The Rapid Exchange of Zinc^{2+} Enables Trace Levels to Profoundly Influence Amyloid-β Misfolding and Dominates Assembly Outcomes in Cu^{2+}/Zn^{2+} Mixtures

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[*]Running Title: *Trace Zinc Disrupts Aβ Fibre Assembly through Rapid Exchange*

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Abbreviations: Aβ, amyloid-beta; APP, amyloid precursor protein; ThT, thioflavin T; TEM, transverse electron microscopy; PTA, phosphotungstic acid; EDTA, ethylenediaminetetraacetic acid; UHQ, ultra-high quality; ZnT3, Zinc transporter-3.

ABSTRACT

The misfolding and self-assembly of amyloid-β (Aβ) into oligomers and fibres is fundamental to Alzheimer's disease pathology. Alzheimer's disease is a multifaceted disease. One factor that is thought to have a significant role in disease aetiology is Zn^{2+} homeostasis, which is disrupted in the brains of Alzheimer's disease sufferers, and has been shown to modulate Alzheimer's symptoms in animal models. Here we investigate how the kinetics of Aβ fibre growth are affected at a range of Zn^{2+} concentrations, and use transmission electron microscopy to characterise the aggregate assemblies formed. We demonstrate that for $A\beta_{(1-40)}$, and $A\beta_{(1-42)}$, as little as 0.01 mole equivalents of Zn^{2+} (100 nM) is sufficient to greatly perturb the formation of amyloid fibres irreversibly. Instead, $A\beta_{(1-40)}$ assembles into short rod-like structures that pack tightly together into ordered stacks, whereas $A\beta_{(1-42)}$ forms short crooked assemblies that knit together to form a mesh of disordered tangles. Our data suggests that a small number of Zn^{2+} ions are able to influence a great many Aβ molecules through the rapid exchange of Zn^{2+} between Aβ peptides. Surprisingly, although Cu^{2+} binds to Aβ ten-thousand times tighter than Zn^{2+}, the effect of Zn^{2+} on Aβ assembly dominates in Cu^{2+}/Zn^{2+} mixtures, suggesting trace levels of Zn^{2+} must have a profound effect on extracellular Aβ accumulation. Trace Zn^{2+} levels profoundly influences Aβ assembly even at concentrations weaker than its affinity for Aβ. These observations indicate inhibitors of fibre assembly do not necessarily have to be at high concentration and affinity to have a profound impact.

INTRODUCTION

It is estimated that there are currently 30 million people worldwide suffering with Alzheimer's disease, with incidence expected to double in the next 20 years [1]. The pathology of Alzheimer's disease is characterised by the misfolding of a small peptide, amyloid-β, and its subsequent aggregation into oligomers, fibres and plaques [2]. Amyloid-β (Aβ) is cleaved from amyloid-precursor protein (APP), largely forming the two alloforms $A\beta_{(1-40)}$ and $A\beta_{(1-42)}$, of which $A\beta_{(1-42)}$ more readily aggregates to form fibres and synaptotoxic oligomers [3, 4].

The aggregation of Aβ into fibres and oligomers is generally accepted to underlie Alzheimer's disease neurotoxicity. However, it is not clear what promotes the initial misfolding of Aβ in sporadic Alzheimer's disease, or what promotes aggregation of the neurotoxic oligomers and fibres over benign amorphous aggregates [5, 6]. One possibility is that aggregation is influenced through elevated Zn^{2+} [7], for which levels in the neuropil are increased 2-fold in patients with Alzheimer's disease [8].

Zn^{2+} is an important intracellular signalling molecule that is thought to be crucial for synaptic plasticity, learning, and memory [9-11]. In the brain, large reserves of free Zn^{2+} are found to be stored in the presynaptic vesicles of glutamatergic neurons, transported there by Zinc transporter-3 (ZnT3) [12-15]. These stores of Zn^{2+} exit the vesicles with glutamate following neuronal depolarisation, and are reported to bind and regulate the function of a range of neurotransmitter receptors, including N-methyl-D-aspartate (NMDA)-type glutamate receptors [16]. A general consensus has been reached that the affinity of Zn^{2+} for Aβ at pH 7.4 is within the range 1 – 20 μM [17-19]; though this is a relatively moderate affinity, the high levels of Zn^{2+} at the synapse especially during depolarisation, when Zn^{2+} is reported to reach concentrations of up to 100 – 300 μM [20-22], implies synaptic Aβ would be expected to bind endogenous Zn^{2+} [23].

There is much evidence to support Zn^{2+} homeostasis being associated with Alzheimer's disease pathology. It has been found that a Cu^{2+}/Zn^{2+} chelator, PBT2, decreases soluble Aβ levels and improves cognition in Alzheimer's disease transgenic mice [24], and in another study, it has been found that for two transgenic mouse models of Alzheimer's disease (Tg2576 and TgCRND8), a Zn^{2+} enriched diet is associated with potentiated Alzheimer's-like spatial memory impairment, as well as a reduction in Aβ plaque deposition, potentially increasing soluble toxic Aβ levels [25]. Furthermore, in *drosophila* models of Alzheimer's disease, tight control of copper and zinc availability reduces disease phenotypes [26]. Conversely, Zn^{2+} supplementation for 3 x TG-AD transgenic mice actually attenuated disease pathology [27]. In support of this mice depleted of synaptic Zn^{2+} through a ZnT3 knockout mutation present age-dependent memory impairments similar to those observed in Alzheimer's disease models [28]; this is particularly significant as the authors found that ZnT3 levels decline in humans with aging, particularly those with Alzheimer's disease [29]. Thus it currently unclear from animal models of AD whether Zn^{2+} is

protective or harmful, as both increases and decreases are seen to both accentuate and potentiate disease phenotypes.

It is thought that Zn^{2+} influences Alzheimer's disease pathology through direct binding to the Aβ peptide. Typically, Zn^{2+} forms tetrahedral complexes, although 5 and 6 coordinate complexes are also possible. Three histidine residues (His6, His13, His14) within Aβ along with carboxylate coordination from Glu11 or Asp1 (possibly with the N-terminal amino group) have been identified as the Zn^{2+} coordinating ligands [17, 18, 30, 31]. The complex is in rapid exchange (on NMR timescales), and these ligands may well be in a dynamic exchange [18, 30]. Furthermore, the histidine residues that complex Zn^{2+} may come from different Aβ peptides, generating cross-linked Aβ [30, 32].

Metal ions binding to Aβ may alter the kinetics of on-pathway amyloid fibre formation, or alternatively, promote formation of off-pathway aggregates [33]. Initially, it was thought that Zn^{2+} promoted formation of off-pathway, amorphous aggregates [34-39]; however, it has also been reported that Zn^{2+} can cause Aβ to form annular protofibrils [40, 41]. Zn^{2+} is thought to induce $A\beta_{(1\text{-}40)}$ to rapidly form disc shaped oligomers that are highly toxic to primary cell culture, but may then further aggregate to form non-toxic assemblies [42]. Conversely, it was recently reported that Zn^{2+} can slow $A\beta_{(1\text{-}40)}$ fibre growth, without affecting the number of fibres ultimately formed [43]. Consequently, though many studies agree that Zn^{2+} has a profound effect on Aβ misfolding, which aggregate species develop from the Aβ-Zn^{2+} interaction is still not fully resolved.

A great amount of research has highlighted the importance of controlling Zn^{2+} levels in the brains of Alzheimer's disease sufferers and those at risk of developing the disease, while emphasising that both an excess of Zn^{2+} and Zn^{2+} deficiency may be pathogenic [24-29, 42, 44]. Thus, it is of importance to understand how Aβ is influenced by Zn^{2+} at a full range of concentrations, and whether glutamate may affect the Aβ-Zn^{2+} interaction, particularly as Zn^{2+} is released at the synapse with glutamate [12-15]. In this study, we have used thioflavin-T (ThT) fluorescence, and transmission electron microscopy (TEM) to clarify the influence of Zn^{2+} on Aβ self-association, for both $A\beta_{(1\text{-}42)}$ and $A\beta_{(1\text{-}40)}$ alloforms, at a range of physiologically relevant conditions, including extremely low substoichiometric levels of Zn^{2+}.

The interaction of a second divalent metal ion, Cu^{2+}, with Aβ has also generated a lot of attention [45]. Cu^{2+} is also released at the synapse during depolarisation [46], and has a 50 pM affinity for Aβ [47]. We have previously shown that while substoichiometric Cu^{2+} accelerates $A\beta_{(1\text{-}40)}$ fibre formation [48], Cu^{2+} stabilises protofibrillar/oligomeric assemblies of $A\beta_{(1\text{-}42)}$, and can even rapidly convert $A\beta_{(1\text{-}42)}$ fibres to protofibrillar/oligomeric assemblies [49]. We have investigated the effect of a range of Cu^{2+} and Zn^{2+} mixtures on Aβ assemblies, as both are present extracellularly at the synapse, and share histidine coordinating ligands. Cu^{2+} is not displaced by the presence of Zn^{2+} but the coordination geometry is influenced by the presence of Zn^{2+} [50, 51].

Here we demonstrate that trace levels of Zn^{2+} (0.01 mole equivalents, 100 nM) profoundly influences the misfolding pathways of both $A\beta_{(1-42)}$ and $A\beta_{(1-40)}$. Our data suggests this occurs through a rapid exchange mechanism, that enables Zn^{2+} to exert more influence over Aβ assembly than Cu^{2+} in Zn^{2+}/Cu^{2+} mixtures, despite the latter having a much higher (10^{11} *versus* 10^{5}) affinity for Aβ.

RESULTS

Zn^{2+} and $A\beta_{(1-42)}$ Assembly – The well-established amyloid-binding ThT fluorescence assay was used to examine the fibre formation kinetics of $A\beta_{(1-42)}$ at a range of Zn^{2+} concentrations, with TEM being used to examine the species of aggregates formed. The fibre growth of Aβ progresses through a nucleation-dependent pathway, for which formation of an oligomeric nucleus precedes elongation of Aβ into mature fibrils [52, 53]. This classic pattern of fibre formation is observed for $A\beta_{(1-42)}$ in the absence of Zn^{2+}, while at 2 mole equivalents, Zn^{2+} is found to abolish ThT binding amyloid assembly (Fig. 1A), as has been observed by others [34-37, 40-42, 44, 45, 48]. We then wanted to determine whether much lower levels of Zn^{2+} are also capable of influencing $A\beta_{(1-42)}$ misfolding. We investigated the effect of substoichiometric levels of Zn^{2+} on $A\beta_{(1-42)}$ fibre growth, and found that as little as 0.01 mole equivalents of Zn^{2+} is sufficient to greatly perturb $A\beta_{(1-42)}$ fibre growth (Fig. 1B), even though at any one time ~ 99 % of Aβ molecules will not be bound to Zn^{2+}.

In vivo, following depolarisation, glutamate is released with large reserves of Zn^{2+} present, we were interested in whether glutamate may modulate the effect of Zn^{2+} upon $A\beta_{(1-42)}$ self-association. Our ThT data shows that in the presence of two-fold as much glutamate as Zn^{2+}, 0.01 mole equivalents of Zn^{2+} (100 nM) is still sufficient to greatly impede the rate of $A\beta_{(1-42)}$ fibre growth and reduce the total amount of ThT fluorescent assemblies by 75% (Fig. 1C). It is important to note that the impact on ThT fluorescence intensity is not a direct quenching effect of Zn^{2+}, as addition of Zn^{2+} to preformed fibres has no effect on the intensity of the ThT fluorescence signal (data shown later in Fig. 6a).

We characterised the morphology of the $A\beta_{(1-42)}$ assemblies formed using TEM, figure 2. Our images show that in the absence of Zn^{2+}, $A\beta_{(1-42)}$ predominantly formed amyloid fibres, with a typical straight unbranched morphology of 10 – 20 nm thickness (depending on the number of filaments stacked together), and many microns in length. However, as little as 0.05 equivalents of Zn^{2+} visibly influences the heterogeneous population of aggregates that develop (Fig. 2b-c), with $A\beta_{(1-42)}$ generally forming very disjointed crooked fibril-like assemblies, 10 nm thick and 50 – 200 nm long, that are non-ThT binding. These short fibrous aggregates self-associate to form large disorderly tangles that are not observed in the absence of Zn^{2+}. These tangles were 100 – 1000 nm wide, densely stained, and had no repeating structure. At 2 equivalents of Zn^{2+} large disorderly tangles, resembling those observed for 0.05 equivalents of Zn^{2+}, predominated, (Fig. 2c-d). Our TEM images also demonstrate that the influence of Zn^{2+} on $A\beta_{(1-42)}$

aggregation is not markedly altered by the presence of glutamate (Fig. 2E-H). Thus, our data show that both substoichiometric and suprastoichiometric levels of Zn^{2+} promote $A\beta_{(1-42)}$ to form disorderly tangles, rather than growth of mature amyloid fibrils, and that this is relevant *in vivo*, as we found glutamate does not prevent this affect. These observations were made consistently, over numerous Zn^{2+}-$A\beta_{(1-42)}$ preparations and TEM grids.

Zn^{2+} and $A\beta_{(1-40)}$ Assembly – It has previously been observed that the effect of Cu^{2+} on $A\beta_{(1-42)}$ and $A\beta_{(1-40)}$ assembly differs substantially [49]. Consequently, we used ThT fluorescence to investigate how $A\beta_{(1-40)}$ amyloid fibre growth is influenced by Zn^{2+}, at a range of Zn^{2+} concentrations (Fig. 3). As for $A\beta_{(1-42)}$, as little as 0.01 equivalents of Zn^{2+} was able to inhibit growth of $A\beta_{(1-40)}$ fibres, with the total amount of ThT binding fibres being consistently reduced by more than a half, but not abolished. We also demonstrate that these low levels of Zn^{2+} were able to perturb $A\beta_{(1-40)}$ fibre growth even in the presence of glutamate (Fig. 3B).

Using TEM we characterised what species of $A\beta_{(1-40)}$ aggregates formed in the presence of varying levels of Zn^{2+}. We found that there are apparent differences between $A\beta_{(1-40)}$ and $A\beta_{(1-42)}$ assembly in the presence of low levels of Zn^{2+}, but not for suprastoichiometric levels (Fig. 4). The images show that in the absence of Zn^{2+}, $A\beta_{(1-40)}$ forms typical straight unbranched amyloid fibres, 10 – 20 nm thick and many microns in length. The presence of 0.05 equivalents of Zn^{2+} greatly reduces the number of mature amyloid fibres that form, and, unlike for $A\beta_{(1-42)}$, promotes the formation of short rod-like fibres, which are 10 – 20 nm thick, 50 – 200 nm long, and pack closely together, to form stacks 50 – 300 nm thick (Fig. 4B). These stacks were observed at greater number for 0.2 equivalents of Zn^{2+} (Fig. 4C). For 2 equivalents of Zn^{2+}, we observe a similar effect as for $A\beta_{(1-42)}$, as large disordered tangles predominate. These tangles were found to be 100 – 1000 nm wide, densely stained, and to have no repeating structure (Fig. 4D).

Notably, we found that the presence of glutamate did not influence the effect of Zn^{2+} upon $A\beta_{(1-40)}$ misfolding (Fig. 4E-H). Thus the differences in behaviour between $A\beta_{(1-40)}$ and $A\beta_{(1-42)}$ at low levels of Zn^{2+} could potentially be relevant *in vivo*. These observations were made consistently, over numerous Zn^{2+}-$A\beta_{(1-42)}$ preparations and TEM grids.

A time course of TEM images of $A\beta_{(1-40)}$ with Zn^{2+} at 0.0, 0.4 and 2.0 mole equivalents was also generated. After 2 hours incubation, at pH 7.4, oligomeric structure (*ca* 5 nm in diameter) were just detectable. At 3 hours the difference between oligomers observed with and without Zn^{2+} were not marked however by 4 hours, differences were apparent between 0.4 equivalents Zn^{2+} loaded and apo $A\beta_{(1-40)}$, Figure 5. For the Zn^{2+} loaded $A\beta_{(1-40)}$, by 4 hours, short irregular assemblies are apparent, which are not observed in the Zn^{2+} free samples which instead form circular shaped oligomers, Figure 5. TEM images of $A\beta_{(1-40)}$ with 2.0 mole equivalents Zn^{2+} shows similar kinetics and morphology of assembly to 0.4

equivalent Zn^{2+}; specifically short irregular assemblies are apparent after 4 hours incubation (data not shown).

Recently a study reported the effect of Zn^{2+} on $A\beta_{(1-40)}$ fibre formation, using ThT to monitor fibre formation kinetics [43]. Interestingly, an increase in lag-times were observed with addition of Zn^{2+}, but no reduction in ThT intensity was reported [43]. We wondered if this conflict with our own data, shown in figure 3, might be due to the use of phosphate buffer, which will compete for Zn^{2+} and form poorly soluble $ZnPO_4$. Repeating this experiment with phosphate buffer; we found there was some reduction in ThT signal but that this was less marked than in the absence of phosphate buffer (data not shown). Even with phosphate buffer there was still a marked extension in lag-times in the presence of Zn^{2+} as recently reported [43]. Our TEM images of those preparations indicate short rod-shaped fibres of Zn-$A\beta_{(1-40)}$ were generated in the presence of substoichiometric Zn^{2+} and phosphate buffer, with a similar appearance to those shown in figure 4, despite the presence of phosphate buffer.

Adding and Removing Zn^{2+} from Aβ Assemblies at Equilibrium – We investigated the effect of adding Zn^{2+} to mature preformed $A\beta_{(1-40)}$ fibres. Using a ThT assay (Fig. 6A), we found that after adding 0.4 mole equivalents of Zn^{2+} to the preformed mature amyloid fibres, ThT fluorescence stayed constant following a further 100 hours of incubation, suggesting that Zn^{2+} does not readily disassemble fully formed amyloid fibres. This data also confirms that Zn^{2+} does not directly quench ThT fluorescence in the presence of amyloid fibre. This was corroborated by TEM data, figure 6B, which showed that the mature $A\beta_{(1-40)}$ fibres to which Zn^{2+} was added retained a typical amyloid fibre structure, and did not adopt the structural features of short rod-like assemblies characteristic of freshly solubilized $A\beta_{(1-40)}$ incubated with Zn^{2+}.

In addition to this, we wanted to see whether aggregates formed in the presence of Zn^{2+} would persist in the absence of Zn^{2+}. For this investigation, we added EDTA (a strong Zn^{2+} chelator) to both $A\beta_{(1-42)}$ and $A\beta_{(1-40)}$ samples which had previously been incubated with Zn^{2+} (Fig. 7). Our data suggests that removal of Zn^{2+} from Aβ does not enable resumption of amyloid fibre formation, as ThT signal did not increase following EDTA addition. Our TEM confirmed that the aggregate species formed when freshly solubilized Aβ is incubated with Zn^{2+} persist once Zn^{2+} is removed with EDTA. This data suggests that Zn^{2+} promotes formation of aggregates that are off-pathway to amyloid fibre formation. Interestingly, even the short rod structures (formed with $A\beta_{(1-40)}$ + Zn^{2+}), whose gross morphology is reminiscent of amyloid fibres, do not seed the formation of ThT binding fibres once Zn^{2+} is removed by EDTA (Fig. 7B).

We wanted to further characterize the Zn^{2+} induced Aβ assemblies. CD spectra (Fig. 8), indicate

the β-sheet content, as assessed by the minima at 217 nm, is comparable to that generated for both apo $A\beta_{(1-40)}$ and $A\beta_{(1-42)}$ fibres, even though the ThT florescence signal for these assemblies is reduced by between half and three-quarters respectively. Regardless of Zn^{2+} inducing off-pathway Aβ aggregates, Zn^{2+} induced assemblies have high β-sheet content similar to Aβ incubated in the absence of Zn^{2+} (Fig. 8). Freshly solubilized Aβ has a negative CD signal at 198 nm typical of an intrinsically disordered peptide, with little CD signal at 217 nm.

Aβ Seeding with Zn^{2+} **-** It is notable that the influence of Zn^{2+} on Aβ formation does not occur in a stoichiometric manner; our data here has shown that even as little as 0.01 equivalents of Zn^{2+} substantially perturbs fibre formation. Thus, it is evident that a single Zn^{2+} ion can influence the assembly of many Aβ molecules, promoting alternative assemblies rather than the strongly fluorescent ThT binding amyloid fibres. This could occur through two potential mechanisms: a seeding mechanism, in which Zn^{2+} binding causes an Aβ nucleus to adopt a conformation capable of recruiting monomeric Aβ to form the non-typical assemblies that we have observed, or a dynamic exchange model, in which the rapid exchange of Zn^{2+} between Aβ molecules allows a greater number of Aβ molecules to be influenced by low levels of Zn^{2+} than one may otherwise expect.

To investigate which mechanism is more likely, we carried out a seeding experiment (Fig. 9) analogous to the protein misfolding cyclic amplification experiments used for studying prion proteins [54]. In our experiment freshly solubilized Aβ was incubated with 0.4 mole equivalents of Zn^{2+} for a week, which was then used to seed a subsequent experiment, consisting of 10 % seed and 90 % freshly solubilized Aβ, which in turn was incubated for a week and used to seed a further experiment.

This was repeated until there was only 0.0004 mole equivalents of Zn^{2+} present in the final experiment. We found that ThT fluorescent signal generally increased between experiments, and by the final experiment the ThT fluorescence had approximately returned to that observed for freshly solubilized Aβ incubated without Zn^{2+} (Fig. 9A). Supporting the ThT data, our TEM images demonstrate that Aβ aggregate species formed in later seeding experiments closely resembles the Aβ(1-40) and Aβ(1-42) fibres produced in the absence of Zn^{2+}, Figure 9B-E.

As the concentration of Zn^{2+} was reduced in sequential seeding experiments, Zn^{2+} induced aggregate species became less evident; this suggests that the dynamic exchange mechanism more accurately models how a small number of Zn^{2+} ions influence a large number of Aβ molecules. If a seeding mechanism was taking place, the Zn^{2+} induced aggregate species formed in the initial experiments would be able to promote formation of similar aggregates in sequential experiments, regardless of reductions in Zn^{2+} levels.

The Effect of Cu^{2+} and Zn^{2+} Mixtures on Aβ Misfolding **-** The presence of Cu^{2+} has also been shown to influence fibre formation [48, 49] [33, 55]. Additionally, both divalent metal ions are released at the synapse and are thought to chelate to Aβ via its histidine residues [18, 30, 47]. Consequently, it is striking that not only does Zn^{2+} influence Aβ fibre formation at much lower equivalents than Cu^{2+}, but that it does so in a markedly different way. This is most evident for $Aβ_{(1-40)}$, for which substoichiometric Cu^{2+} accelerates fibre formation [48, 49], while Zn^{2+} prevents fibre formation. As we were interested in whether either of these metal ions could modulate or mask the other's effect, we used ThT fluorescence to follow $Aβ_{(1-40)}$ fibre growth in the presence of a variety of Cu^{2+}:Zn^{2+} mixtures. As is apparent in figure 10A, even when there was only a quarter as much Zn^{2+} as there was Cu^{2+}, the effect on fibre aggregation of Zn^{2+} predominated. In particular, ThT signal is reduced four-fold, and there is no acceleration in fibre growth. As the concentration of Zn^{2+} (100 – 300 μM) is generally thought to be higher than that of Cu^{2+} (15 – 250 μM) at the synapse [46, 56], our data suggests that *in vivo* the effect of Zn^{2+} may exert the greater influence on Aβ misfolding. This is supported by our TEM data, figure 10B; here the assembly outcome for mixtures of Zn^{2+} and Cu^{2+} (0.2 and 0.4 equivalents respectively) is dominated by the presence of Zn^{2+}. $Aβ_{(1-40)}$ assembled into short fibril-like rods, that packed together in bundles, as is observed when $Aβ_{(1-40)}$ is incubated with Zn^{2+} in the absence of Cu^{2+}.

DISCUSSION

Trace Zn^{2+} and Aβ Fibre Assembly – We have demonstrated that supra-stoichiometric levels of Zn^{2+} completely abolish Aβ fibre growth, as has previously been reported [34-38, 44, 48], and induces $Aβ_{(1-40)}$ and $Aβ_{(1-42)}$ to form disordered tangled aggregates. We have shown here that as little as 0.01 mole equivalents of Zn^{2+} (100 nM) also significantly influenced the misfolding pathway of both $Aβ_{(1-40)}$ and $Aβ_{(1-42)}$. It is notable that the marked influence of such very low amounts of Zn^{2+} on Aβ assembly is even more remarkable when we consider the affinity of Zn^{2+} for Aβ. The effect is observable with 100 nM of Zn^{2+}, but Zn^{2+} affinity for Aβ is much weaker, at 10 μM [17-19]. Only a fraction, 0.005 mole equivalents of Zn^{2+}, will be bound to Aβ at any one time, at an Aβ concentration of 10 μM. We were surprised that a single Zn^{2+} ion can influence the aggregation of so many Aβ molecules, as each Zn^{2+} ion can only be bound to one or two Aβ molecules, at one time.

There are a number of potential mechanisms through which trace levels of Zn^{2+} could influence large numbers of Aβ molecules. Although Zn^{2+} cross-linking Aβ may contribute, it is insufficient to explain the very low substoichiometric amount of Zn^{2+} that effects fibre formation. Alternatively, the length of fibres may be restricted by Zn^{2+} bound Aβ peptide, capping the end of fibres and limiting fibril length, or introducing sharp kinks into the fibre (Fig. 11). However, the stoichiometric ratio of Zn^{2+} to Aβ

does not correlate to fibre length, Zn^{2+} promoted aggregates have a similar appearance, irrespective of Zn^{2+} stoichiometry; 0.05 or 2 mole equivalent. A more likely explanation is the rapid exchange of Zn^{2+} between Aβ peptides. For fibrillogenesis, Aβ monomers need to adopt a specific conformation to bind to a growing fibre [57-59]; however, Zn^{2+} may rapidly exchange between Aβ peptides, altering the population of conformations typically available to them, and restricting the population of the necessary fibre forming conformation. This metal ion induced entropic expansion of conformational space, for disordered proteins, has recently been described [60]. A fourth possibility would be that Zn^{2+} binding promotes the formation of a unique seed, which may direct downstream Aβ aggregation through a template mechanism, however our seeding experiment (Fig. 9) shows this to be unlikely. Instead our data supports a dynamic exchange of Zn^{2+} between Aβ peptides as the most credible explanation; this is consistent with ^{1}H NMR data that suggests a rapid exchange of Zn^{2+} between Aβ peptides on a milli- to micro-second time-scale [18, 30].

Our data shows that trace levels of Zn^{2+} influence $A\beta_{(1-40)}$ and $A\beta_{(1-42)}$ misfolding in distinct ways; with $A\beta_{(1-40)}$, Zn^{2+} induced formation of short rod-like structures that stack together in bundled structures, whereas with $A\beta_{(1-42)}$, typically Zn^{2+} promotes the formation of short crooked disordered fibrous assemblies that knit together. The dissimilarity in how substoichiometric levels of Zn^{2+} influence $A\beta_{(1-40)}$ and $A\beta_{(1-42)}$ isoforms may arise from differences in the fibril assembly pathways and fibre structure [61-63].

Our time-course TEM images indicate Zn^{2+} generates distinctive assembles of $A\beta_{(1-40)}$ early in the assembly pathway, at 4 hours. Interestingly, although assemblies generated with Zn^{2+}-$A\beta_{(1-40)}$ have a markedly reduced ThT fluorescence, studies using soild-state NMR suggest the basic cross-β structure is maintained [64], and we have demonstrated here a large proportion of β-sheet content to be retained.

Several experiments were carried out to contextualise our results to an *in vivo* environment. *In vivo,* Zn^{2+} is released at the synapse bound to glutamate [12-15], so it was important to determine whether glutamate modulated the effect of Zn^{2+} upon Aβ. We found that glutamate had no influence, indicating glutamate's affinity (K_{d1} = 600 μM at pH 7.4) [65] for Zn^{2+} is not sufficient to prevent Zn^{2+} influencing Aβ assembly. Additionally, as levels of Zn^{2+} fluctuate in the brain with depolarisation [20, 46], it was of interest to observe the effect of Zn^{2+} upon preformed mature fibrils and also the reversibility of Zn^{2+} induced aggregation. We found that Zn^{2+} did not influence mature Aβ fibrils, nor did removal of Zn^{2+} enable aggregates formed with Zn^{2+} to revert and form fibres. This suggests the misfolding pathway of Zn^{2+} induced assemblies is distinct from that of amyloid fibril growth. In contrast, protofibril assemblies of $A\beta_{(1-42)}$ generated by the presence of Cu^{2+} will rapidly seed fibre formation upon Cu^{2+} chelation [49].

Contrasting and Dominating Influence of Zn^{2+} over Cu^{2+} in Fibre Assembly – Another divalent metal ion that is thought to be important in Alzheimer's disease pathology is Cu^{2+} [45, 48, 49, 66-68]. Interestingly, although the two divalent metal ions share a number of the same coordination ligands [17, 18, 30, 47, 50, 51, 69], there are marked differences in the influence of Zn^{2+} and Cu^{2+} on Aβ misfolding, as outlined in Table 1. In particular, substoichiometric Cu^{2+} accelerates $A\beta_{(1-40)}$ fibre growth [48, 49], while substoichiometric Zn^{2+} shows a reduced total fibre growth for both alloforms. A further difference between Zn^{2+} and Cu^{2+} is the amount that is required to disrupt Aβ misfolding, with Zn^{2+} having a much greater influence at lower levels than Cu^{2+}. In particular, the influence of Cu^{2+} on Aβ assemblies is only apparent at stoichiometries of greater than 0.25 [48, 49], 25 times greater than the 0.01 levels of Zn^{2+} required to disrupt fibre growth. The differences in the effects of Cu^{2+} and Zn^{2+} are presumably in part due to differences in the metal binding exchange rate with Aβ.

As Cu^{2+} and Zn^{2+} are elevated in Alzheimer's disease, and both metal ions are released at the synapse, but differ in their relationship to Aβ, we sought to determine the net effect when $A\beta_{(1-40)}$ was incubated with mixtures of Zn^{2+} and Cu^{2+}. We were surprised to find that when both ions were present, Cu^{2+} had little influence upon $A\beta_{(1-40)}$ fibre growth. Although the affinity of Cu^{2+} for Aβ is much higher than Zn^{2+} by more than 5 orders of magnitude, (50 pM and 10 μM respectively) [18, 47]. Zn^{2+} has the ability to rapidly exchange (ms-μs) between Aβ molecules [18, 30] and influence a large number of Aβ peptide conformations, this results in Zn^{2+} dominating Aβ assembly in mixtures of substoichiometric Cu^{2+} and Zn^{2+}. In contrast, the off rate of Cu^{2+} binding to Aβ will be relatively slow with its tight pM affinity.

This highlights that Zn^{2+} may be important in Alzheimer's disease pathology, not only through directly influencing Aβ misfolding, but through attenuating the effect of other factors, such as Cu^{2+}. For instance, *in vivo*, Cu^{2+} levels may become elevated, but have no effect due to a dominant influence of Zn^{2+}, or alternatively, Cu^{2+} levels may remain stable, but changes in Zn^{2+} levels result in an associated change in the relationship between Cu^{2+} and Aβ. The fact that substoichiometric levels of Zn^{2+} influenced Aβ misfolding differently to suprastoichiometric levels, and that Zn^{2+} may influence the effect of Cu^{2+}, provides an explanation as to why Zn^{2+} supplementation has been found to both attenuate and potentiate Alzheimer's like symptoms in animal studies [24, 25, 27, 29], as not only is Aβ misfolding extremely sensitive to Zn^{2+}, but also to the ratio of Aβ and Zn^{2+} as well as its interplay with Cu^{2+}. It has recently been shown that physiologically relevant N-terminally truncated forms of Aβ have a femto-molar affinity for Cu^{2+} [70], it remains to be established what impact Zn^{2+} might have on these very tightly bound Cu^{2+} complexes.

To conclude, Zn^{2+} and Cu^{2+} homeostasis has been implicated in Alzheimer's disease pathology, through a number of animal studies [24, 25, 29, 66-68]. Additionally, Zn^{2+} is prevalent at the

glutamatergic neurons, often described as zincergic neurons [71, 72]. This study highlights the many variables to be considered in delineating the role of Zn^{2+} and Cu^{2+} in Alzheimer's disease pathology, as effect will be dependent on Aβ isoform, metal ion-Aβ stoichiometry, and relative metal ion concentrations. This complexity is underscored by finding that shifts of 0.01 equivalents of Zn^{2+} (100 nM) bound to glutamate are sufficient to change the misfolding landscape of Aβ. However, a complex system also provides many routes for intervention, in this particular case, chelation/supplementation of Zn^{2+}/Cu^{2+} could provide such a route. A high-throughput screening in a yeast model of Aβ toxicity found that a Zn^{2+}/Cu^{2+} chelator, clioquinol, ameliorated Aβ toxicity [73]. Clioquinol has also been found to inhibit Aβ deposition and ameliorate symptoms, in a mouse model of AD [74], further emphasising the potential importance of regulating metal homeostasis in Alzheimer's disease.

The observation that Zn^{2+} can profoundly impact the assemblies of Aβ at concentrations two orders of magnitude weaker than its affinity for Aβ highlights the possibility that small molecules can profoundly influence Aβ assembly even when simple considerations of low affinity and low stoichiometry might suggest that their likely impact on Aβ assembly would be minor. It is clear the binding exchange-rate of potential therapeutic amyloid modulators may be very important in influencing assembly, rather than simply their concentration and affinity for Aβ.

MATERIALS AND METHODS

Aβ production and solubilisation – Lyophilised $A\beta_{(1\text{-}40)}$ and $A\beta_{(1\text{-}42)}$ was purchased commercially from Cambridge Research Biochemicals and Zinser. Peptides were synthesised using solid phase F-moc chemistry, and HPLC indicated a single peak at the expected molecular mass. Characterisation of the peptides was carried out using ^{1}H NMR, which confirmed that Met^{35} was un-oxidised. Lyophilised $A\beta_{(1\text{-}40)}$ and $A\beta_{(1\text{-}42)}$ were solubilised at a concentration of 0.7 mg/ml in water at pH 10.5, using NaOH, and then left at 5 °C for 12 hours, as described by [75]. It is clear that solubilised preparations of Aβ have low levels of fibre nucleating assemblies as at pH 7.4 solubilised Aβ preparations typically have a clear lag-phase. Furthermore, negative stain TEM images at pH 7.4 show no detectable oligomer assemblies directly after solubilisation. Furthermore a single elusion peak is observed in size exclusion chromatograph. Aβ concentration was determined using the Tyrosine absorbance at 280 nm, $\varepsilon_{280} = 1280$ $M^{-1}\,cm^{-1}$.

Fibre growth assay – The kinetics of amyloid formation were monitored using binding of Thioflavin T (ThT) to amyloid fibres, which induces ThT to fluoresce at 487 nm; this signal is typically directly proportional to the amount of amyloid fibrils present [76]. BMG-Galaxy and BMG-Omega FLUOstar fluorescence 96-well plate readers were used for the measuring of ThT fluorescence.

Fluorescence readings were typically taken every 30 mins, following 30 seconds of mild agitation. Fluorescence excitation and emission detection were at 440 nm and 490 nm respectively. Well-plates were sealed with clear polyolefin film (STARLAB) to stop evaporation.

Fibre growth kinetics are sensitive to a number of factors, including pH, concentration, agitation, ionic strength and temperature; consequently, measures were taken to reduce variance in these parameters, and the identical Aβ stock solution was split when making direct kinetic comparisons. Fibre growth experiments were incubated at 30 ^{0}C and in 30 mM HEPEs buffer (due to its low affinity for Zn^{2+} and Cu^{2+} ions) and in 10 or 160 mM NaCl (Sigma-Aldrich; <20 ppm of trace metals). The pH, a critical factor in rate of fibre growth, was adjusted to pH 7.4 with small additions of 10 mM NaOH and HCl; variation between samples was measured to be 0.05 pH units or less. Typically one molar equivalents of ThT was added using a fresh 2 mM stock. Cu^{2+} and Zn^{2+} stock solutions were 10 mM $CuCl_2.2H_2O$ and 10 mM $ZnCl_2$ (Sigma-Aldrich; <20 ppm of trace metals). Contaminate levels of Zn^{2+} (<50 nM) and Cu^{2+} (<500 nM) are shown not to have a detectable impact on fibre growth kinetics or fibre morphology for the types of *in vitro* experiment described [48, 49]. Thus any Zn^{2+} and Cu^{2+} contaminant from the buffer will not impact these studies. Ethylenediaminetetraacetic acid (EDTA) stock was 1 mM (UHQ (ultra-high quality) water (10^{-18} Ω^{-1} cm^{-1} resistivity) was used for all experiments. The final volume in each well was 200 µl, with 96 wells per plate.

Growth curve analysis – Conversion of essentially monomeric Aβ to Aβ fibres follows a characteristic growth curve, consisting of first a lag-phase (nucleation; dominated by both primary and secondary nucleation, and fragmentation) and then a growth-phase (elongation; dominated by monomer addition to growing fibres).

A growth curve can be fitted to the data, to obtain a number of empirical parameters, using the following equation [77]:

$$Y = (\upsilon_i + m_i x) + \frac{(\upsilon_{f} + m_f x)}{1 + exp^{-((x - x_0)/\tau)}} \quad \text{(eq 1)}$$

Y represents fluorescent intensity, and x represents time. Initial fluorescence intensity is represented by υ_i, υ_f represents the final fluorescence intensity, and x_0 is the time at which half maximal fluorescence is reached (t_{50}). The apparent fibre growth rate (k_{app}), is obtained by $1/\tau$ and the lag-time (t_{lag}) by x_0-2τ. This equation allows for a slope in the initial and final parts of the growth curve, ($\upsilon_i + m_i x$), ($\upsilon_f + m_f x$), rather than forcing these to be horizontal. Data was processed using KaleidaGraph 4.0 graphing and data analysis software.

Transmission electron microscopy (TEM) – 5 μl samples were added to flow-discharged carbon-coated 300-mesh grids using the droplet method, with UHQ H_2O washes before and after addition of negative-stain. Phosophotungstic acid (2 % w/v), adjusted to pH 7.4, was used to negatively stain the sample. Selected images are representative of multiple images (~50) that were taken over multiple fields and grids. Control TEM images of buffer show no detectable staining. Images were recorded using a JEOL JEM-1230 electron microscope operated at 80 keV, and the Olympus iTEM software package.

Circular Dichroism (CD) – Spectra were recorded at 25 ^{o}C, between 195 and 260 nm, on an Applied Photophysics Chirascan instrument. We sampled points every 2 nm, and carried out 3 repeat scans per spectrum, using a 0.1 cm path-length cuvette. A baseline spectrum was subtracted from each spectrum, and a 3 nm smoothing window applied, after which the data was zeroed at 260 nm. CD ellipticity values (θ millidegrees) were converted to molar CD, Δε (M^{-1} cm^{-1}), using the following equation:

$$\Delta\varepsilon = \theta/(33{,}000.c.l) \quad \text{(eq 2)}$$

In the above equation *c* represents molar concentration (M) and *l* represents path-length (cm). Data was processed using Photophysics Chirascan Viewer, Microsoft Excel, and Kaleidagraph 4.0.

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Author contributions: CJM, NDY and JHV designed the investigation, analysed the results and prepared the manuscript. CJM and NDY carried out the experiments. All authors approved the final version of the manuscript.

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FOOTNOTES

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FIGURE 1. **Aβ(1-42) fibre growth with Zn^{2+}.** *A.* $A\beta_{(1-42)}$ fibre growth, monitored by Thioflavin T fluorescence, with no Zn^{2+} (red) and 2 equivalents of Zn^{2+} (blue). *B.* $A\beta_{(1-42)}$ fibre growth with no Zn^{2+} (red), 0.01 equivalents of Zn^{2+} (orange), and 0.05 equivalents (green). *C.* $A\beta_{(1-42)}$ fibre growth with glutamate and Zn^{2+}. No Zn^{2+} (red); 0.01 equivalents of Zn^{2+} (orange); 0.05 equivalents of Zn^{2+} (green), and 2 equivalents of Zn^{2+} (blue). Ratio of Zn^{2+}:glutamate; 1:2. As little as 0.01 equivalents of Zn^{2+} strongly inhibited ThT fluorescent fibre formation. Fibre growth was carried out at pH 7.4, with 10 µM $A\beta_{(1-42)}$, 30 mM HEPES, 160 mM NaCl and mild agitation, at 30 °C. (typically N = 6 traces).

FIGURE 2. **TEM Images of $A\beta_{(1-42)}$ with Zn^{2+} and glutamate**. *A-H.* representative TEM images of $A\beta_{(1-42)}$ samples incubated with: No Zn^{2+} (A); 0.05 equivalents of Zn^{2+} (B and C); 2.0 equivalents of Zn^{2+} (D); no Zn^{2+} (E); 0.05 equivalents of Zn^{2+} (F); 0.4 equivalents of Zn^{2+} (G); and 2.0 equivalents of Zn^{2+} (H). E – H also have glutamate present; ratio Zn^{2+}:glutamate is 1:2 . All samples were negatively stained with phosphotungstic acid. Scale bars are 250 nm.

FIGURE 3. **Aβ(1-40) fibre growth with Zn^{2+}.** *A.* $A\beta_{(1-40)}$ fibre growth, monitored by Thioflavin T fluorescence, with no Zn^{2+} (red), 0.05 equivalents of Zn^{2+} (orange), 0.2 equivalents of Zn^{2+} (green), 0.4 equivalents of Zn^{2+} (blue) and 2 equivalents of Zn^{2+} with (purple). *B.* $A\beta_{(1-40)}$ fibre growth with Zn^{2+} and glutamate, where the Zn^{2+}:glutamate ratio is 1:2. No Zn^{2+} (red), 0.01 equivalents of Zn^{2+} with (orange), 0.05 equivalents of Zn^{2+} (green), and 0.4 equivalents of Zn^{2+} (blue). Fibre growth was carried out at pH 7.4, with 10 µM $A\beta_{(1-40)}$, 30 mM HEPES, 10 mM NaCl and agitation, at 30 °C. (N = 4 – 6 traces).

FIGURE 4. **TEM Images of $A\beta_{(1-40)}$ with Zn^{2+} and glutamate**. *A-H.* Representative TEM images of $A\beta_{(1-40)}$ samples, incubated with no Zn^{2+} (A), 0.05 equivalents of Zn^{2+} (B), 0.2 equivalents of Zn^{2+}(C), 2 equivalents of Zn^{2+} (D), 4 equivalents of glutamate no Zn^{2+} (E), 0.05 equivalents of Zn^{2+} (F), 0.2 equivalents of Zn^{2+} (G), and 0.4 equivalents of Zn^{2+} (H). E-H as Zn^{2+}:glutamate, ratio is 1:2. All samples were stained with phosphotungstic acid. Scale bars are 250 nm.

FIGURE 5**: Influence of Zn^{2+} on $A\beta_{(1-40)}$ assembly over time:** $A\beta_{(1-40)}$ fibres formed in the absence of Zn^{2+} (top row) and in the presence of 0.4 mole equivalents Zn^{2+} (bottom row) monitored over time using TEM. Samples were incubated for 2 weeks with aliquots taken at different time points. Images are shown at 3 hrs, 4 hrs and 2 weeks. At four hours there are marked differences in the assemblies due to the presence of Zn^{2+}. $A\beta_{(1-40)}$ (10 µM) was grown at pH 7.4 in 30 mM HEPES buffer and 160 mM NaCl at 25 °C with no agitation.

FIGURE 6. **Zn^{2+} added to mature $A\beta_{(1\text{-}40)}$ fibres.** *A*. $A\beta_{(1\text{-}40)}$ fibre growth, monitored by Thioflavin T fluorescence, to which buffer (red) and 0.4 equivalents of Zn^{2+} (blue) was added at 250 hours. Fibre growth was carried out at pH 7.4, with 10 µM Aβ(1-42), 30 mM HEPES, 10 mM NaCl and agitation, at 30 °C. (N = 3 Traces). *B*. Representative TEM image of mature $A\beta_{(1\text{-}40)}$ fibres, which after fibre growth were incubated with 0.4 equivalents of Zn^{2+} for 100 hours. The addition of Zn^{2+} to mature fibres has no effect on content or appearance. Samples were stained with phosphotungstic acid. Scale bar is 250 nm.

FIGURE 7. **EDTA added to Aβ fibres incubated with Zn^{2+}.** *A*. $A\beta_{(1\text{-}40)}$ fibre growth, monitored by Thioflavin T fluorescence, for samples with no Zn^{2+} (red), 0.05 equivalents of Zn^{2} (green), and 0.4 equivalents of Zn^{2+} (blue), to which 50 µM EDTA was added at 250 hours (n = 3). *B*. Representative TEM image of $A\beta_{(1\text{-}40)}$ incubated with 0.4 equivalents Zn^{2+}, to which 50 µM EDTA was added after 250 hours, and incubated for a further 100 hours. *C*. ThT fluorescence for $A\beta_{(1\text{-}42)}$ with no Zn^{2+} (red), 1 equivalents of Zn^{2+} (green), and 2 equivalents of Zn^{2+} (blue), to which 50 µM EDTA was added at 265 hours (n = 6). *D*. Representative TEM image of $A\beta_{(1\text{-}42)}$ incubated with 2 equivalents Zn^{2+}, to which 50 µM EDTA was added after, and incubated for a further 70 hours. fibre growth was carried out at pH 7.4, with 10 µM Aβ, 30 mM HEPES, 10 mM NaCl and agitation, at 30 °C. Samples were stained with phosphotungstic acid. Scale bar is 500 nm. Once $A\beta_{(1\text{-}40)}$ or $A\beta_{(1\text{-}42)}$ form Zn^{2+} induced aggregates EDTA has little effect.

FIGURE 8. **Circular dichroism spectra of Aβ incubated with Zn^{2+}.** *A*. $A\beta_{(1\text{-}40)}$ incubated with either no Zn^{2+} (red) or 0.05 mole equivalents of Zn^{2+} (blue). *B*. $A\beta_{(1\text{-}42)}$ incubated with either no Zn^{2+} (red) or 0.05 mole equivalents of Zn^{2+} (blue). 20 µM Aβ samples were incubated with 5 mM HEPES, at pH 7.4, at 30 °C under agitated conditions for over 200 hours. Although Zn^{2+} profoundly effects the morphology of Aβ assemblies, apparent β-sheet content is retained.

FIGURE 9. **Aβ and Zn^{2+} cross-seeding.** *A*, Maximal ThT fluorescence of $A\beta_{(1\text{-}40)}$ (grey) and $A\beta_{(1\text{-}42)}$ (black) with 0.4 equivalents of Zn^{2+}, which after a week were used to seed monomeric Aβ (1st seeding), which in turn were also used to seed monomeric Aβ (2nd seeding), which again were used to seed monomeric Aβ (3rd seeding). Seeds were 10 % of final volume. Fluorescence is presented as a percentage of ThT fluorescence for mature $A\beta_{(1\text{-}40)}$ and $A\beta_{(1\text{-}42)}$ fibres grown in the absence of Zn^{2+} the dotted line. Fibre growth was carried out at pH 7.4, with 10 µM Aβ, 30 mM HEPES, 10 mM NaCl and agitation, at 30 °C. *B – E*. Representative TEM images of $A\beta_{(1\text{-}42)}$ and $A\beta_{(1\text{-}40)}$ grown with 0.4 equivalents of Zn^{2+} (B,C); 2nd seeding for $A\beta_{(1\text{-}42)}$ and $A\beta_{(1\text{-}40)}$ contains 0.004 equivalents of Zn^{2+} (D,E). Samples were stained with

phosphotungstic acid. Scale bars are 500 nm. Zn^{2+} generated assemblies could not seed similar assemblies once Zn^{2+} was diluted.

FIGURE 10. **$A\beta_{(1-40)}$ fibre growth with mixtures of Cu^{2+} and Zn^{2+}.** *A*. $A\beta_{(1-40)}$ fibre growth, monitored by Thioflavin T fluorescence, with no metal ions (red), 0.4 equivalents of Cu^{2+} (blue), 0.4 equivalents of both Cu^{2+} and Zn^{2+} (green), 0.4 equivalents of Cu^{2+} and 0.2 equivalents of Zn^{2+}(purple), and 0.4 equivalents of Cu^{2+} and 0.1 equivalents of Zn^{2+} (orange). Fibre growth was carried out at pH 7.4, with 10 μM $A\beta_{(1-40)}$, 30 mM HEPES, 10 mM NaCl and agitation, at 30 °C, (N = 3 – 6 Traces). *B*. Representative TEM image of $A\beta_{(1-40)}$ incubated with 0.4 mole equivalents of Cu^{2+} and 0.2 equivalents of Zn^{2+}. Samples were stained by phosphotungstic acid. Scale bar 200 nm. Assembly outcome is dominated by presence of Zn^{2+}, with short rods and weak ThT signal observed, rather than long fibres.

FIGURE 11. **Schematic of Zn^{2+} Induced Assemblies.** *A*. Binding of Zn^{2+} to Aβ peptide in rapid exchange prevents a large number of Aβ molecules from forming fibres. Alternatively a single Zn^{2+} ion may influence a great number of Aβ peptides through capping the ends of fibres, and inducing formation of bundles of crooked assemblies (*B*) or short rods (*C*). Although scenario B and C are less probable as increasing ratios of Zn:Aβ do not affect the appearance of the cross-seeded assemblies. TEM Scale bars, 200 nm.

TABLE 1. **Aβ aggregate species in the presence of metal ions**. This table shows the predominant species of Aβ aggregate formed upon reaching equilibrium, after 200 hours, in the presence of Zn^{2+} and Cu^{2+}, at pH 7.4, with 10 μM Aβ, 30 mM HEPES, 10 mM NaCl and mild agitation, at 30 °C.