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Journal
FEBS Letters, 591(1)

ISSN
0014-5793

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Publication Date
2017

DOI
10.1002/1873-3468.12526

Peer reviewed

HHS Public Access

Author manuscript

FEBS Lett. Author manuscript; available in PMC 2018 January 01.

Published in final edited form as:

FEBS Lett. 2017 January ; 591(1): 177–185. doi:10.1002/1873-3468.12526.

Cross-seeding between Aβ40 and Aβ42 in Alzheimer's disease

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Abstract

Aβ42 is the major component of parenchymal plaques in the brain of Alzheimer's patients, while Aβ40 is the major component of cerebrovascular plaques. Since Aβ40 and Aβ42 co-exist in the brain, understanding the interaction between Aβ40 and Aβ42 during their aggregation is important to delineate the molecular mechanism underlying Alzheimer's disease. Here, we present a rigorous and systematic study of the cross-seeding effects between Aβ40 and Aβ42. We show that Aβ40 fibril seeds can promote Aβ42 aggregation in a concentration-dependent manner, and vice versa. Our results also suggest that seeded aggregation and spontaneous aggregation may be two separate pathways. These findings may partly resolve conflicting observations in the literature regarding the cross-seeding effects between Aβ40 and Aβ42.

Keywords

Amyloid; protein aggregation; fibrillization kinetics

INTRODUCTION

Brain deposition of Aβ protein in the form of amyloid plaques is a pathological hallmark of Alzheimer's disease [1]. There are two major species of Aβ: the 40-residue Aβ40, and the 42-residue Aβ42. Aβ42 differs from Aβ40 by having two extra amino acids at the C-terminal end. In the brain, Aβ40 is severalfold more abundant than Aβ42 [2–4]. However, Aβ42 is the major species of the parenchymal plaques [5–8]. On the other hand, Aβ40 is the major component in the cerebrovascular plaques [5–9]. Given the high sequence similarity between Aβ40 and Aβ42, it is logical to postulate that Aβ40 and Aβ42 interact with each other during the process of their aggregation. Indeed, Aβ40 inhibits amyloid deposition of Aβ42 in vivo [10] and slows down the aggregation of Aβ42 in vitro [11,12]. Pre-formed fibrils of Aβ40 and Aβ42 can promote each other's aggregation [12–19]. Furthermore, Gu and Guo [20] show that Aβ40 and Aβ42, when mixed together, form interlaced fibrils, supporting that Aβ40 and Aβ42 interact at molecular level. Similarly, surface plasma resonance studies also suggest strong specific binding between Aβ40 and Aβ42 [19]. Using

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AUTHOR CONTRIBUTIONS

JT and DC performed seeding experiments; JT, DC, FH and HW prepared Aβ proteins; ZG conceived and supervised the study, and designed experiments; JT and ZG wrote the paper; DC, FH and HW made manuscript revisions. All authors gave final approval for publication.

Aβ42/Aβ40 ratio, rather than simply the concentration of Aβ42, improves diagnosis or prediction of Alzheimer's disease [21–23]. Some contradictory results, however, were also reported suggesting an absence of cross seeding between Aβ40 and Aβ42 [24–26]. It is important to resolve this contradiction because it may lead to confusion regarding the roles of Aβ42 and Aβ40 in Alzheimer's disease, and may also lead to misleading conclusions on subsequent studies that build upon the knowledge of Aβ40 and Aβ42 interaction.

The key question this work addresses is the cross seeding effects between Aβ40 and Aβ42. Fibrillization kinetics of Aβ is typically represented by a sigmoid curve, which includes a lag phase, a growth phase, and a plateau phase. The lag phase can be shortened by adding fibrils of the same protein, and this phenomenon is known as "self seeding". In some cases, the lag time can be shortened by adding fibrils of a different protein, and this phenomenon is called "cross seeding". Understanding the cross seeding effects between Aβ40 and Aβ42 may have important implications in Alzheimer's research. For example, cross seeding between Aβ40 and Aβ42 may provide a molecular explanation for the importance of Aβ42/Aβ40 ratio in the pathogenesis of Alzheimer's disease. A recent development in Aβ research is on brain-derived amyloid fibrils, which are seeded using brain plaques from Alzheimer's patients [26]. The plaques from brain may contain both Aβ40 and Aβ42. Due to cross seeding between Aβ40 and Aβ42, care must be taken to ensure that the resulting fibrils are derived from the perceived source. Furthermore, cross seeding between Aβ40 and Aβ42 has in vivo implications as it provides a biochemical basis for in vivo seeding of brain amyloid [27].

In a broader context, cross seeding between Aβ and other amyloid proteins may be one of the mechanisms linking Alzheimer's disease to type 2 diabetes and Parkinson's disease. Depositions of amylin and α-synuclein are involved in type 2 diabetes and Parkinson's disease, respectively. Aβ fibrils have been shown to be capable of seeding the aggregation of amylin and α-synuclein both in vivo [28,29] and in vitro [14,30]. Stable complexes between Aβ and tau, whose deposit is another pathological hallmark of Alzheimer's disease, have also been reported [31]. Molecular dynamics simulations have been used to reveal molecular interactions among Aβ, tau, amylin, and α-synuclein aggregates [32–36]. These studies suggest a complex relationship between different amyloid proteins and between different amyloid disorders that involve these proteins. Cross seeding effects may play an integral role in the molecular interactions between these amyloid proteins. This further emphasizes the importance of resolving the conflicting observations on Aβ40 and Aβ42 cross seeding: if Aβ40 and Aβ42 indeed do not seed each other, how could they seed other proteins with much less sequence similarity?

In this work we performed a systematic study on the cross seeding between Aβ40 and Aβ42 aggregation. To distinguish from previous studies, we used recombinant Aβ protein without additional residues to obtain sigmoid kinetics curves with well-resolved lag phase and to show concentration-dependent cross seeding effects for both Aβ40 and Aβ42 aggregation. Our study suggests that seeded nucleation and spontaneous nucleation are parallel processes in the lag phase, and cross seeding effects may not be detected if the spontaneous nucleation is too fast. This may partially explain the absence of cross seeding effects in some of the previous reports.

RESULTS

Self and cross seeding of Aβ40 fibrillization

The Aβ protein used in this study was produced in *E. coli* as a fusion protein [37]. After purification, the fusion protein partner was cleaved off, and full-length Aβ40 was obtained without any extra residues. All aggregation experiments were performed at 37°C in PBS buffer (pH 7.4) without agitation. To ensure the results were not due to batch variations, the same fibril seeds and Aβ stock solutions were used in all the self and cross seeding experiments. Four repeats were performed for each experimental condition. For quantitative analysis of the kinetics data, we extracted lag time and half time directly from the kinetics traces. The lag time was defined as the time when thioflavin T fluorescence reaches 5% of the plateau amplitude in the growth phase. The half time is defined as the time when thioflavin T fluorescence reaches 50% of the plateau amplitude.

We first performed a self seeding experiment for Aβ40. As shown in Figure 1A, in the absence of seeds, spontaneous Aβ40 fibrillization at 50 μM has a lag time of 17.7 h, and a half time of 18.7 h. The lag time was shortened by the presence of Aβ40 fibril seeds in a concentration-dependent manner (Figure 1A). Remarkably, the presence of just 0.25% Aβ40 fibril seeds shortened the lag time by more than half.

To study cross seeding, we added Aβ42 fibril seeds to Aβ40 aggregation. Similar to Aβ40, Aβ42 fibril seeds were also able to reduce the lag time of Aβ40 fibrillization in a concentration dependent manner (Figure 1B). Notably, the cross seeding effect was less dramatic than self seeding. It required 2% Aβ42 fibril seeds to halve the lag time of Aβ40 fibrillization.

Quantitatively, the effects of self seeding and cross seeding on lag time and half time are shown in Figures 1C and 1D. To achieve the same lag time or half time of Aβ40 fibrillization, it requires approximately 4 times more Aβ42 fibril seeds than Aβ40 fibril seeds. In this sense, Aβ40 is four times more efficient than Aβ42 in seeding Aβ40 fibrillization.

We have also performed the cross seeding of Aβ40 fibrillization using a different batch of Aβ40. The lag time was shortened by addition of Aβ42 seeds in a seed concentration-dependent fashion (Figure S1A). The exact lag time is different between batches, but the effect of cross seeding remains true.

Self and cross seeding of Aβ42 fibrillization

Spontaneous Aβ42 aggregation has a lag time of 1.5 h at a concentration of 5 μM (Figure 2A, C). In contrast, Aβ40 at 50 μM has a lag time of 17.7 h (Figure 1). These kinetics data are consistent with the common notion that Aβ42 aggregates faster than Aβ40. In self seeding experiments, we added sonicated Aβ42 fibrils at 2, 4, 8, and 16% of monomer equivalent concentration to seed Aβ42 aggregation. The results show a concentration-dependent decrease of both lag time and half time (Figure 2A, C, D). The self seeding effect for Aβ42 is much less prominent than Aβ40 self seeding. Even 8% fibril seeds did not reduce the lag time by half.

For cross seeding, we added Aβ40 fibril seeds at 2, 4, 8, and 16% of the monomer equivalent concentration. Aβ40 fibril seeds also accelerated Aβ42 aggregation in a concentration dependent manner (Figure 2B). The lag time and half time of cross seeding are shown in Figure 2C, D. At seed concentrations of 8% or lower, the seeding effect is similar for Aβ40 and Aβ42 seeds. Only at 16% seed concentration, we observed a dramatic difference between Aβ40 and Aβ42 seeds. Aβ42 seeds at 16% essentially eliminated the lag phase of Aβ42 aggregation. Meanwhile Aβ40 seeds at 16% resulted in a similar seeding effect as 8% Aβ40 seeds, suggesting that there may be an upper limit for the seeding effect of Aβ40 seeds.

To check batch-to-batch variations, we performed another cross seeding experiment with a different batch of Aβ42. And we observed similar concentration-dependent seeding effects (Figure S1B).

DISCUSSION

Whether there exists cross seeding between Aβ40 and Aβ42 is critical to the mechanistic understanding of Aβ aggregation in Alzheimer's disease. Therefore, it is important to resolve the conflicting observations on this subject so that proper conclusions can be made. Our literature search showed that there are eleven previous reports [12–19,24–26] that investigated the cross seeding between Aβ40 and Aβ42, with conflicting conclusions. We believe that four technical points are critical for a rigorous investigation on this topic: (1) Source of Aβ should be recombinant expression, instead of chemical synthesis. Synthetic Aβ has been shown to have different aggregation propensity from recombinant Aβ due to small amounts of impurities [38]; (2) The kinetics curve should be sigmoidal with a clear lag phase, which allows quantification of lag time; (3) Cross seeding effects should be demonstrated by reduction of lag time in a seed concentration-dependent manner, which suggests specific interactions; (4) There should be multiple repeats for each seeding experiment. This is because aggregation kinetics may be affected by miscellaneous factors comprising the aggregation environment, which include, but not limited to, pipetting inaccuracy and surface characteristics of the plastics (microplate, Eppendorf tubes, etc). Doing multiple repeats will not remove these uncertainties, but will provide an estimate of these uncertainties. Among the eleven previous studies (Table 1), only one report by Cukalevski et al. [24] satisfied all four technical points (with the caveat of having an extra methionine at the N-terminus of Aβ sequence). Cukalevski et al. [24] reported that the half time of Aβ40 aggregation was not affected by Aβ42 seeds, and vice versa. Along the similar line, Pauwels et al. [19] and Xiao et al. [25] showed aggregation experiments where Aβ42 fibrils did not accelerate the aggregation of Aβ40. Lu et al. [26] mentioned that "polymorphic Aβ42 fibrils prepared in vitro do not seed the growth of Aβ40 fibrils", but no data were shown. On the other hand, seven other reports concluded that Aβ42 fibrils can seed the aggregation of Aβ40, but these reports [12–18] did not meet all four technical points (Table 1). In terms of Aβ42 cross seeding, Cukalevski et al. [24] showed that the half time of Aβ42 aggregation was not affected by Aβ40 seeds, while four other studies [12–14,19] support an opposite view (Table 1).

It is in the best interest of Aβ researchers that we resolve these conflicting observations immediately, so that the Aβ community can move forward on a common ground. Here we present a systematic study of Aβ40/Aβ42 cross seeding that satisfies the four technical points outlined above. For these experiments, we used recombinant Aβ that does not contain any extra residues. Our kinetics experiments show sigmoid curves with a well-resolved lag phase. We performed four repeats of each seeding experiments. And we also used multiple concentrations of fibril seeds to demonstrate concentration-dependent effects. Our results show that Aβ42 fibril seeds can cross seed Aβ40 aggregation in a concentration-dependent manner (Figure 1). Vice versa, Aβ40 fibril seeds can also cross seed Aβ42 aggregation in a concentration-dependent manner (Figure 2). Generally speaking, the effect of cross seeding is less pronounced than the effect of self seeding. We have also repeated the cross seeding experiments with different batches of Aβ40 and Aβ42 and observed similar cross seeding effects (Figure S1).

We noted that Aβ42 requires a much higher concentration of seeds to show seeding effect, and this is the case even for self seeding. A significant decrease in lag time of Aβ42 aggregation was achieved with 4% (or higher) Aβ42 fibril seeds (Figure 2). In contrast, a dramatic decrease in the lag time of Aβ40 aggregation was observed with 0.25% Aβ40 fibril seeds (Figure 1). These results led us to propose that fibril seeds promote aggregation via a separate pathway from the spontaneous aggregation, rather than simply accelerating the spontaneous aggregation pathway (Figure 3). Thus the total rate of aggregation is the sum of seeded aggregation and spontaneous aggregation. In order to see a significant effect of seeding, the rate of seeded aggregation needs to be faster than the rate of spontaneous aggregation. If the rate of spontaneous aggregation is too fast compared to seeded aggregation, it would be difficult to observe seeding effects because most Aβ would aggregate via spontaneous aggregation. For Aβ40, the rate of spontaneous aggregation is slow, with a lag time of 17.7 h for 50 μM of Aβ40 (Figure 1). So a small amount of seeds can have a dramatic seeding effect. On the other hand, Aβ42 has a very fast rate of spontaneous aggregation, with a lag time of 1.5 h for 5 μM of Aβ42 (Figure 2). Therefore, a relatively large amount of seeds are required to observe seeding effects. This may partly explain the lack of cross seeding effect in some of the previous studies. For example, Pauwels et al. [19] used 25 μM Aβ40 and observed a lag time of ~3 h. Cukalevski et al. [24] used 10 μM Aβ40 and obtained a lag time of ~3 h. Both of these two studies have considerably faster Aβ40 aggregation than our 17.7 h of lag time with 50 μM of Aβ40. In the cross seeding aggregation experiments by Xiao et al. [25], the authors used repeatedly seeded Aβ42 fibrils to seed Aβ40 aggregation, so the absence of cross seeding effects may be due to the enrichment of a rare fibril polymorph. For the cross seeding of Aβ42, the report by Cukalevski et al. [24] is the only study that concluded that Aβ40 seeds did not promote the aggregation of Aβ42, in contrast to four previous studies by Hasegawa et al. [12], Jan et al. [13], Ono et al. [14], and Pauwels et al. [19]. It is worth pointing out that, in the study by Cukalevski et al. [24], pH seemed to affect cross seeding between Aβ40 and Aβ42, as the authors reported cross seeding effects at pH 8.0, although not at pH 7.4. Other studies [12–14,19] reporting cross seeding effects were all performed at pH 7.4 or 7.5. We have compiled other experimental details of the cross seeding studies in supplementary Table S1. It is not obvious what other factors directly contribute to the conflicting

observations. Altogether, the evidence for cross seeding between Aβ40 and Aβ42 is overwhelmingly strong.

The basis of Aβ40/Aβ42 cross seeding may be the structural plasticity of Aβ40 and Aβ42 proteins. In other words, Aβ40 and Aβ42 are capable of adopting each other's structure in the fibrils. The structure of Aβ40 fibrils has been studied extensively with various techniques. Structural models based on solid-state NMR constraints [39,40] mainly consist of two β-sheets: one at residues 17–20 (β1), and the other one at residues 31–36 (β2). Some other studies [41–43] suggest longer stretches of β-strands with the core regions still at 17–20 and 31–36. This β1-turn-β2 motif is the core structure of Aβ40 fibrils. For the structure of Aβ42 fibrils, our own studies using electron paramagnetic resonance suggest that the same β1-turn-β2 motif also exists in Aβ42 fibrils [17]. We also found that Aβ42 fibrils contain a turn at residues 37–38 and a C-terminal β-sheet at residues 39–41, which constitute the major difference between Aβ40 and Aβ42 fibrils. Hydrogen exchange studies also support the β1-turn-β2 motif in Aβ42 fibrils [44,45]. In contrast, solid-state NMR studies [25,46,47] suggest different β-sheet patterns in Aβ42 from those in Aβ40 fibrils. CryoEM studies show yet another structural model of Aβ42 fibrils [48]. These studies suggest that Aβ42 is capable of adopting different structures. Some of these structures resemble Aβ40 fibrils in some way, while others may adopt totally different folds. Therefore, this structural plasticity may underlie the cross seeding between Aβ40 and Aβ42.

MATERIALS AND METHODS

Preparation of Aβ protein

The DNA constructs of wild-type GroES-ubiquitin-Aβ [37] and the deubiquitylating enzyme Usp2cc [49] were kindly provided by Dr. Il-Seon Park at Chosun University (South Korea) and Dr. Rohan T. Baker at Australian National University (Australia). Protein expression and purification of Aβ40 and Aβ42 were performed as previously described [17,20,50]. Briefly, Aβ was expressed in *E. coli* as a fusion protein, GroES-ubiquitin-Aβ. After purification using a nickel column, the fusion protein was cleaved off with a deubiquitylating enzyme to obtain full-length Aβ without any extra residues. After purification, Aβ was stored at −80°C as lyophilized powder.

Preparation of Aβ fibril seeds

Lyophilized Aβ40 and Aβ42 powders were dissolved in 1,1,1,3,3,3 hexafluoro-2-propanol (HFIP) to a final concentration of 500 μM and incubated for 30 minutes at room temperature. Then HFIP was evaporated in a chemical hood overnight. To make fibrils, HFIP-treated Aβ40 and Aβ42 proteins were dissolved in CG buffer (20 mM CAPS, 7 M guanidine hydrochloride, pH 11). Then Aβ samples were diluted 20-fold to PBS buffer (50 mM phosphate, 140 mM NaCl, pH 7.4) and incubated at 37°C for 5 days. The final Aβ concentration was 50 μM for Aβ40 and 10 μM for Aβ42. The progress of aggregation was monitored using thioflavin T. The concentration of fibrils was considered to be the same as the starting monomer concentration, assuming the monomer concentration is negligible at the completion of fibrillization. To make fibril seeds, Aβ40 and Aβ42 fibrils were sonicated

for 200 s using a Branson Digital Sonifier model 450 (microtip, 10% amplitude, pulse mode). The sample was put on ice after every 50 s of sonication to avoid overheating.

Aβ40 seeding experiments

Aβ40 was first dissolved in 10 mM NaOH, and then 8 volumes of PBS buffer and 1 volume of 10 mM HCl were added to the sample. The concentration was determined using absorbance at 280 nm and an extinction coefficient of 1.28 $mM^{-1} \cdot cm^{-1}$ [51]. For different concentrations of fibril seeds, the stock solution of seeds was first diluted using the same buffer containing the seeds so that the same volume of seeds was added to each seeding experiment. Because the seeds contain 0.35 M guanidine hydrochloride, 82.6 mM guanidine hydrochloride was present in all the aggregation reactions. The final Aβ40 monomer concentration was 50 μM. For Aβ40 fibril seeds, the final seeds concentration was 0.25, 0.5, 1, and 2%. For Aβ42 fibril seeds, the final seeds concentration was 1, 2, 4, and 8%. Aggregation with each seed concentration was run in quadruplicate. The results of these seeding experiments are shown in Figure 1.

Aβ40 cross seeding experiments were repeated using another batch of Aβ40. In this experiment, Aβ40 was dissolved in CG buffer to a concentration of 1.5 mM. Then it was diluted 20-fold into PBS buffer containing 0, 1, 2, 3, 4% of Aβ42 fibril seeds. Aggregation with each seed concentration was run in triplicates. The results of these experiments are shown in Figure S1.

The final sample volume for aggregation was 50 μL. Aggregation was performed on a 384-well Nonbinding Surface microplate with clear bottom (Corning product# 3655), and sealed with a polyester based sealing film (Corning product# PCR-SP). Samples were kept on ice during preparation. Finally, the microplate was transferred to a Victor 3V plate reader (Perkin Elmer) to start aggregation at 37°C without agitation. The ThT fluorescence was measured through the bottom of the plate approximately every 3.5 min, with excitation filter of 450 nm and emission filter of 490 nm.

Aβ42 seeding experiments

Aβ42 was first dissolved in CG buffer to approximately 25 μM concentration, and then buffer exchanged to PBS buffer using a 5-mL HiTrap desalting column (GE Healthcare). The concentration of Aβ42 was determined using fluorescamine fluorescence with hen egg white lysozyme as standards. For different concentrations of fibril seeds, the stock solution of seeds was first diluted so that the same volume of seeds was added to each seeding experiment. This is to ensure that the same concentration of guanidine hydrochloride, 56 mM in this case, was present in all aggregation reactions. The final Aβ42 monomer concentration was 5 μM. For both Aβ40 and Aβ42 fibril seeds, the final seeds concentration was 2, 4, 8, and 16%. Aggregation volume for all the samples is 50 μL. The aggregation setup is the same as described for Aβ40 seeding experiments. The results of these experiments are shown in Figure 2.

Aβ42 cross seeding experiments were repeated using another batch of Aβ42. The experiments were formed exactly as described above and the results are shown in Figure S1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Christine Xue and Yoon Kyung Lee at University of California, Los Angeles, for help with protein preparation and data analysis.

FUNDING

This work was supported by the National Institute of Health (Grants R01GM110448 and R25GM055052).

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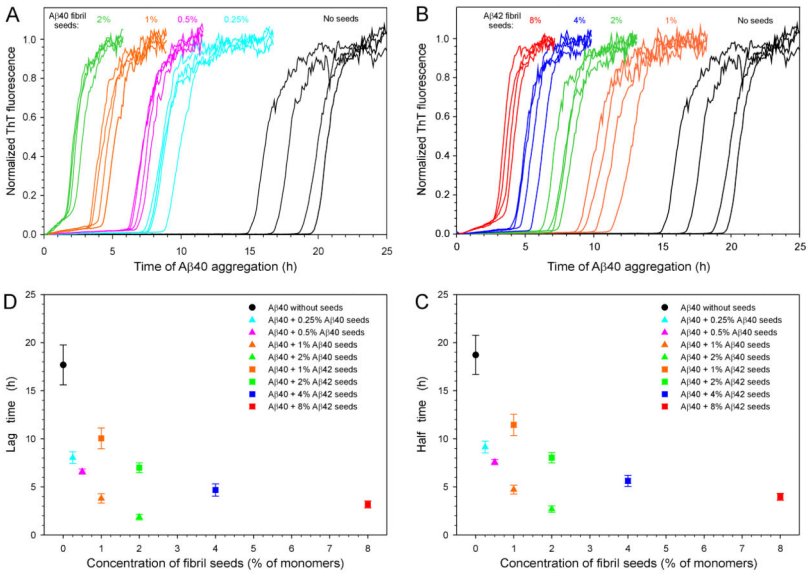

Figure 1. Self and cross seeding of Aβ40 aggregation

(*A, B*) Kinetics of Aβ40 aggregation in the absence and presence of Aβ40 (*A*) or Aβ42 (*B*) fibril seeds. Aggregation experiments were performed in quadruplicates. Aβ40 monomer concentration is 50 μM. The aggregation was performed in PBS buffer at 37°C without agitation. The kinetics traces were normalized to the ThT fluorescence at aggregation plateau. (*C, D*) Lag time (*C*) and half time (*D*) of Aβ40 aggregation in the absence and presence of fibril seeds. Symbols and error bars are the average and standard deviation of four kinetics experiments.

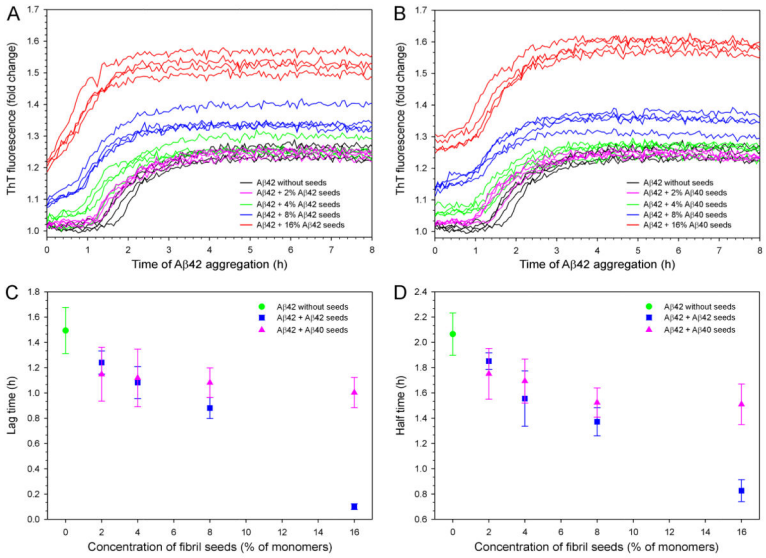

Figure 2. Self and cross seeding of Aβ42 aggregation

(*A, B*) Kinetics of Aβ42 aggregation in the absence and presence of Aβ42 (*A*) or Aβ40 (*B*) fibril seeds. Aggregation experiments were performed in quadruplicates. Aβ42 monomer concentration is 5 μM. The concentration of fibril seeds is expressed as a percentage of monomer concentration. The aggregation was performed in PBS buffer at 37°C without agitation. Due to the extensive overlap, these aggregation traces were not normalized and are presented as the fold change over ThT background fluorescence at each time point of aggregation. (*C, D*) Lag time (*C*) and half time (*D*) of Aβ42 aggregation in the absence and presence of fibril seeds. Symbols and error bars are the average and standard deviation of the four kinetics experiments.

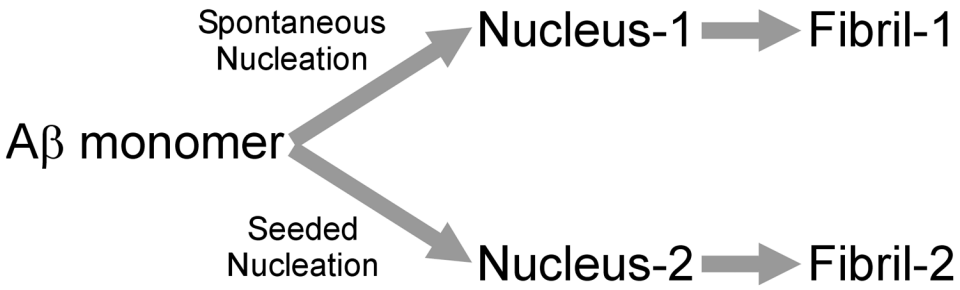

Figure 3. A proposed model for parallel pathways of spontaneous and seeded Aβ aggregation

In the absence of fibril seeds, Aβ monomer aggregates through spontaneous nucleation, followed by elongation to form amyloid fibrils. Fibril seeds promote aggregation via a seeded nucleation pathway that is parallel to spontaneous nucleation. The resulting nuclei and fibrils from spontaneous and seeded nucleation pathways may have the same or different structures, depending on what fibril seeds are used and the condition of the aggregation.

Table 1

Major technical points and conclusions of previous and present Aβ40/Aβ42 cross seeding studies.

Publication	Source of Aβ	Kinetics curve shows clear lag phase?	Seed concentration dependence?	Multiple repeats of each aggregation?	Results support cross seeding?
Aβ40 aggregation with Aβ42 seeds					
Jarrett et al. 1993	Synthetic	Yes	No	No	Yes
Snyder et al. 1994	Synthetic	No	No	No	Yes
Hasegawa et al. 1999	Synthetic	Yes	Yes	No	Yes
Jan et al. 2008	Synthetic	Yes	Yes	No	Yes
Ono et al. 2012	Synthetic	Yes	No	No	Yes
Pauwels et al. 2012	Recombinant	Yes	No	No	No
Yamaguchi et al. 2013	Recombinant	Yes	No	No	Yes
Xiao et al. 2015	Synthetic	Yes	No	No	No
Cukalevski et al. 2015	Recombinant, with N-Met[a]	Yes	Yes	Yes	No
Gu et al. 2016	Recombinant	Yes	No	Yes	Yes
This work	**Recombinant**	**Yes**	**Yes**	**Yes**	**Yes**
Aβ42 aggregation with Aβ40 seeds					
Hasegawa et al. 1999	Synthetic	No	Yes	No	Yes
Jan et al. 2008	Synthetic	Yes	Yes	No	Yes
Ono et al. 2012	Synthetic	Yes	No	No	Yes
Pauwels et al. 2012	Recombinant	Yes	No	No	Yes
Cukalevski et al. 2015	Recombinant, with N-Met[a]	Yes	Yes	Yes	No
This work	**Recombinant**	**Yes**	**Yes**	**Yes**	**Yes**

[a]There is an extra methionine residue at N-terminus.