subsequently stirred at room temperature for an additional hour. The solution was then maintained at 4 °C for 30 min to avoid solvent evaporation during aliquot collection. Finally, aliquots of soluble A $\beta$ (1–40) were collected and HFIP was evaporated under a gentle stream of nitrogen. The samples were stored at -20 °C.

*Aggregation studies:* The A $\beta$  aliquots were re-suspended in 50  $\mu$ L of DMSO and the monomers were solubilized using sonication for 10 min. 50  $\mu$ L of 500  $\mu$ M ThT and the appropriate amount of Cu(II) salt, stock solution of peptide and PBS (pH 7.4) were then added to obtain final solutions with a concentration of 25  $\mu$ M A $\beta$ (1–40) and of 31.2  $\mu$ M ThT in PBS containing 5% (v/v) DMSO. For the kinetic assays, the samples were placed in a 96-well plate at 37 °C and stirred at 700 rpm with double orbital mode. The course of the aggregation was followed *via* ThT fluorescence using a FLUOstar OMEGA plate reader (BMG Labtech GmbH), equipped with excitation and emission filters ( $\lambda_{\text{exc}}$  440 nm;  $\lambda_{\text{em}}$  490 nm).

Cu-induced oligomeric A $\beta$ (1–40) species were generated by the incubation of monomeric A $\beta$  with an equimolar amount of CuCl<sub>2</sub> in PBS for 2 h at 37 °C, under double orbital agitation at 700 rpm. Their formation was confirmed through ThT emission and by TEM analysis.

### **Consumption of ascorbate**

Absorbance measurements at 265 nm (maximum absorption of ascorbate) were carried out at room temperature in 100 mM phosphate buffer at pH 7.4, under aerobic conditions. The buffer solution was previously treated with Chelex 100 resin (Bio-Rad) to remove metal traces. The absorbance was registered over time for 30 minutes.

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### **Author Contributions**

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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### **ABBREVIATIONS**

AD: Alzheimer's disease

MPAC: Metal-Protein Attenuating Compounds

ATCUN: Amino Terminal Copper and Nickel binding motif

ThT: Thioflavin T

### **ASSOCIATED CONTENT**

**Supporting Information.** A PDF file containing Figures S1-S14 and Tables S1 and S2, is available free of charge.

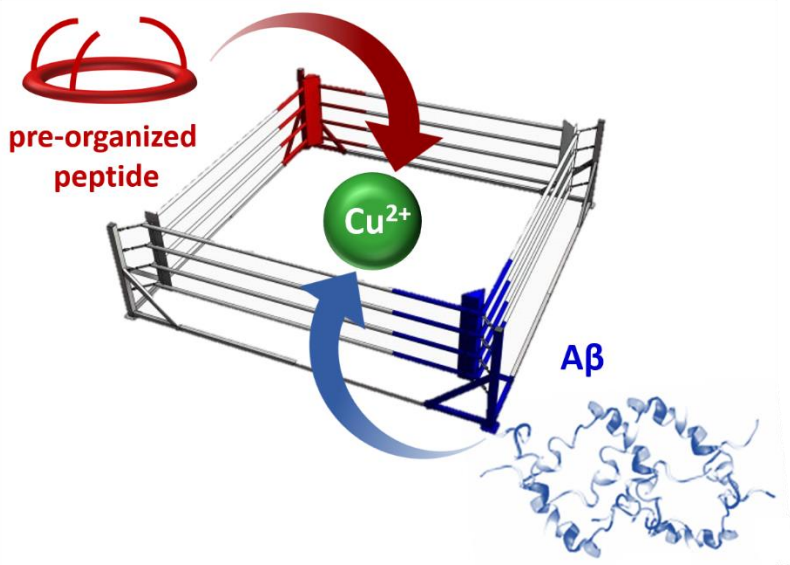
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## TABLE OF CONTENTS (TOC)



**SYNOPSIS:** The ability of five peptidic scaffolds to compete with Aβ has been assessed, aimed to lessen the deleterious interaction of Cu(II) with Aβ. The present study shows that (cyclic) peptides, pre-organized for Cu(II) binding, may be applied for the development of potential copper-Aβ attenuating compounds.