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## Current understanding of metal-dependent amyloid- $\beta$ aggregation and toxicity

Yelim Yi and Mi Hee Lim \*

The discovery of effective therapeutics targeting amyloid- $\beta$  (A $\beta$ ) aggregates for Alzheimer's disease (AD) has been very challenging, which suggests its complicated etiology associated with multiple pathogenic elements. In AD-affected brains, highly concentrated metals, such as copper and zinc, are found in senile plaques mainly composed of A $\beta$  aggregates. These metal ions are coordinated to A $\beta$  and affect its aggregation and toxicity profiles. In this review, we illustrate the current view on molecular insights into the assembly of A $\beta$  peptides in the absence and presence of metal ions as well as the effect of metal ions on their toxicity.

### Introduction

Globally, over 55 million people are living with dementia associated with population aging.<sup>1,2</sup> Alzheimer's disease (AD) is the most common form of dementia that is neuropathologically characterized by the accumulation of protein aggregates, such as amyloid- $\beta$  (A $\beta$ ) aggregates.<sup>3</sup> The amyloid cascade hypothesis claims A $\beta$  as a primary causative factor of AD and, thus, research efforts have been made towards developing therapeutic agents that can control A $\beta$  species.<sup>4,5</sup> The development of human monoclonal antibodies that target A $\beta$  aggregates further supports this hypothesis. For example, aducanumab was approved as the first disease-modifying treatment for AD by the United States Food and Drug Administration.<sup>5,6</sup> Unfortunately, the use of aducanumab against AD is questionable. Very recently, lecanemab was tested in the phase III clinical trial, demonstrating that it has better safety and efficacy than aducanumab.<sup>7-9</sup> In addition, there has been a call for the expansion or modification of the amyloid cascade hypothesis based on emerging evidence on the inter-relationship between A $\beta$  and other pathogenic elements found in AD-affected brains.<sup>3,10,11</sup>

Given that high concentrations of metals (*e.g.*, 0.4 mM for copper and 1.0 mM for zinc) are observed in senile plaques mainly composed of A $\beta$  aggregates, metal ions and A $\beta$  are suggested to be mutually involved in the pathogenesis of AD.<sup>3,12-17</sup> Labile metal ions, such as Cu(I/II) and Zn(II), can be released into the synaptic cleft and bind to A $\beta$  peptides forming metal-A $\beta$  complexes.<sup>18-20</sup> Such complexation between metal ions and A $\beta$  can affect its aggregation pathways producing toxic A $\beta$  aggregates (*e.g.*, soluble and structured oligomers).<sup>21-26</sup>

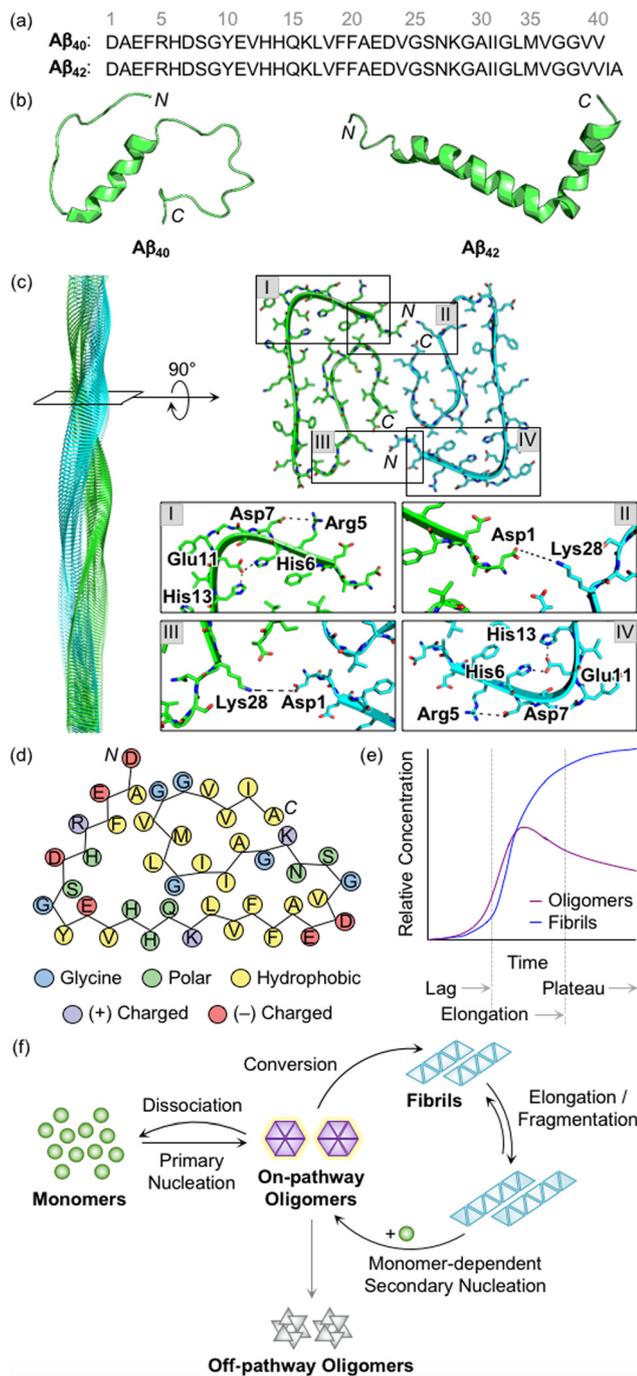
Furthermore, redox-active metal ions [*e.g.*, Cu(I/II)] bound and unbound to A $\beta$  can catalytically generate reactive oxygen species (ROS) and induce oxidative stress leading to neuronal cell death. Based on these reactivities, numerous studies have attempted to elucidate the A $\beta$ -related pathology associated with metal ions at the molecular level.<sup>3,10-12,18</sup> In this review, we illustrate the aggregation pathways of A $\beta$  in the absence and presence of metal ions, with an emphasis on the molecular mechanisms underlying its self-assembly. The influence of metal ions on the toxicity profile of A $\beta$  is also described. Overall, this review provides insight into the roles of metal ions in the pathogenic characteristics of A $\beta$  based on bio-inorganic chemistry.

### Aggregation of A $\beta$ peptides

A $\beta$  peptides with 38–43 amino acid residues in length are generated by the proteolysis of amyloid precursor protein (APP).<sup>3,27,28</sup>  $\beta$ - and  $\gamma$ -Secretases cleave APP at extracellular and intracellular regions, respectively, mainly producing two A $\beta$  isoforms, A $\beta_{40}$  and A $\beta_{42}$  (Fig. 1a).<sup>29</sup> A $\beta$  peptides are intrinsically disordered and, thus, their three-dimensional structures have not been fully determined.<sup>3,29</sup> A large amount of research has been dedicated to finding optimal experimental conditions that can stabilize A $\beta$  monomers. For example, Ramamoorthy's group reported a structure of monomeric A $\beta_{40}$  in aqueous media that was identified by high-resolution solution nuclear magnetic resonance (NMR) spectroscopy.<sup>30</sup> As presented in Fig. 1b, the central region (*i.e.*, His13–Asp23) of A $\beta$  was revealed to form a  $3_{10}$  helix. The *N*- and *C*-terminal regions relatively lacked structured conformations, but they were not completely unstructured, as local interactions between the side chains of amino acid residues beside the helical region

Department of Chemistry, Korea Advanced Institute of Science and Technology (KAIST), Daejeon 34141, Republic of Korea. E-mail: miheelim@kaist.ac.kr





**Fig. 1** Structures of Aβ peptides and their aggregation. (a) Amino acid sequences of Aβ<sub>40</sub> and Aβ<sub>42</sub>. (b) Examples of previously reported Aβ monomers (for Aβ<sub>40</sub>, PDB 2LFM,<sup>30</sup> for Aβ<sub>42</sub>, PDB 1IYT<sup>31</sup>). (c) Example of a previously reported Aβ<sub>42</sub> fibril (PDB 1IYT<sup>31</sup>). The salt bridges that contribute to the stabilization of fibrillar forms are indicated in I–IV. (d) Schematic view of a fibrillar structure of Aβ<sub>42</sub> with amino acid residues colored according to their polarity and charge states. Three His residues in Aβ are classified into polar amino acid residues based on its chemical environment at pH 7.4. (e) Schematic representation of relative concentrations of oligomeric and fibrillary Aβ as a function of time.<sup>52</sup> The increase in the concentration of fibrils in a sigmoidal manner presents the macroscopic aggregation of Aβ that is typically divided into lag, elongation, and plateau phases. Reproduced with permission from ref. 52. Copyright© 2020 Springer Nature. (f) Schematic illustration of microscopic steps involved in Aβ aggregation.<sup>37</sup> Reproduced with permission from ref. 37. Copyright© 2022 AIP Publishing.

generated twists and turns. In addition, the structure of monomeric Aβ<sub>42</sub> was characterized in the cellular membrane-mimicking apolar environment by solution NMR and circular dichroism (CD) spectroscopies.<sup>31</sup> In a mixture of water and hexafluoroisopropanol, the Aβ<sub>42</sub> monomer showed two helical regions at Ser8–Gly25 and Lys28–Gly38 that are connected by a β-turn. Aβ monomers aggregated into amyloid fibrils with a conformational transition of helical regions into β-strand structures.<sup>3,28,29</sup> Each β-strand was perpendicularly organized to a fibril axis, as shown in Fig. 1c. Multiple driving forces, including hydrophobic contacts between hydrophobic amino acid residues in the central and C-terminal regions, intermolecular hydrogen bonds, and salt bridges, can contribute to forming and stabilizing Aβ fibrils, as illustrated in Fig. 1c and d.<sup>28,29,32,33</sup>

During the aggregation processes, heterogeneous Aβ aggregates are generated with variable sizes and morphologies.<sup>3,12,28</sup> As indicated in Fig. 1e, profiling the aggregation kinetics of Aβ at the macroscopic level portrays three stages: lag, elongation, and plateau phases.<sup>3</sup> The overall aggregation pathways of Aβ can be further analyzed at the microscopic level, as depicted in Fig. 1f.<sup>34–39</sup> The assembly of monomers is initiated by the primary nucleation step that generally presents the step for forming nuclei from monomers. Nuclei are often delineated as the smallest aggregates for which the addition of monomers preferably occurs rather than the loss of monomers.<sup>40,41</sup> The definition of oligomers, which possibly correspond to nuclei, is different depending on the literature. The aggregates, except for monomers and fibrils, are broadly regarded as oligomers.<sup>19</sup> In particular, oligomers may refer to aggregates with certain sizes (*e.g.*, from dimer to triacontamer), aggregation rates, or morphologies, distinct from those of fibrils.<sup>40,42</sup> In some reports, oligomers can be specific depending on experimental systems, such as preparation procedures and analytic methods.<sup>40,43,44</sup> It has not been clear which oligomeric species could be nuclei. Following the aggregation, oligomers can be converted into fibrils that are β-sheet-rich aggregates.<sup>37</sup> Through the elongation and fragmentation steps (Fig. 1f), the size of fibrils is varied by providing new ends of the length extension and breaking down the fibrils, respectively.<sup>34–38</sup> These fibrils can be offered as surfaces that catalyze the generation of new nuclei (*e.g.*, oligomers) *via* secondary nucleation.<sup>45–47</sup> The aggregation of Aβ could include a process of monomer-dependent secondary nucleation, whereby monomers preferentially generate nuclei on the surface of preformed aggregates.<sup>35,36,48</sup>

In the case of oligomers as intermediates for Aβ aggregation, they can be subdivided into two classes: on-pathway or off-pathway oligomers (Fig. 1f).<sup>26,49,50</sup> When oligomers aggregate into fibrils, the process can be regarded to follow the on-pathway aggregation. On the other hand, oligomers generated through alternative aggregation pathways are accepted to be classified as off-pathway oligomers. It should be noted that this binary definition for oligomers could be unclear because oligomers in a heterogeneous population undergo multiple fates that cannot be definitely specified.<sup>51</sup> For example, some oligomers can be dissociated back to monomers, categorized as neither on-pathway oligomers nor off-pathway oligomers. The competition between the dissociation of oligomers and the



formation of fibrils was supported by the studies of aggregation kinetics that measure the concentrations of oligomers and fibrils, as represented in Fig. 1e.<sup>52,53</sup> The results obtained by the mathematical fitting of rate equations to aggregation curves denote that less than 10% of oligomers are converted into fibrillar species, whereas the others disassemble into the monomers. Knowles and coworkers showed a non-binary and quantitative definition in which an oligomer is assigned with a value (*e.g.*, between 0 and 1) that describes its relative contribution to fibril formation, rather than the binary definition for oligomers, which may be more appropriate to establish a general concept for the aggregation of A $\beta$ .<sup>51</sup> Moreover, research progress has been made towards connecting the results from macroscopic and microscopic analyses for the mechanisms of A $\beta$  aggregation, which suggests that multiple microscopic steps can take part in one macroscopic aggregation phase.<sup>40</sup> It should be noted that in addition to the heterogeneous nature of intrinsically unstructured A $\beta$ , the analysis of its self-assembly is challenging because of its different aggregation behaviors depending on experimental conditions (*e.g.*, the purity and concentration of A $\beta$ , pH, ionic strength, and temperature).<sup>41</sup>

## Change in the properties of A $\beta$ through interactions with metal ions

### Metal coordination to A $\beta$

The amino acid residues in A $\beta$  responsible for metal binding have been identified by multiple biophysical methods (Fig. 2a–d). In the case of Cu(II) with the d<sup>9</sup> electronic configuration, two components of Cu(II)–A $\beta$  complexes were determined at different pHs by electron paramagnetic resonance (EPR), CD, electronic absorption, X-ray absorption, and NMR spectroscopies.<sup>19,54,55</sup> At physiological pH, the *N*-terminal primary amine, two imidazole nitrogen (N) donor atoms from His6 and His13 or His14, and the oxygen (O) donor atom from the backbone carbonyl group between Asp1 and Ala2 coordinate to the Cu(II) center, as shown in component I (3N1O coordination; Fig. 2a).<sup>54,56–60</sup> Cu(II)–A $\beta$  complexes have a distorted square planar or square pyramidal geometry possibly with a weakly bound carboxylate group from the side chain of Asp1, Glu3, Asp7, or Glu11 or a water molecule at the apical position as a fifth ligand.<sup>54,56–58,61,62</sup> The dissociation constant ( $K_d$ ) value of the Cu(II)–A $\beta$  complex was reported in a nanomolar range.<sup>63–65</sup> The second Cu(II) binding to A $\beta$  could occur with at least two orders of magnitude weaker binding affinity, relative to the first Cu(II) coordination; however, the second Cu(II)-binding site has not been established.<sup>66–69</sup> At relatively high pH (*ca.* pH  $\geq$  8), several potential Cu(II)-binding sites in component II were suggested that include a deprotonated backbone amide moiety between Asp1 and Ala2: (i) 4N coordination with His6, His13, and His14 and either the *N*-terminal primary amine or the deprotonated backbone amide group between Asp1 and Ala2;<sup>67,70</sup> (ii) 3N1O coordination with three N donor atoms (imidazole N donor atoms from His6, His13, and His14 or the *N*-terminal primary amine, the deprotonated backbone amide group between Asp1 and Ala2, and one

imidazole N donor atom from His6, His13, or His14) and one O donor atom from the backbone carbonyl moiety between Ala2 and Glu3.<sup>54,56–58,60</sup> The carboxylate group from the side chain of Asp1, Glu3, Asp7, or Glu11 is proposed to be at the apical position as the fifth ligand.<sup>54,56,57,71</sup> This variation in the coordination sphere of Cu(II)–A $\beta$  complexes depending on the pH can be a factor for altering A $\beta$  aggregation (*vide infra*).

Cu(I) coordination to A $\beta$  may occur *via* three different combinations of His6, His13, and His14 in a linear geometry, as illustrated in Fig. 2b.<sup>55,59,72–74</sup> Since there are distinct geometries between Cu(II)–A $\beta$  (*i.e.*, distorted square planar geometry) and Cu(I)–A $\beta$  (*i.e.*, linear geometry), a large reorganization energy ( $\lambda = ca.$  1.4 eV) is required for efficient electron transfer.<sup>75</sup> To overcome this energy demand, an intermediate species (Fig. 2c) that is in equilibrium between Cu(II)–A $\beta$  and Cu(I)–A $\beta$  has been proposed as a redox-competent state in which binding modes of Cu(I) and Cu(II) are similar and, consequently, the reorganization energy for one-electron transfer ( $\lambda = 0.3$  eV) is much less. In the cellular environment that includes reducing agents (*e.g.*, ascorbate and glutathione), Cu(II) with and without A $\beta$  can be reduced to Cu(I), as shown in Fig. 2e.<sup>59,73,76,77</sup> Cu(I)–A $\beta$  can react with O<sub>2</sub> and, subsequently, produce ROS (*e.g.*, O<sub>2</sub><sup>•-</sup>, H<sub>2</sub>O<sub>2</sub>, and •OH) that can oxidatively modify A $\beta$  with a consequent change in its aggregation.<sup>71,78–81</sup> A wide range of  $K_d$  values for Cu(I)–A $\beta$  (femtomolar to submicromolar) was reported depending on experimental conditions.<sup>82,83</sup>

Zn(II) is a d<sup>10</sup> metal ion that exhibits less structural variations upon complexation with A $\beta$ , compared to Cu(II). Possible binding modes of Zn(II)–A $\beta$  complexes have been determined by NMR and X-ray absorption spectroscopies.<sup>55,84,85</sup> As displayed in Fig. 2d, Zn(II) binding to A $\beta$  at pH 7.4 can form a tetrahedral geometry through two imidazole N donor atoms from His6 and His13 or His14 and two O donor atoms from the carboxylate groups in the side chains of Asp1, Glu3, or Asp7 and Glu11.<sup>84,85</sup> A water molecule is able to replace Asp1, Glu3, or Asp7.<sup>84</sup> The  $K_d$  value of Zn(II)–A $\beta$  was reported to be in a micromolar range.<sup>65,86</sup>

It should be noted that the amino acid residues participating in metal coordination can be varied depending on the aggregation states of A $\beta$ . Very limited studies indicated that the ligands that do not participate in metal coordination in monomers, such as carboxylate groups from the side chain of Glu3, Glu11, Glu22, or Asp23 and the *C*-terminus, are possibly involved in metal binding to A $\beta$  aggregates.<sup>87,88</sup> In the case of A $\beta$  aggregates, metal ions may have distinct binding modes, compared to those found in A $\beta$  monomers, since the alignment of peptides in the aggregates can induce intermolecular interactions, which may form metal-binding sites between peptides.<sup>88–93</sup> The structural analysis of metal-bound A $\beta$  aggregates would be further carried out in detail to advance our understanding of metal-associated A $\beta$  aggregation.

### Metal-to-peptide stoichiometry

The aggregation of A $\beta$  is shown to be significantly varied depending on the metal-to-A $\beta$  ratio. For example, the sub-equimolar



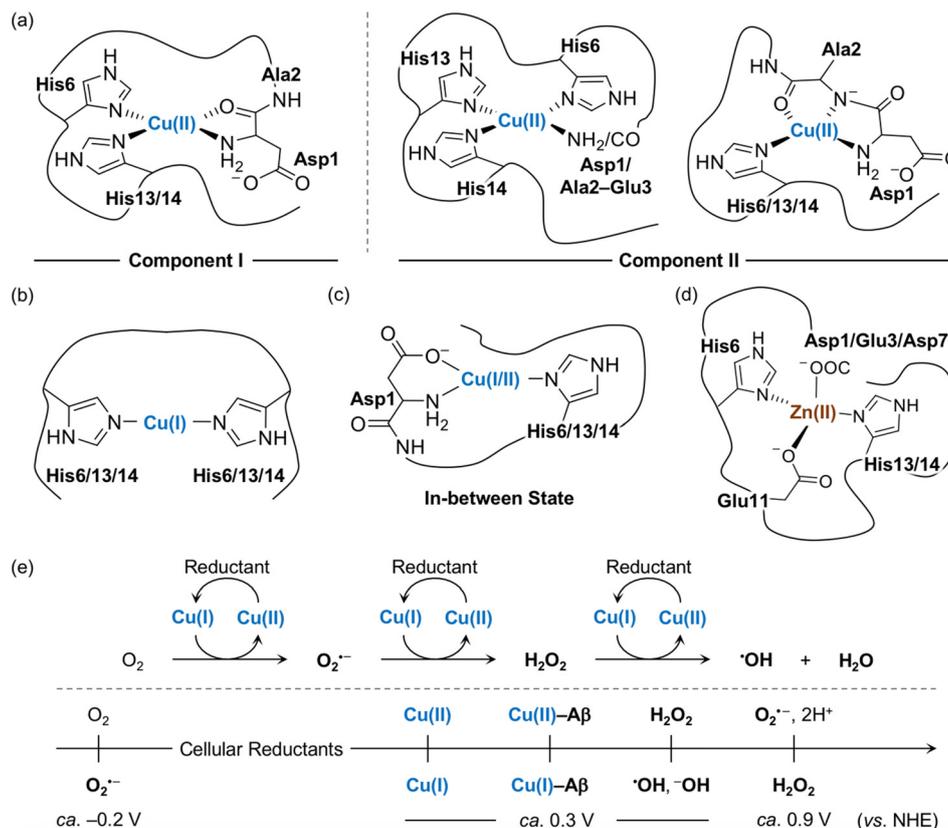


Fig. 2 Metal-binding properties of A $\beta$ . Examples of structures of (a) Cu(II)-A $\beta$ , (b) Cu(I)-A $\beta$ , (c) their in-between state, and (d) Zn(II)-A $\beta$ . Possible fifth ligands on the metal centers are omitted in the figure for clarity. (e) Scheme of ROS formation catalyzed by Cu(I/II) in the presence of a reductant and redox potentials of the species involved in the reactions with respect to the normal hydrogen electrode (NHE).<sup>71</sup> Cellular reductants include ascorbate and glutathione.

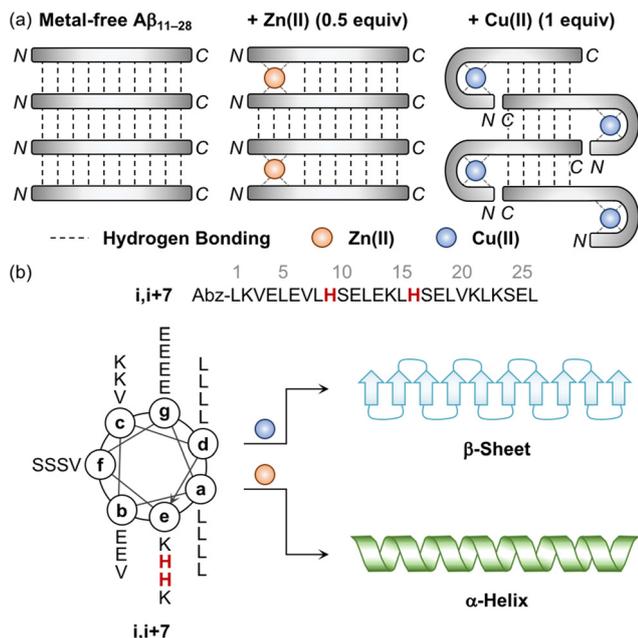
metal ion possibly bridges two peptides, which triggers the dimerization of A $\beta$ .<sup>90</sup> These dimers can serve as a nucleus that facilitates the aggregation of A $\beta$ . This concept was experimentally demonstrated employing a model peptide, A $\beta_{11-28}$ , that contains a hydrophobic region (*i.e.*, Leu17-Ala21) responsible for initiating the aggregation by hydrophobic interactions and metal-binding sites.<sup>94-96</sup> As presented in Fig. 3a, when sub-stoichiometric Zn(II) was treated with A $\beta_{11-28}$ , the formation of the A $\beta_{11-28}$  dimer bridged by Zn(II) through His13 and His14 was observed by X-ray absorption and NMR spectroscopies.<sup>95,96</sup> Upon incubation, amyloid fibrils were produced, confirmed by transmission electron microscopy (TEM) as well as the turbidity and fluorescence assays. This suggests that the fast exchange of Zn(II) between monomers and aggregates enables the generation of nuclei, accelerating the formation of amyloid fibrils. In the presence of an equimolar concentration of metal ions, two metal-A $\beta$  complexes could be conceptually aligned by hydrogen bonds between the backbone amide groups, which could eventually form  $\beta$ -sheets. When 1 equivalent of Cu(II) was bound to the NH<sub>2</sub>-Xxx-Xxx-His motif at the N-terminal region of A $\beta_{11-28}$ , however, the number of hydrogen bonds decreased and, consequently, lowered the fibril formation, as monitored by the turbidity and fluorescence assays and TEM.<sup>94</sup> When the metal-to-A $\beta$  stoichiometry was above 1, an excess amount of metal ions can nonspecifically bind

to A $\beta$  peptides, which could deform hydrogen-bond networks between backbone amide groups and, thus, inhibit the production of amyloid fibrils, resulting in amorphous aggregates.<sup>90</sup> It should be noted that pathophysiologically relevant metal-to-peptide stoichiometry was not clearly defined because of the difficulty in determining exact concentrations of heterogeneous A $\beta$  species and metal ions that are dependent on the diseased state.<sup>13,94,97,98</sup> Further investigations would be valuable to elucidate the metal-binding properties of various-sized A $\beta$  peptides with different metal-to-peptide ratios.

### Charge state of A $\beta$

Given that hydrophobic interactions between A $\beta$  peptides are the main driving force towards their self-assembly, the peptides with an overall charge close to 0 would be more favorable for their rapid aggregation.<sup>12,19,90</sup> At physiological pH, the overall charge state of A $\beta$  is 3<sup>-</sup> (Fig. 1d) and, thus, Cu(I/II) or Zn(II) binding to A $\beta$  results in a net charge state nearer to 0, which can make metal-A $\beta$  complexes more prone to aggregation. In addition, metal binding to A $\beta$  lowers the pK<sub>a</sub> values (K<sub>a</sub>, acidity constant) of the ligands from the peptide and, consequently, deprotonates the amino acid residues (*e.g.*, backbone amide group), decreasing the net charge, as depicted in component II (Fig. 2a).<sup>67,70</sup> Zn(II) is a weaker Lewis acid than Cu(II) and, thus,





**Fig. 3** Influence of metal ions on the alignment and conformation of model peptides. (a) Possible metal-binding modes of  $A\beta_{11-28}$  depending on the type of metal ions and the metal-to-peptide stoichiometry.<sup>95</sup> The sub-equimolar concentration of  $Zn(II)$  can bridge two  $A\beta_{11-28}$  peptides, stabilizing their interaction and preferably forming  $\beta$ -sheets. In the presence of an equimolar amount of  $Cu(II)$ ,  $A\beta_{11-28}$  can wrap around the metal ion and, consequently, reduce the number of hydrogen bonds, which possibly hinders the alignment required to form  $\beta$ -sheets. Reproduced with permission from ref. 95. Copyright © 2010 Springer Nature. (b) Conformational change of the coiled coil peptide (*i.e.*,  $i,i+7$ ) upon the addition of metal ions. A helical wheel diagram presents the amino acid residues of the coiled coil peptide in a heptad repeat labeled as (a–g). The His residues that participate in metal binding are highlighted in red. Abz introduced at the *N*-terminal of the peptide indicates *o*-aminobenzoic acid.  $Cu(II)$  contributes to the conversion of the coiled coil peptide into the  $\beta$ -sheet-rich peptide, while  $Zn(II)$  stabilizes the  $\alpha$ -helical structure of the peptide.

it can detach protons from ligands upon coordination in a manner less than  $Cu(II)$ . The overall charge state is expected to be less negative when  $Zn(II)$  is bound to  $A\beta$ , compared to  $Cu(II)$ . Thus,  $Zn(II)$  may induce  $A\beta$  aggregation more efficiently than  $Cu(II)$ .<sup>19,90</sup> Therefore, both metal binding and ligand deprotonation are associated with the degree of  $A\beta$  aggregation.

### Metal-induced variation in the secondary structure of $A\beta$

Distinct effects of  $Cu(II)$  and  $Zn(II)$  on the change in the secondary structure of  $A\beta$  were monitored using a model peptide. Brezesinski and coworkers designed a coiled coil peptide (*i.e.*,  $i,i+7$ ) to mainly adopt the  $\alpha$ -helical conformation, as shown in Fig. 3b.<sup>89</sup> Two His residues capable of metal binding were included at the *i* and  $i+7$  positions of the peptide, considering the metal-binding mode of  $A\beta$  (Fig. 2a and d). The His6 and His13 residues of  $A\beta$  responsible for metal binding were also placed at the same positions. Three Val residues rendered the coiled coil peptide prone to transformation into the  $\beta$ -sheet structure within hours to days. Upon treatment of  $Cu(II)$ , the structural transition from  $\alpha$ -helix to  $\beta$ -sheet was

observed in the CD spectra in a time-dependent manner, while  $Zn(II)$  did not allow the peptide to form a  $\beta$ -sheet and maintain the  $\alpha$ -helical conformation. Mechanistic details have not been reported, but their distinct binding modes may induce a different influence on the secondary structure of the peptide.

## Impact of metal ions on the aggregation and toxicity of $A\beta$

The roles of metal ions in the aggregation of  $A\beta$  still remain elusive. The influence of metal ions on  $A\beta$  aggregation is valid based on the timescales for the reactions of the metal exchange between  $A\beta$  peptides (microseconds to seconds *in vitro*) as well as their aggregation (minutes to hours *in vitro*).<sup>70,90,99–102</sup> When metal ions bind to  $A\beta$ , the secondary structure of the peptide is altered, which can modify its aggregation forming structured or amorphous aggregates.<sup>19,103</sup> Depending on the aggregation extent and morphology, the resultant  $A\beta$  aggregates can exhibit toxic events in cellular environments. Furthermore,  $A\beta$  bound to redox-active  $Cu(I/II)$  catalytically produces ROS leading to oxidative stress and cell death.<sup>78–80</sup>

### Alteration in the aggregation pathways of $A\beta$ by metal ions

In this review, we introduce some examples of *in vitro* studies that illustrate the variation of  $A\beta$  aggregation by metal ions under physiologically relevant conditions (*e.g.*, pH 7.4 at 37 °C). Notionally, the formation of  $A\beta$  fibrils occurs more rapidly with sub-equimolar concentrations of metal ions, compared to that under metal-free conditions (*vide supra*). Yuan and coworkers demonstrated this concept according to the results obtained by the thioflavin-T (ThT) fluorescence assay and TEM.<sup>104</sup> ThT is a fluorescence dye that detects  $\beta$ -sheet-rich aggregates.<sup>13</sup> The ThT fluorescence intensity of  $A\beta_{40}$  with a sub-equimolar concentration of  $Cu(II)$  [ $Cu(II):A\beta = 0.25:1$ ] increased at the early stage of its aggregation, relative to that of metal-free  $A\beta_{40}$ . The resultant  $A\beta$  aggregates were observed to be fibrils, indicating that  $Cu(II)$  accelerated  $A\beta$  aggregation producing  $\beta$ -sheet-rich fibrils. The studies employing  $A\beta_{42}$  also displayed fibrillary morphology upon incubation with sub-equimolar  $Cu(II)$  [ $Cu(II):A\beta = 0.1:1$ ].<sup>105</sup> The counterexamples were reported, however. Hemmingsen's and Goto's groups determined that the generation of  $\beta$ -sheet-rich  $A\beta_{40}$  aggregates was retarded in the presence of sub-equimolar amounts of  $Cu(II)$  [ $Cu(II):A\beta = 0.1–0.55:1$ ] with the extension of the lag phase.<sup>106,107</sup> In addition, the studies with atomic force microscopy (AFM) presented the resultant  $A\beta$  aggregates with amorphous characteristics.<sup>107</sup> TEM studies with  $A\beta_{42}$  showed some moderate results with a mixture of filamentous and amorphous morphologies with sub-equimolar  $Cu(II)$  [ $Cu(II):A\beta = 0.6:1$ ].<sup>108</sup> In the case of  $Zn(II)$ , microscopic events upon  $A\beta_{40}$  aggregation were characterized by analyzing the fluorescence-based aggregation kinetics.<sup>109</sup> As a result, sub-stoichiometric concentrations of  $Zn(II)$  were revealed to reduce the elongation rate of  $A\beta_{40}$  [ $Zn(II):A\beta = 0.025–0.125:1$ ], but not completely inhibit the formation of amyloid fibrils.<sup>109</sup> NMR studies further elucidated that the



*N*-terminal region in A $\beta$ <sub>40</sub> could be transiently folded surrounding Zn(II), forming a metastable Zn(II)–A $\beta$  complex, which could subsequently modify the ends of fibrils where elongation takes place and retard amyloid fibrillization.<sup>109</sup>

Upon increasing the concentration of metal ions up to equimolar and supra-equimolar amounts, the metal-mediated dimerization of A $\beta$  could occur *via* bridging, but nonspecific metal binding to A $\beta$  could form amorphous aggregates (*vide supra*). These phenomena were monitored by the ThT and turbidity assays, CD spectroscopy, and microscopies.<sup>104,105,110–113</sup> Compared to the observations under metal-free conditions, the ThT fluorescence intensity in the overall macroscopic aggregation steps of both A $\beta$ <sub>40</sub> and A $\beta$ <sub>42</sub> was decreased in the presence of Cu(II) or Zn(II) with a metal-to-A $\beta$  ratio not less than 1 [Cu(II):A $\beta$  = 1–25:1; Zn(II):A $\beta$  = 1–3:1]. On the other hand, the turbidity of A $\beta$  samples increased, suggesting that metal ions could induce the generation of ThT-undetectable aggregates.<sup>104</sup> CD spectroscopic studies further manifested that metal ions lowered the  $\beta$ -sheet contents of A $\beta$  aggregates.<sup>110,112</sup> The morphology of the resultant A $\beta$  aggregates, confirmed by TEM and AFM, was unstructured.<sup>104,105,110,112,113</sup> Exley and coworkers reported that A $\beta$ <sub>42</sub> incubated with equimolar Cu(II) indicated fibrils that could be converted to be amorphous when supra-equimolar Cu(II) [Cu(II):A $\beta$  = 10:1] is treated with the peptide.<sup>108</sup> Further research to elucidate the mechanisms of A $\beta$  aggregation with a range of concentrations of Cu(II) has been carried out based on a quantitative analysis of aggregation kinetics. Heegaard and coworkers proposed the aggregation models of A $\beta$ <sub>40</sub> in the presence of Cu(II) [Cu(II):A $\beta$  = 0.25–5:1] by spectroscopies, microelectrophoresis, mass spectrometry, and microscopies.<sup>102</sup> A $\beta$  was bound to Cu(II) (timescale, milliseconds to seconds), forming a Cu(II)–A $\beta$  complex with 1:1 stoichiometry. This complex either aggregated (timescale, minutes) or remained soluble over a long time (timescale, hours to days) depending on the metal-to-peptide stoichiometry. Oligomers containing Cu(II) can have multiple conformations in a dynamic equilibrium, resulting in diverse morphologies of A $\beta$  aggregates at the endpoint of aggregation studies. Esbjörner and coworkers investigated the aggregation mechanisms of A $\beta$ <sub>42</sub> with Cu(I/II) [Cu(I/II):A $\beta$  = 0.5–2:1] by the global fitting of specific mathematical models to experimental data from the ThT assay with and without A $\beta$ <sub>42</sub> seeds.<sup>114</sup> As a result, Cu(I) and Cu(II) were revealed to inhibit the primary nucleation mildly and elongation of A $\beta$ <sub>42</sub>, respectively, but TEM images still showed amyloid fibrils. It should be noted that the results were unpredictable based on the aforementioned roles of metal ions in changing A $\beta$  aggregation and the inconsistency in previously reported observations could be due to the heterogeneous nature of A $\beta$  samples and various experimental conditions, including the concentration and source of A $\beta$  (*e.g.*, synthetic and recombinant);<sup>41</sup> an apparent fluorescence quenching in the presence of Cu(II) needs to be carefully interpreted due to the Cu(II)-induced inner filter effect.<sup>102,114,115</sup>

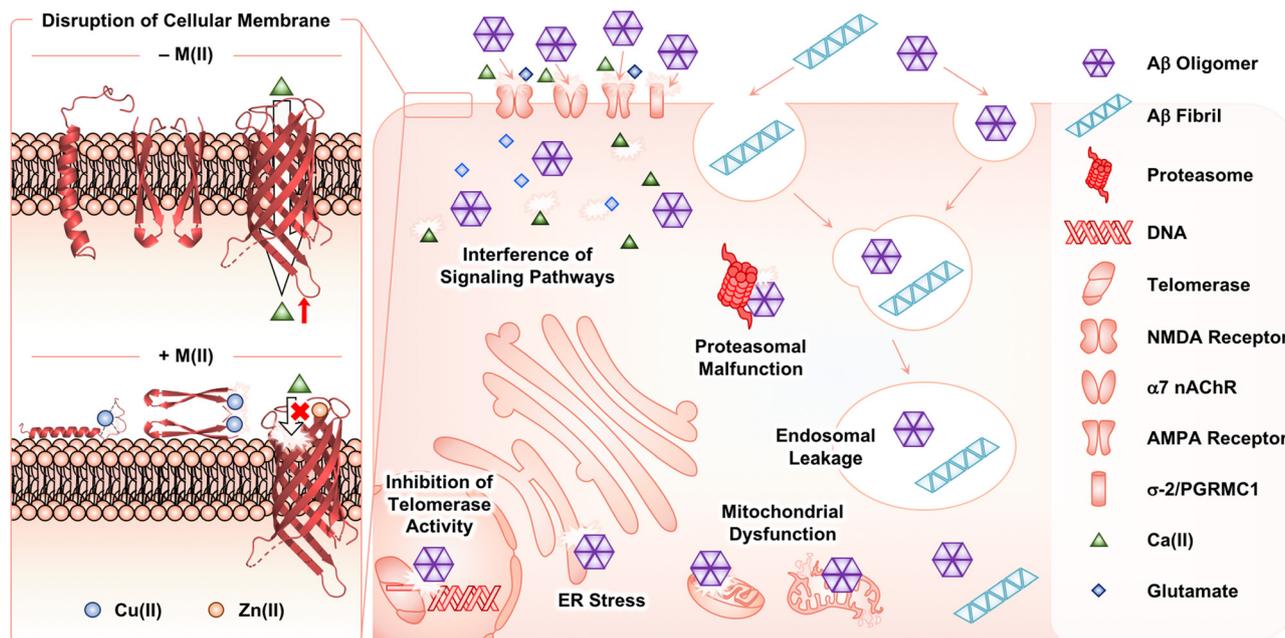
More specifically, according to the toxicity of soluble and structured A $\beta$  oligomers (*vide infra*), the effects of metal ions on the formation of oligomeric species should be further studied.

The aforementioned fluorescence assay and microscopies (*e.g.*, ThT assay and TEM) have limitations in detecting relatively small oligomers over large A $\beta$  aggregates such as fibrils.<sup>19,26,116,117</sup> Other methods can be used for monitoring A $\beta$  oligomers.<sup>118</sup> For example, gel electrophoresis with Western blotting is commonly used to analyze A $\beta$  species based on their molecular weight distribution.<sup>116,119</sup> Low molecular weight oligomers (*e.g.*, less than 70 kDa) that may be produced at the early aggregation stage can be resolved by this method.<sup>120</sup> The conformation of A $\beta$  aggregates can be further differentiated in Western blotting or dot blotting employing different antibodies [*e.g.*, anti-A $\beta$  antibody (6E10), anti-amyloid oligomer antibody (A11); anti-amyloid fibril antibody (OC)].<sup>26,118,120–122</sup> Moreover, electrospray ionization–ion mobility–mass spectrometry (ESI–IM–MS) is a technique capable of characterizing heterogeneous oligomers.<sup>118,123</sup> The mass and cross-sectional area of A $\beta$  species, observed by ESI–IM–MS, provide information on the size and conformation of A $\beta$  oligomers. The size distribution of A $\beta$  aggregates, including low molecular weight oligomers, can also be obtained by dynamic light scattering experiments.<sup>124</sup> The studies that demonstrate the formation of toxic A $\beta$  oligomers upon treatment of metal ions complementarily using the aforementioned techniques are very limited. These detailed investigations can assist in advancing our understanding of metal-associated A $\beta$  aggregation.

#### Disruption of cellular events by metal-associated A $\beta$ aggregates

Among A $\beta$  aggregates, soluble and structured oligomers are the main species that trigger toxicity in cellular environments.<sup>26</sup> Through secondary nucleation (Fig. 1f), A $\beta$  fibrils can contribute to aggravating the cytotoxicity by proliferating the production of oligomers.<sup>45–47</sup> The influence of metal ions on the A $\beta$ -induced toxicity has been mainly proposed by investigating the interactions of A $\beta$  with membranes in the absence and presence of metal ions. As illustrated in Fig. 4, A $\beta$  species are known to contact cellular membranes, compromising their structural integrity.<sup>26,125,126</sup> Under metal-free conditions, the interaction between A $\beta$  peptides and membranes results in an equilibrium shift from the  $\alpha$ -helical conformation to the  $\beta$ -sheet structure.<sup>125,126</sup> This change in their secondary structure triggers the aggregation of A $\beta$  near or within membranes, inducing the generation of membrane-penetrable pore structures, which can stimulate the leakage of Ca(II) that is a neurotransmitter essential for signal transduction.<sup>26,127,128</sup> Metal binding to A $\beta$  near membranes is also suggested to govern the structural perturbation of membranes leading to toxicity. In the presence of negatively charged unilamellar vesicles (LUVs) as a model system mimicking cellular membranes, EPR studies indicated that Cu(II) could bind to the *N*-terminal region of A $\beta$ <sub>42</sub>.<sup>129,130</sup> Upon Cu(II) binding to A $\beta$  in a solution of LUVs, its secondary structure was converted from  $\beta$ -sheet to  $\alpha$ -helix, which possibly penetrates membrane bilayers, as monitored by CD spectroscopy.<sup>129,130</sup> Dissimilar results were also reported depending on the type of artificial membranes. <sup>31</sup>P and <sup>2</sup>H solid-state NMR spectroscopic studies indicated that Cu(II) without A $\beta$  could destabilize the lipid





**Fig. 4** Possible toxic events induced by A $\beta$  oligomers and fibrils with and without metal ions in cellular environments. In the absence of metal ions, A $\beta$  species can interact with cellular membranes, changing their secondary structures at the surface or within the cell membranes. Consequently, A $\beta$  can aggregate near the membrane and form a membrane-permeable pore structure that possibly controls the influx and efflux of neurotransmitters [e.g., Ca(II)]. Such processes can be altered upon interaction of Cu(II) and Zn(II) with A $\beta$  species near the membranes. Extracellular A $\beta$  aggregates can be internalized by endocytosis and the receptors in cellular membranes. The intracellular accumulation of A $\beta$  aggregates can disrupt subcellular events.

layers of multilamellar vesicles and cause the formation of smaller vesicles.<sup>131</sup> In the presence of A $\beta_{42}$ , this effect of Cu(II) on the model membrane disappeared, possibly due to the competitive binding of Cu(II) between lipid layers and A $\beta$ . The studies using <sup>31</sup>P solid-state NMR spectroscopy further revealed that Cu(II)-A $\beta_{42}$  could interact with the surface of negatively charged phospholipid membranes.<sup>132</sup>

Moreover, Cu(II) can contribute to the dimerization of A $\beta_{42}$  via the formation of a His bridge.<sup>129,133</sup> When Cu(II) was treated with A $\beta$  at a metal-to-peptide ratio greater than 0.6, EPR spectra exhibited characteristic *g* and *A* values, indicative of producing an A $\beta$  dimer with a dinuclear Cu(II) center. These spectral patterns were not observed for A $\beta$  containing three His residues methylated at either the  $\pi$ - or  $\tau$ -N atom of the imidazole side chain, highlighting the importance of His residues in the dimerization of A $\beta$ . Solid-state NMR spectroscopic studies with LUVs that include <sup>31</sup>P-labeled phospholipid head groups under experimental conditions generating the Cu(II)-mediated His bridge presented the interaction between Cu(II)-A $\beta_{42}$  and the head groups of lipid membranes.<sup>133</sup> This suggests the binding of Cu(II)-A $\beta_{42}$  at the surface of membranes rather than the insertion within the bilayers. Cu(II)-bound His-bridged A $\beta$  dimers are shown to be toxic in primary cortical neurons.<sup>133</sup>

When A $\beta$  is further aggregated near membranes, and subsequently forms oligomers that can constitute a Ca(II) channel inserted into membranes, Zn(II) can interact with the channel. Rojas and coworkers illustrated that Zn(II) could bind to A $\beta_{40}$  oligomers incorporated into artificial membranes as a pore structure and inhibit the channel conductance, probably

attenuating the toxicity induced by A $\beta$  channels.<sup>134</sup> Such behaviors of Zn(II) toward A $\beta$  oligomers were reversed by the addition of *o*-phenanthroline as a Zn(II) chelator, corroborating the Zn(II)-mediated blockade of Ca(II) channels composed of A $\beta$  oligomers. Similar results have been reported under various experimental conditions (e.g., the type of membranes and pore structures composed of diverse lengths of A $\beta$ ).<sup>135–140</sup> Upon further aggregation of A $\beta$  oligomers into fibrils, the membranes were severely disrupted and, thus, the Ca(II) selectivity of the A $\beta$  channel was abolished. Zn(II) did not stop such uncontrollable leakage of Ca(II) from the channel.<sup>141</sup>

In addition to the surface of membranes, extracellular A $\beta$  aggregates produced in the absence and presence of metal ions can interact with transmembrane proteins [e.g., *N*-methyl-D-aspartate (NMDA) receptors,  $\alpha 7$  nicotinic acetylcholine receptor ( $\alpha 7$  nAChR),  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA receptor), and  $\sigma$ -2 receptor and progesterone receptor membrane component 1 ( $\sigma$ -2/PGRMC1)], perturbing cellular signaling pathways.<sup>142–146</sup> Upon internalization by the receptors and endocytosis, A $\beta$  aggregates can lead to the dysfunction of cellular organelles and components, such as endosomes, mitochondria, endoplasmic reticulum (ER), proteasome, and telomerase.<sup>147–151</sup>

#### Oxidative stress triggered by Cu(II)-A $\beta$ complexes

In the presence of a reducing agent, Cu(II)-A $\beta$  can participate in the generation of ROS (*vide supra*; Fig. 2e) and, consequently, impair cellular components through multiple pathways.<sup>3,10–12,18,152,153</sup> In the brains of AD patients, an increased level of the products



from the oxidation of lipids, proteins, and deoxyribonucleic acids (DNAs) is observed, compared to age-matched controls, which supports the relationship of oxidative damage to the pathology of AD.<sup>154</sup> In particular, oxidative modifications of A $\beta$  in a site-specific manner (*e.g.*, Asp, His, Phe, Tyr, and Met) are detected in the presence of Cu(II) and reductants, which could modulate its aggregation and toxicity profiles.<sup>78,155–157</sup> The Cu(II)-triggered oxidation of A $\beta$ <sub>40</sub> is also available in membrane-mimicking environments, implicating the possibility of lipid peroxidation through ROS generated from Cu(I/II)–A $\beta$  near membranes with the consequent neuronal cell death.<sup>158–160</sup> At the cellular level, the downstream response to ROS generation includes the impairment of protein expression, cell signaling, mitochondrial functions, and autophagy, as well as the promotion of inflammation and apoptosis.<sup>154,161–164</sup>

## Conclusions

A long journey in developing therapeutics targeting A $\beta$  has only prompted the exploration of the intertwined pathology associated with A $\beta$  and multiple pathological factors.<sup>3</sup> On the basis of heterogeneous A $\beta$  aggregates that are generated upon its self-assembly, a relationship between microscopic and macroscopic processes involved in A $\beta$  aggregation has been recently investigated to establish the aggregation mechanisms.<sup>34–39</sup> Increasing evidence suggests different facets of A $\beta$  connected with metal ions: metal coordination to A $\beta$  and metal-mediated aggregation and toxicity of A $\beta$ .<sup>3</sup> This highlights bioinorganic aspects of metal–A $\beta$  complexes to illuminate the roles of metal ions in the A $\beta$ -related pathology. Research endeavors discussed in this review can pave the way for elucidating the pathology of AD as well as developing effective therapeutics in the future.

## Conflicts of interest

There are no conflicts to declare.

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