Metal ion coordination delays amyloid-β peptide self-assembly by forming an aggregation-inert complex

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Running title: Mechanistic insights into Aβ self-assembly by metal ions

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ABSTRACT

A detailed understanding of the molecular pathways for amyloid-β (Aβ) peptide aggregation from monomers into amyloid fibrils, a hallmark of Alzheimer’s disease, is crucial for the development of diagnostic and therapeutic strategies. We investigate the molecular details of peptide fibrillization in vitro by perturbing this process through addition of differently charged metal ions. Here, we used a monovalent probe, the silver ion, that, similarly to divalent metal ions, binds to monomeric Aβ peptide and efficiently modulates Aβ fibrillization. On the basis of our findings, combined with our previous results on divalent zinc ions, we propose a model that links the microscopic metal ion binding to Aβ monomers to its macroscopic impact on the peptide self-assembly observed in bulk experiments. We found that substoichiometric concentrations of the investigated metal ions bind specifically to the N-terminal region of Aβ, forming a dynamic, partially compact complex. The metal ion bound state appears to be incapable of aggregation, effectively reducing the available monomeric Aβ pool for incorporation into fibrils. This is especially reflected in a decreased fibril-end elongation rate. However, since the bound state is significantly less stable than the amyloid state, Aβ peptides are only transiently redirected from fibril formation and eventually almost all Aβ monomers are integrated into fibrils. Taken together, these findings unravel the mechanistic consequences of delaying Aβ aggregation via weak metal ion binding, quantitatively linking the contributions of specific interactions of metal ions with monomeric Aβ to their effects on bulk aggregation.

Association and dissociation of protein-protein and metal-protein complexes are highly relevant in biological systems, both for function and misfunction related to diseases (1). The interplay between proteins and metal ions, including the stability/lifetime of such complexes, might be key for an understanding of the disease-associated
molecular processes. One central event in Alzheimer’s disease (AD) is the formation of cerebral senile plaques consisting of aggregated amyloid-β (Aβ) peptides (2, 3). The self-assembly of the Aβ peptide can be described as a nucleation-dependent process and detailed information about the fibrillation and nucleation mechanisms may be of importance for an understanding of the disease pathology (4–7). Metal ions have been shown to be potent modulators of Aβ aggregation (8–10), exhibiting specific binding to the intrinsically disordered Aβ monomer (8–14). Their small size and variable charge makes them suitable agents to elucidate the effect of modulating the Aβ monomer structure upon metal ion binding on the overall Aβ fibrillation process. In general, metal ions such as copper, zinc and iron are implicated in AD pathology (15–17) and are also abundantly sequestered in senile plaques (18). But also other metal ions may take key roles, e.g. through exogenous contamination and competition with the same binding ligands as endogenous metal ions (13, 19–23). Here we use metal ions in substoichiometric concentrations as amyloid aggregation modulators to disturb the aggregating system to obtain further insights into the fundamental self-assembly mechanisms.

The monomeric Aβ peptides are predominantly unstructured (24, 25), yet are prone to self-assembly into larger aggregates and eventually they form fibrils with a stable cross-β structure (26, 27). The main coordinating metal ligands in the Aβ peptide are in the hydrophilic N-terminal part (12, 28–30). Findings from nuclear magnetic resonance (NMR) and molecular modeling studies suggest one main binding mode consisting of three histidines and the N-terminal aspartic acid (28–30), where Glu11 is an alternative potential fourth ligand (20). A specific metal-binding mode affects the structure of the Aβ peptide and hence its properties. The net charge of the Aβ peptide at physiological pH is around −3, and interactions with cations decrease the net charge and the intrapeptide electrostatic repulsions. The metal ion effects on the Aβ aggregation kinetics are concentration-dependent. Super-stoichiometric concentrations of Cu(II) and Zn(II) easily induce rapid formation of amorphous aggregates without amyloid structures, where the metal ion might bind to a second binding site and possibly bridge between Aβ peptides (9, 31, 32). In contrast, substoichiometric Cu(II) (33, 34) or Zn(II) concentrations retard the overall fibrillation process, and our previous study on Zn(II) revealed the specific reduction of the fibril-end elongation (14). Besides Cu(II) and Zn(II), several other transition metal ions bind to the Aβ peptide, competing for similar ligands with slightly different coordination modes (13, 19–21).

Silver ions are well-known protein-binding ions and substantially used in the past as a protein-staining agent (35, 36). Besides changes in charge, the ionic radii of Ag(I) and divalent metal ions differ, together with small deviations in preferred binding ligands and coordination geometry (Table S1). Both in vitro and in vivo studies showed Cu(I) replacement by Ag(I) ions in copper-containing proteins (37–39). Ag(I) ions exhibit a larger Pauling radius and a smaller charge density than Cu(I) ions (Table S1), but due to the same electric charge number the more stable Ag(I) ions have been used as a probe for the readily redox-active Cu(I) ions in in vitro studies (40–42). In fact, a recent study investigated Cu(I) and Ag(I) binding towards the model peptide Aβ16 and showed similar but slightly different binding modes (42). Furthermore, silver ions are not paramagnetic, in contrast to Cu(II), and are thus a useful substitute for interaction studies using NMR.

Studying misfolding and aggregation of amyloidogenic proteins by varying the experimental conditions such as pH, electrostatics, ionic strength, temperature and local concentration, is a valuable tool, as the aggregation processes can be understood in more detail (43–47). Modulation of electrostatic repulsion of Aβ has been shown to greatly influence the Aβ self-assembly mechanism by promoting surface-catalyzed secondary nucleation reactions (46, 47). Hence, in contrast to the divalent ions Zn(II) and Cu(II), the net charge of +1 makes Ag(I) ions a valuable comparative agent for studying the impact of electrostatics for metal ion modulation of peptide/protein aggregation.

In this project we used a combination of biophysical methods to study the characteristics of Aβ metal ion binding to Ag(I) ions by
Mechanistic insights into Aβ self-assembly by metal ions

characterizing the exchange dynamics and thermodynamics of the binding reaction and how this binding affects the fibrillation kinetics. Further, together with our previous results on Zn(II) (14), we rationalized a model for the determinants of modulation of Aβ self-assembly by transition metal ions. In short, (1) monomeric Aβ binds Ag(I) ions specifically in the N-terminal part, forming a dynamic metal-ion bound complex. (2) The weak metal ion-binding prevents monomeric Aβ from incorporation into fibrils. This leads to (3) attenuation of the Aβ fibrillation kinetics in particular by reduction of the fibril-end elongation rate. Remarkably, these results are strikingly similar to effects of Zn(II) ions (14), which suggests a common mechanism of interaction of monovalent Ag(I) and divalent Zn(II) ions with Aβ peptides. Taken together, the metal ion binding redirects Aβ monomers from fibril formation, retarding the overall Aβ fibrillation, in particular by reducing fibril-end elongation. This study hence links quantitatively the microscopic perturbation of metal ion binding to Aβ monomers with its effect on the bulk peptide aggregation process.

Results and discussion

Ag(I) ions predominately retard Aβ fibril-end elongation

Fibrillation kinetics can be monitored using different fluorescent dyes that detect amyloid formation (48, 49). In this study, we simultaneously measured the aggregation kinetics of 20 μM Aβ40 and 5 μM Aβ42 at +37 °C under quiescent conditions using pentameric formyl thiophene acetic acid (pFTAA) (49) and Thioflavin T (ThT) (48) (Figs. S1 and S2). With pFTAA we observed the typical sigmoidal aggregation kinetic traces (50, 51), while the monitoring of aggregation by ThT is interfered by interactions of Ag(I) and ThT (52, 53) (Fig. S1), which makes ThT unsuitable for aggregation kinetics in the presence of Ag(I) (Supporting Information text, page S4).

From pFTAA fluorescence experiments, we observed that the fibrillation kinetics of Aβ40 and Aβ42 are retarded by Ag(I) ions in a concentration-dependent manner (Fig. 1A-C, Fig. S1). We quantitatively analyzed the effect of Ag(I) ions on Aβ aggregation and found a clear Ag(I) concentration dependence of the aggregation halftime, τ₁/₂, and the maximum growth rate, rₘₐₓ (Fig. 1D,E), obtained from fitting a sigmoidal function to the aggregation trace. To obtain insights into the microscopic nucleation process of Aβ we analyzed the aggregation kinetics applying a global fit analysis using an integrated rate law (50, 51, 54, 55) (Fig. 1A-C). The fibrillation process can selectively be differentiated into distinct nucleation events with their related microscopic rate constants, such as primary nucleation (kₙ), surface-catalyzed secondary nucleation (kₛ) and fibril-end elongation (kₑ) (5, 54, 55) and we assumed that similar microscopic nucleation events occur also in the presence of Ag(I).

To test the contribution of each microscopic rate constant we globally fitted the kinetic curves with one single microscopic rate constant as an effective free fitting parameter by fixing the other two rate constants to constant values (Fig. 1A-C). We found that the aggregation behavior could not be described with kₙ as the sole fitting parameter. While letting kₑ be free better explains the observed aggregation data, using kₛ as free parameter yields a better fit than for kₙ but significantly worse than for kₑ (Fig. 1 and Fig. S3).

To confirm that it is the elongation rate that is most affected by Ag(I) ions, we performed kinetics experiments in the presence of preformed seeds (Fig. 1F, Figs. S4 and S5). The initial slope of such kinetic trace is directly proportional to the elongation rate and hence the isolated effect on kₑ by silver ions can be estimated (5, 14, 50, 51). A high concentration of seeds was added to a monomeric peptide solution in the presence of different Ag(I) concentrations, and we found that the relative elongation rates obtained from these experiments agree very well with those from the global fit (Fig. 1G), providing further evidence that indeed the elongation rate is the rate constant mainly modulated by Ag(I). Noteworthy, these findings do not exclude that there may be minor effects of Ag(I) on kₙ and kₛ as well.

Additionally, the experimental data were also fitted to a model, where the metal-bound population of Aβ monomers is assumed to be unavailable for fibril formation. The reduced aggregation-prone Aβ
Mechanistic insights into Aβ self-assembly by metal ions

monomer pool can be described by an apparent free Aβ monomer concentration for each Ag(I) concentration. Similarly to a global fit analysis of aggregation kinetics with different Aβ concentrations (50, 51), the combined microscopic rate constants were globally fitted and constrained to the same values across all Ag(I) concentrations. In addition, an apparent dissociation constant $K^\text{app}_D$ was included as a global fit parameter, reflecting the apparently reduced free Aβ monomer concentration due to the Ag(I)-binding. This model describes reasonably well the observed aggregation kinetics, with $K^\text{app}_D = 14.5 \pm 0.2$ µM (Fig. 1H).

To investigate whether Ag(I) affects the final state of the Aβ fibrils we used atomic force microscopy (AFM) and recorded images of the end-point samples from the aggregation experiments (Fig. 1I,J and Fig. S6). No detectable difference in the fibril morphology was found. This was further supported by CD, which exhibit spectra showing similar $\beta$-structures of the aggregated state at all Ag(I) concentrations (Fig. S1G,H).

We conclude that Ag(I) ions predominantly retard fibril-end elongation, while the structural state and the amount of the end-point fibrils are not affected (Fig. S1C). Hence, these metal ion interactions only modulate the fibrillation process, solely resulting in a delay of the aggregation process, while the aggregation mechanism and the final products are not altered.

Interaction between Aβ monomers and Ag(I) ions

While aggregation experiments showed a reduction in the fibril-end elongation rate, these kinds of experiments do not reveal any details on the mechanism of metal ion-binding to monomeric peptide. To obtain high resolution information on the binding mechanism, we opted for nuclear magnetic resonance spectroscopy (NMR) and recorded 2D $^1$H-$^1$H-N-HSQC and $^1$H-$^1$C-HSQC experiments (Fig. 2 and Fig. S7) at various silver ion concentrations. Addition of 20 µM Ag(I) ions to 80 µM $^{13}$C-$^1$N-labeled Aβ$_{40}$ (Aβ(I):Aβ$_{40}$ ratio of 1:4) resulted in an immediate attenuation of signal intensities of crosspeaks corresponding to N-terminal residues in both the $^1$H-$^1$N- (Fig. 2A,B) and the $^1$C-edited spectra (Fig. S7). In addition, induced chemical shift changes were observed (Fig. 2E,F and Fig. S7), indicating Ag(I)-induced conformational changes in the N-terminus of Aβ. To exclude specific effects from Ag(I)-buffer interactions, we performed experiments in different buffers and found similar binding patterns (Fig. S8). The Ag(I) ion binding is reversible, as probed by an added chelator (1,10-Phenantroline) to a sample containing both Aβ and Ag(I) ions (Fig. S9).

The loss of signal intensity and chemical shift differences induced by Ag(I) ions immediately returned to the original values in the presence of the chelator. SDS micelles constrain Aβ in a monomeric state while keeping the metal binding N-terminus disordered in solution (56) and, indeed, while bound to SDS micelles similar binding pattern as in buffer solution was found (Fig. S10). Hence, we can conclude that Ag(I) ions bind to Aβ monomers (Supporting information text, page S6, and Fig. S10). The $^1$H-$^1$N-HSQC crosspeak intensities of the histidine residues (His6, His13, His14) in Aβ are only very weak under present conditions, while crosspeak intensities both in the aliphatic and in the aromatic region are detectable in the $^1$H-$^1$C-HSQC spectra, (Fig. S7). All histidines are clearly affected by the presence of Ag(I) ions and show about 75-80% reduction of the initial signal intensity at 20 µM Ag(I). Neighboring residues to the histidines in the sequence such as Arg5, Ser8, Val12, and Gln15 are also affected, as well as Asp1, similar to the effect of Zn(II) ions (28).

To further verify that the histidines are the metal-binding ligands we performed $^1$H-$^{15}$N-HSQC titration experiments on a histidine-free H6A,H13A,H14A,$^{15}$N-Aβ$_{40}$ variant (Aβ$_{40}^{\text{noHis}}$), and we found that the addition of more than 10-fold excess of Ag(I) ions neither caused any signal loss nor any chemical shift changes (Fig. 2C,D). Moreover, 1D proton experiments for non-labeled Aβ$_{40}$ were conducted in D$_2$O (Fig. S11A). Here the non-exchangeable imidazole proton signals are observed as three peaks around 7.7 ppm and upon titration of Ag(I) ions these signals were broadened. We conclude that the three histidines His6, His13 and His14 are involved in silver ion coordination.

The dissociation constant can be calculated from the signal loss in NMR HSQC data, assuming that the line broadening effect is linearly coupled to the
bound population, and is around 3 µM in 20 mM sodium phosphate buffer, pH 7.4 (Table S2 and Fig. S12). Calculating the dissociation constant from induced chemical shifts instead gives consistent values (Table S2 and Fig. S12). In addition, intrinsic Tyr10 fluorescence experiments were conducted where Ag(I) ions decrease the Tyr10 fluorescence intensity and this phenomenon was used for a direct estimation of the dissociation constant (Supporting information text, page S4). The obtained values agree well with the values determined by NMR data and from analysis of the kinetics data (Fig. 1H). The values for dissociation constant vary thus in a relatively narrow interval between 3 and 15 µM depending on the applied technique and experimental conditions (Table S2).

**Silver ions induce a more compact structure in Aβ**

To examine whether the Ag(I) ion interaction causes the Aβ peptide to fold upon coordination of the metal ion, pulse field gradient (PFG) diffusion experiments (57) were conducted at different Ag(I) concentrations. The translational diffusion coefficient for 80 µM Aβ40 peptide increases in a concentration-dependent manner from 6.7 to 7.0 x 10^{-11} m^2/s upon increasing Ag(I) concentration from 0 to 50 µM (Fig. 3A). From the translational diffusion coefficient, the hydrodynamic radius ($R_H$) can be calculated using the Stokes-Einstein equation (58, 59). The apparent hydrodynamic radius decreases from 17.0 Å for Aβ40 alone to 16.3 Å in the presence of 50 µM Ag(I) ions, suggesting a slightly more compact Aβ peptide structure once bound to the Ag(I) ion, without significant changes in the secondary structure content (Fig. S11B). As the observed $R_H$ is the population-weighted mean of the free and bound state, the hydrodynamic radius of the bound state can be estimated to be 16.0 Å, indicating a significant compactification upon metal induced folding.

**Chemical exchange between Aβ-Ag(I) complex and free peptide**

The loss of NMR signal of $^{15}$N-Aβ40 upon Ag(I) addition presumably originates from chemical exchange effects. Transient structures and dynamical features on the µs to ms timescale (typically underlying exchange broadening) are suitable to quantify using NMR relaxation dispersion experiments (60). We applied $^{15}$N Carr–Purcell–Meiboom–Gill (CPMG)-based pulse schemes (61–63) to characterize the influence of Ag(I) ions on 80 µM Aβ40 (Fig. 3B-F), and we observed relaxation dispersion profiles in the presence of 4 and 6 µM Ag(I) at four different temperatures (278-287 K) (Figs. S13, S14 and Table S3).

Especially, the N-terminal residues exhibit high-amplitude relaxation dispersion profiles, where 7 residues show significant relaxation dispersion (F-test P-value <0.01) and these residues were used for further analysis (Table S3). The 7 residues are in close proximity to the histidines, the metal binding ligands. Notably, also in the presence of SDS micelles, constraining Aβ as a monomer, the same N-terminal residues show relaxation dispersion profiles confirming that the chemical exchange process can be attributed to Ag(I) binding to monomeric Aβ (Supporting information text, page S6 and Figs. S10 and S15). In contrast, we have previously reported that Aβ40 peptides without metal ions do not show any relaxation dispersion profiles in this NMR time regime (14).

The relaxation dispersion profiles fit to a two-state exchange model (14, 64, 65), which allows determination of the population of the free and bound states, ($p_f$) and $p_B$, the chemical exchange rate, $k_{ex}$, between the two states, the absolute value of chemical shift differences, $|Δδ_N|$, and intrinsic transverse relaxation rate $R_2^0$ (Fig. 3E,F and Table S4). While the latter two parameters, $R_2^0$ and $|Δδ_N|$, are residue-specific parameters, the exchange rate and populations can be applied as global fitting parameters and constrained to the same values for all residues. We found that a model with a temperature dependent $p_B$ (referred to as model 1) best describes the data (Table S5). In this model, the exchange rate $k_{ex}$ linearly increases and the population $p_B$ decreases with increasing temperatures. Under the chosen experimental conditions, around 6 to 9% of the Aβ population is bound to a silver ion at any given time point (Fig. 3F) and exchanges between the Ag(I)-bound and the free state with an exchange rate of 200 to 500 s^{-1} (Fig. 3E).
The chemical shift differences correlate well with the values obtained from the titration experiments monitored by $^1$H-$^15$N-HSQC experiments, indicating that the same structural state from the same process is observed (Fig. 3G). Interestingly, the exchange and population parameters for Ag(I) are in the same order as the ones previously determined for zinc ions (14). When calculating $K_D$ values from relaxation dispersion data these values were estimated to approximately 1 µM at 281 K (Table S4). This value is in the same order of magnitude compared to the $K_D$ values determined by HSQC titration (Table S2). To further confirm the model with a two-state exchange process, two different Ag(I) ion concentrations were compared. The exchange rate determined from the global fit at 6 µM Ag(I) ions does not significantly differ from the one at 4 µM Ag(I) ions and can be constrained to the same value in the fitting procedure (Tables S4 and S5). In contrast, the populated state ($p_B$) does increase with approximately 30% as expected for this increase in concentration (Table S4). This observation shows that the signal intensity loss and chemical shift changes in HSQC spectra upon Ag(I) ions titration are linearly coupled to the bound Aβ population. The $p_B$ parameter is temperature dependent and related to the Gibbs free energy difference, $\Delta G$, between the free and the bound state via the equilibrium constant (Supporting information text, page S5 and Fig. 3H). Notably, at higher temperature (≥281 K) the data exhibits a linear temperature dependence, which reflects non-temperature dependent contributions of the enthalpy and entropy. However, the whole data set fits best to temperature dependent enthalpy and entropy terms, reflected in a heat capacity difference between the free and the bound state (Supporting information text, page S5-S6). We found that the binding reaction is favored by enthalpy but disfavored by entropy. Together, the Gibbs free energy differences are small, yielding an unstable final fold at all temperatures (Supporting information text, page S5-S6, Fig. 3H and Tables S4 and S6).

Insights into metal ion binding mechanisms and effects on aggregation comparing Ag(I) and Zn(II)

The weak Ag(I) binding transiently removes Aβ monomers from the pool of aggregation-prone monomeric species, which are available to be incorporated into the fibrils (Figs. 1 and 3). Since the metal ion binding is weak, eventually all Aβ peptides fibrillate. This analysis hence directly links the metal ion interactions with monomeric Aβ to the overall retardation effect of fibril formation.

To distinguish if this is solely a Ag(I) ion effect or if it is a general metal ion modulation effect of the Aβ fibrillization, we compared the results from monovalent Ag(I) with our previously reported findings from divalent Zn(II) (14) to be able to elucidate the impact of charge of the transition metal ion on the nucleation mechanism and binding characteristics. An induced folding of the N-terminus upon metal ion binding is a shared feature, reflected in a decreased hydrodynamic radius of the Zn(II)-Aβ (14) and Ag(I)-Aβ complexes (Fig. 3A and Table S7). In fact, normalized diffusion data of Ag(I) and Zn(II) can be fitted together, constraining the normalized diffusion coefficient for the bound state, $D_B/D_{free}$, to the same values for both metal ions. This analysis revealed an increased value of $D_B/D_{free} = 1.087\pm0.002$, reflecting a decreased hydrodynamic radius by a factor 0.92 for the metal ion-bound/‘folded’ state (Fig. 4A). Interestingly, unlike in the presence of Zn(II), we observed significant chemical shift changes in $^1$H-$^15$N-HSQC resonances for Ag(I), indicating somewhat modulated exchange kinetics. Overall, both ions display similar binding regions with similar exchange dynamics, yet Zn(II) ions act at lower metal ion:Aβ ratios.

In fact, when plotting the population of the metal-bound states estimated by the $K_D$ values against the metal ion:Aβ ratio, a linear relation is evident (Fig. 4B). Notably, the bound population of Ag(I), as determined by relaxation dispersion experiments agrees well with this prediction (Fig. 4B). For the bound/‘folded’ state this suggests that the association mechanisms are very similar and determined by the respective dissociation constant. Comparing the thermodynamics for the binding, it turned out that both metal ions induce an enthalpy favorable, yet unstable, final Aβ fold, where the heat capacity in the presence of Ag(I) is consistent with the more accurately determined value for
Zn(II) (Table S7). Hence, these findings suggest strikingly similar binding mechanisms for both metal ions, where the lower binding efficiency for Ag(I) ions may stem from lower charge and larger ionic radius.

Specific interaction with monomeric Aβ, resulting in reduction of the elongation rate is seemingly a common mechanism for the investigated metal ions. Indeed, the relative elongation rates for both Ag(I) and Zn(II) ions (14) can be fitted with a model for metal ion binding to Aβ monomers as a function of an apparent binding constant, revealing $K_{\text{app}}$ = 4.1 ± 0.4 μM and $K_{\text{app}}$ = 1.2 ± 0.2 μM for Ag(I) and Zn(II), respectively. These values are similar as obtained here by other methods for Ag(I) (Table S2) and reported previously for Zn(II) (28). This analysis hence suggests that the reduction of free Aβ monomer population by metal ion-bound state causes the decrease of the apparent fibril-end elongation rates.

**Concluding remarks**

Taken together, we rationalized the mechanisms of action of transition metal ion binding to Aβ in a schematic model (Fig. 4D). Metal ion binding causes a N-terminal fold in Aβ with a histidine coordination of the metal ion, where the Aβ peptide exchanges at a ms time-scale between the free and bound, ‘folded’ state. This ‘folded’ state is inert to fibril elongation. Hence, the pool of monomeric species available to be incorporated into the fibrils is reduced, retarding the overall Aβ fibrillization. While also primary and secondary nucleation are dependent on the Aβ monomer concentration, the metal ion interaction mainly affects the process of fibril-end elongation, since the elongation reaction involves a folding event when integrating Aβ into the fibril. This elongation event presumably includes multiple steps, where in a first step the peptide binds to fibril end, followed by a folding event representing the conversion from a predominantly unstructured state to a β-structure conformation (66). In contrast, primary and secondary nucleation do not require a full β-structure formation to form the amyloid-state. Hence the elongation event is particularly dependent on modulation of the folding thermodynamics, where the metal bound state is unable to adopt a productive fold on the fibril end. However, since the metal ion binding is weak, presumably much weaker than the affinity of the monomer to the fibrillar state, eventually almost all Aβ peptides are incorporated into the fibrils. This is also reflected when comparing the Gibbs free energy values of the fibril elongation event, which was determined to ca. −38 kJ/mol (67), and the value of the metal-bound state in the range of 5 to 7 kJ/mol.

To conclude, our analysis hence establishes the link between microscopic metal ion interactions with monomeric Aβ and its macroscopic retardation effect of fibril formation, providing detailed mechanistic insights into modulation of Aβ self-assembly. We hence could further develop our previous model for Zn(II) (14) for both mono- and divalent metal ions, and were able to show here in a strictly quantitative manner that the population of the aggregation-inert metal ion-bound state causes the retardation of Aβ fibrillation. These insights might be beneficial to interfere with specific Aβ nucleation events, which potentially prevents toxic pathways (68–70), and thereby find efficient ways for treatment of protein/peptide misfolding related disorders.

**Experimental procedures**

**Sample preparation** Recombinant amyloid-β (Aβ) peptides with the sequence DAEFR$_5$ HDGSY$_{10}$ EVHHQ$_{15}$ KLVF$_{20}$ AEDVG$_{25}$ SNKGA$_{30}$ IIGLM$_{35}$ VGGV$_{40}$ (IA) were used in this study. Non-, $^{13}$N- and $^{15}$C,$^{15}$N-labeled Aβ$_{40}$ peptides were bought lyophilized from AlexoTech AB and non-labeled Aβ$_{42}$ peptides were purchased from rPeptide. Silver(I) acetate, silver(I) nitrate, and 1,10-Phenanthroline were purchased from Sigma Aldrich, Inc. and the Ag(I) ion concentration was determined by weight. For NMR experiments, the lyophilized peptides were dissolved in 10 mM NaOH pH 12 at 1 mg ml$^{-1}$, sonicated in an ice-water bath and diluted to the desired concentration in the selected buffer. For kinetics experiments, the lyophilized peptides were dissolved in 6 M Guanidium hydrochloric acid pH 7.2 and prepared with size exclusion chromatography (SEC) using a Superdex 75 10/300 GL column from GE.
Mechanistic insights into Aβ self-assembly by metal ions

pFTAA and ThT fibrillation kinetics using fluorescence spectroscopy For aggregation kinetics experiments, 20 μM Aβ40 in 10 mM MOPS buffer pH 7.2 was supplemented with 0.3 μM pentameric formyl thiophene acetic acid (pFTAA) (49) or 40 μM Thioflavin T (ThT) (48, 71) and different concentrations of Ag(I) ions (0-30 μM). The fluorescence intensity was monitored over time with a FLUOstar Omega microplate reader from BMG LABTECH (Germany) each minute (for seeded samples) or each third minute in a 384-well plate, 30-40 μl per well, at +37 °C. 3-4 replicates per condition were measured. Both raw data and weighted average kinetic traces were analyzed using sigmoidal curve fitting (72) (Supporting information text, page S4). For each type of kinetic experiments at least three measurements were performed with qualitatively similar results.

Global fit analysis of the kinetic curves using the model presented by Meisl et al. (50) was performed where the Aβ fibrillation process under quiescent conditions is described as a monomer-dependent process with three different microscopic rate constants (51), k_n as the primary nucleation rate constant with reaction order n=2, k_+ as the elongation rate constant and k_2 as the secondary nucleation rate constant with the reaction order n=2 (50, 51). The kinetic curves from 20 μM Aβ40 were globally fitted to a multistep secondary nucleation model with a Michaelis constant K_M of 12.5 μM (14). Two of the rate constants k_+ , k_2, and k_n were held constant while the third rate constant was allowed to vary. The global fit analysis was performed with IgorPro 7 (WaveMetrics) and the Amylofit interface (73).

Seeding experiments were conducted with a fixed concentration of seeds of 1 μM supplemented to 20 μM monomeric Aβ40. The seeds were prepared from homogenized fibrillated samples by sonication and the seed concentration was determined from the initial Aβ40 monomer concentration. The relative elongation rate constant, k_+, was determined from the initial rate of the derived concave kinetic curves.

We applied a model where the kinetic traces at different Ag(I) concentrations are described by an apparent free Aβ monomer concentration, which is determined by an apparent dissociation constant, K_D^app. For a two-state exchange K_D^app can be described by Eq. 1 based on the Ag(I)-bound population, p_B, and the initial concentrations of Aβ monomers and Ag(I) ions (14).

\[ K_D^{app} = \frac{(1-p_B)\cdot([Aβ_0]-p_B[Aβ]_0)}{p_B} \]  

(Eq. 1)

The apparent Aβ monomer concentration is then given by [Aβ]^app = [Aβ]_0 (1 - p_B) where p_B is a function of K_D^app, which can be derived from Eq. 1. The aggregation traces were then globally fitted applying a secondary nucleation model (51), where k_+, k_2, and K_D^app are global fit parameters, which were constrained to the same value across all values of [Aβ]^app. Hence, all fitting parameters are globally constrained, and this approach facilitates to test the model of an apparent free Aβ monomer concentration that is determined by K_D^app.

Solid-state AFM imaging and Circular Dichroism spectroscopy Samples from the end of a fibrillation kinetic experiment were used for atomic force microscopy (AFM) imaging and CD measurements. CD spectra were also recorded for a titration series of Ag(I) ions onto monomeric Aβ40 in 20 mM sodium phosphate buffer pH 7.4 (Supporting information text, page S4).

NMR spectroscopy Most NMR experiments were performed on a 700 MHz Bruker Avance spectrometer equipped with a cryogenic probe, if not stated differently. For 2D NMR 1H-15N-HSQC experiments 80 μM wildtype 13C-15N-Aβ40 or 80 μM 15N-labeled mutant Aβ40 (H6A,H13A,H14A) (referred to as Aβ40_mimicking system, sodium dodecyl sulphate (SDS) micelles were used, applying 50 mM d-SDS (>critical micelle concentration) and 170 μM 15N-Aβ40. For the 2D spectra, the relative intensities for each amide crosspeak were determined from the amplitude of
the crosspeaks. The combined chemical shift changes were calculated from Eq. 2 (74, 75).

\[ \Delta \delta = \left( \frac{(\Delta \delta^N)^2 + (\Delta \delta^H)^2}{2} \right)^{\frac{1}{2}} \quad \text{(Eq. 2)} \]

The spectra were referenced to the \(^1\)H signal of trimethylsilylpropanoic acid (TSP). The \(\text{A\beta}_{40}\) amide crosspeak assignment in the HSQC spectra was performed by comparison with previously published work (24, 28, 56).

Pulse field gradient (PFG) diffusion experiments were performed on a 600 MHz Bruker Avance spectrometer with 80 \(\mu\)M \(\text{A\beta}_{40}\) in 20 mM sodium phosphate buffer pH 7.4 in D\(_2\)O at 281 K with different Ag(I) concentrations. A standard sample of 1% H\(_2\)O, 0.1% DSS and 0.1 mg ml\(^{-1}\) GdCl\(_3\) in D\(_2\)O was used to calibrate the pulse field gradients.

The translational diffusion coefficient \((D_t)\) was determined by one-component analysis and hydrodynamic radius \((r_H)\) were determined from the diffusion coefficient (76) using the Stokes-Einstein relation relation (58, 59). The diffusion data were further analyzed using a two-state model:

\[ D_{\text{obs}} = p_{\text{free}}D_{\text{free}} + p_BD_B \quad \text{(Eq. 3)} \]

in which \(D_{\text{free}}\) and \(D_B\) are the diffusion coefficient of the free and the bound state, respectively, with the respective populations. Additionally, a global fit analysis of the diffusion data from Ag(I) and Zn(II) (14) was performed on normalized diffusion data (Fig. 4A), where the coefficients \(D_{\text{free}}\) and \(D_B\) were constrained to the same value for Ag(I) and Zn(II) and the values of \(p_n\) were calculated by resolving Eq. 1 using the respective apparent dissociation constants, here 1 \(\mu\)M for Zn(II) and 3.5 \(\mu\)M for Ag(I) (from HSQC analysis at low temperature, Ref.(28) and Table S2).

\(^{15}\)N-CPMG relaxation dispersion experiments (61–63) were recorded on 80 \(\mu\)M \(^{15}\)N-\(\text{A\beta}_{40}\) peptides with 6 \(\mu\)M or 4 \(\mu\)M Ag(I), in 20 mM sodium phosphate or 10 mM HEPES buffer at pH 7.4. The Ag(I) concentration was adjusted and recalculated to the same signal attenuation upon Ag(I) addition based on the 2D NMR HSQC titration experiments. The experiment was repeated at four different temperatures (278-287 K) with 3 K interval for each Ag(I) ion concentration. The \(^{15}\)N-CPMG relaxation dispersion experiments were performed as pseudo 3D experiments. 7 or 11 different CPMG frequencies were used as delays for the re-focusing pulse with a mixing time, \(T_{\text{CP}}\), of 120 ms. Transverse relaxation rate \((R_2^{\text{obs}})\) values were determined from the signal ratios by \(R_2^{\text{obs}} = 1/T_{\text{CP}} \cdot \ln(I/I_0)\). The significance of each relaxation dispersion profiles was assessed based on an F-test (p-value<0.01). A global fit analysis using a two-state exchange model (14, 64, 65) was performed by fitting the residues displaying significant relaxation dispersion profiles. The intrinsic transverse relaxation rates \(R_2^0\) and the chemical shift changes \((\Delta \delta^S)\) are residue-specific parameters, while the population \(p_f\) and the exchange rate \(k_{\text{ex}}\) were set to the same value for all residues. The chemical shift changes \((|\Delta \delta^S|)\) were assumed to be independent of the temperature, \(T\), \(\Delta \delta^S(\text{HSQC})\) and constrained to the same value for all temperatures in the global fit analysis. We applied two models where in Model 1 \(p_n\) is temperature-dependent and in Model 2 \(p_n\) is temperature-independent. The models were assessed with an F-test and using the Akaike information criterion (AIC, Table S5), yielding Model 1 as the preferred one. In Model 1 the exchange rate for the two Ag(I) concentrations can be constrained to the same value (referred to as Model 1b), resulting in the same quality of the fits (Table S5). From Model 1 thermodynamic parameters for the binding and folding reaction were calculated (Supporting information text, page S5).

NMR data were processed with the Topspin version 3.2 software or NMRPipe. The diffusion data were analyzed using MatLab and the \(^{15}\)N-CPMG relaxation dispersion data was analyzed and fitted using IgorPro 7 (WaveMetrics).

**Dissociation constant determination for Ag(I) binding** Apparent dissociation constants of the \(\text{A\beta}\)-Ag(I) complex were determined for different conditions with different techniques. Relative intensities and chemical shift changes from \(^1\)H-\(^{15}\)N-HSQC spectra were extracted and plotted against the Ag(I) ion concentration. The data were globally fitted to a model assuming one binding site (Eq. 4) (77). Additionally, the apparent dissociation...
constant was determined from fluorescence spectroscopy data ([Supporting information text, page S4]). No buffer corrections were made.

\[
I = I_0 + \frac{I_{\infty} - I_0}{2[A\beta]} \left( K_{D}^{app} + [Ag(I)] + [A\beta] - \sqrt{(K_{D}^{app} + [Ag(I)] + [A\beta])^2 - 4 \cdot [Ag(I)] \cdot [A\beta]} \right)
\]

(Eq. 4)

where \( I_0 \) is the intensity upon saturation, \( I_\infty \) the initial intensity without Ag(I) ions, and \( K_D \) is the apparent dissociation constant. From NMR relaxation dispersion experiments an apparent \( K_D^{app} \) value was determined using the bound population \( p_B \).

The relative elongation constants in Fig. 4C were fitted using an equation that describes the effect of metal ion binding to A\( \beta \) monomers on the elongation rate, in terms of an apparent dissociation constant (78).

\[
k_+/k_0^0 = 1/(1 + [Me]/K_{D}^{app})
\]

(Eq. 5)

**Data availability**

All supporting data are available from the corresponding author upon request: Axel Abelein, Department of Neurobiology, Care Sciences and Society, Center for Alzheimer Research, Division of Neurogeriatrics, Karolinska Institutet, 141 52, Huddinge, Sweden; axel.abelein@ki.se

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**Author contribution:** Conceptualization: C.W., J.D., A.G., and A.A.; Formal Analysis: C.W., J.D., and A.A.; Funding acquisition: A.G.; Investigation: C.W. and A.A.; Methodology: C.W., J.D., and A.A.; Project administration: C.W. and A.A.; Resources: J.J., H.B., and S.W.; Supervision: J.D., A.G., and A.A.; Validation: J.J., H.B., and S.W.; Visualization: C.W. and A.A.; Writing – original draft: C.W. and A.A.; Writing – review & editing: J.J., H.B., S.W., J.D., and A.G.

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The abbreviations used are: Alzheimer’s disease, AD; amyloid-β, Aβ; pentameric formyl thiophene acetic acid, pFTAA; Thioflavin T, ThT; Carr–Purcell–Meiboom–Gill, CPMG
Mechanistic insights into Aβ self-assembly by metal ions

**Figure 1.** Aβ fibrillization retardation in the presence of Ag(I) ions. (A-C) Global fit analysis of aggregation traces of 20 µM monomeric Aβ40 incubated in the presence of 0-30 µM Ag(I) in 10 mM MOPS buffer pH 7.2 at +37 °C under quiescent conditions. The global fits were constrained such that only one nucleation rate \(k_n\), \(k_2\), or \(k_+\) is the single free fit parameter, revealing the best fit for \(k_+\) (normalized MSE 1.0), followed by \(k_2\) (norm. MSE 2.0) and \(k_n\) (norm. MSE 9.2). (D,E) Parameters from sigmoidal curve fitting of the kinetic traces in (A-C). The error bars represent standard deviation values from individual fits to 4 replicates (Fig. S1). (F) Elongation rates obtained from the initial slopes of highly seeded aggregation kinetics experiments, where 1 µM seeds were added to 20 µM monomeric Aβ40 with 0-30 µM Ag(I). The weighted average values calculated from four replicates are shown in (A-C and F). (G) Relative \(k_+\) values obtained from the global fit analysis in (C) compared to values from seeding experiments in (F), showing the same Ag(I)-dependence of \(k_+\). (H) Global fit analysis applying a model, where the apparent free Aβ monomer concentration is determined by the dissociation constant \(K_{D,app}\), revealing \(K_{D,app} = 14.5±0.2 \) µM (norm. MSE 3.7). (I,J) Images from solid-state AFM of samples taken after fibrillization kinetic experiments showing similar Aβ40 fibril structures in the absence (I) and in the presence of Ag(I) ions at 1:1 ratio (J). The scale bar represents 1 µm.
Figure 2. Ag(I) ions bind specifically to the N-terminal part of monomeric Aβ peptide. (A) $^1$H-$^{15}$N-HSQC spectra of 80 µM monomeric $^{13}$C-$^{15}$N-labeled Aβ$_{40}$ peptides alone (blue) and with 20 µM Ag(I) ions, 4:1 Aβ$_{40}$:Ag(I) (red) in 20 mM sodium phosphate buffer pH 7.4 recorded at 278 K. (B) Relative intensities determined from the Ag(I) ion titration. (C) $^1$H-$^{15}$N-HSQC spectra of $^{15}$N-labeled Aβ$_{40}$ H6A,H13A,H14A mutant peptides (Aβ$_{noHis}$) (blue) and in the presence of 1:30 Aβ$_{40}$:Ag(I) ions (green), showing no changes in crosspeak intensities (D) and chemical shifts (C) in the presence of Ag(I) ions. (E) Magnification of the chemical shift changes observed in (A). (F) Combined chemical shift changes from data in (A). Hence, signal attenuation and chemical shift changes upon addition of Ag(I) are most prominent for the N-terminal residues.
**Figure 3.** Chemical exchange between free Aβ and an Aβ-Ag(I) ion complex. (A) Translational diffusion coefficients of 80 µM monomeric Aβ₄₀ in the absence and presence of 5-50 µM Ag(I) ions at 281 K. The average and standard deviation values were determined from five repeated measurements. (B-F) Relaxation dispersion was measured for 80 µM ¹⁵N-labeled Aβ₄₀ with two different Ag(I) concentrations (4 and 6 µM). (B) Chemical shift differences from an individual data set with 6 µM Ag(I). Blue bars show the residues exhibiting significant chemical exchange (p<0.01), which were included in the full analysis, while red bars correspond to residues with no significant chemical exchange. Residues assigned with an open circle (O) were not observed due to low signal intensity and residues assigned with an asterisk (*) exhibited too fast exchange with the solvent or spectral overlap. (C,D) Relaxation dispersion profiles from two selected N-terminal residues at four different temperatures with 6 µM Ag(I). (E,F) The temperature dependence of the global fit parameters, the chemical exchange rate, $k_{ex}$, and bound population, $p_B$. (G) Chemical shift changes from $^1$H-$^15$N-HSQC experiments plotted against the chemical shift differences from relaxation dispersion, displayed in (B), revealing good correlation. (H) Gibbs free energy for the four different temperatures shown for 6 µM Ag(I). At higher temperatures ($\geq$281 K) the data points could be fitted linearly, while the whole data set exhibits a non-linear dependence (green), described by an equation including a heat capacity difference (Supporting information text, page S5-S6).
Mechanistic insights into Aβ self-assembly by metal ions

Figure 4. Model for the role of Aβ:metal ion complexes in Aβ fibrillization using sub-stoichiometric concentrations of Ag(I) and Zn(II) ions. (A) Global fit of diffusion data where data from Ag(I) (blue) were fitted together with previously reported data for Zn(II) (red) using a two-state model, revealing an increased diffusion coefficient of the bound/‘folded’ state by $D_B/D_{free} = 1.087 \pm 0.002$. (B) The metal-bound populations, $p_B$, are determined by the apparent dissociation constants for the respective metal ions and exhibit a linear dependence on the metal ion:Aβ ratio, here shown for the population corresponding to the diffusion data in (A). The bound populations from relaxation dispersion experiments of Ag(I) (turquoise) confirm this relation. (C) The relative elongation rates, $k_+$, of Zn(II) (red) and Ag(I), from seeding (blue circles) and global fit analysis (blue squares), are plotted against the metal ion:Aβ ratio, and fitted with Eq. 5, revealing an apparent dissociation constant of $K_D^{app} = 4.1 \pm 0.4 \mu M$ for Ag(I) ions and $K_D^{app} = 1.2 \pm 0.2 \mu M$ for Zn(II) ions (inserted graph). These values are similar as determined by other methods for Ag(I) (Table S2) and determined previously for Zn(II) (28). These findings indicate that Ag(I) and Zn(II) interact with monomeric Aβ in a remarkably similar manner. (D) Monomeric Aβ binds transition metal ions at the N-terminus, forming a compact, histidine-coordinated fold. This metal-peptide complex is not stable and exchanges with the free peptide on the ms timescale. Aβ fibrils are formed through secondary nucleation mechanisms, in addition to fibril-end elongation, where the latter is predominantly attenuated by the presence of metal ions. Hence, these binding processes reduce the apparent available pool of free monomeric Aβ, resulting in a retardation of the overall fibrillization.
Metal ion coordination delays amyloid-β peptide self-assembly by forming an aggregation-inert complex

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