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Alzheimer's A β 42 and A β 40 form mixed oligomers with direct molecular interactions

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ABSTRACT

Formation of A β oligomers and fibrils plays a central role in the pathogenesis of Alzheimer's disease. There are two major forms of A β in the brain: A β 42 and A β 40. A β 42 is the major component of the amyloid plaques, but the overall abundance of A β 40 is several times that of A β 42. *In vitro* experiments show that A β 42 and A β 40 affect each other's aggregation. In mouse models of Alzheimer's disease, overexpression of A β 40 has been shown to reduce the plaque pathology, suggesting that A β 42 and A β 40 also interact *in vivo*. Here we address the question of whether A β 42 and A β 40 interact with each other in the formation of oligomers using electron paramagnetic resonance (EPR) spectroscopy. When the A β 42 oligomers were formed using only spin-labeled A β 42, the dipolar interaction between spin labels that are within 20 Å range broadened the EPR spectrum and reduced its amplitude. Oligomers formed with a mixture of spin-labeled A β 42 and wild-type A β 42 gave an EPR spectrum with higher amplitude due to weakened spin-spin interactions, suggesting molecular mixing of labeled and wild-type A β 42. When spin-labeled A β 42 and wild-type A β 40 were mixed to form oligomers, the resulting EPR spectrum also showed reduced amplitude, suggesting that wild-type A β 40 can also form oligomers with spin-labeled A β 42. Therefore, our results suggest that A β 42 and A β 40 form mixed oligomers with direct molecular interactions. Our results point to the importance of investigating A β 42–A β 40 interactions in the brain for a complete understanding of Alzheimer's pathogenesis and therapeutic interventions.

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1. Introduction

Deposition of A β aggregates plays a central role in the pathogenesis of Alzheimer's disease [1]. A β is produced from the proteolytic digestion of amyloid precursor protein by β - and γ -secretases [2]. The cleavage by γ -secretase is not specific, generating a number of A β proteins with various C-terminal ends. The most abundant A β species is the 40-residue A β 40, followed by the 42-residue A β 42. Although A β 40 is several fold more abundant than A β 42 in the brain [3–5], the main A β species in amyloid plaques is A β 42 [6–8]. Several studies have shown that A β 40 is present only in a subset of amyloid plaques [6,7,9], suggesting that A β 42 aggregation may precede A β 40 aggregation. Overexpression of A β 40 in mouse models of Alzheimer's disease reduced the plaque pathology, suggesting a protective role for A β 40 [10]. Therefore, studying interactions between A β 42 and A β 40 may provide

mechanistic insight into the pathogenesis of Alzheimer's disease and facilitate development of therapeutic interventions.

In vitro studies show that A β 42 and A β 40 interact at molecular level and affect each other's aggregation. Surface plasmon resonance experiments showed direct interaction between A β 40 and A β 42 monomers [11]. NMR studies suggest that A β 40 inhibits the aggregation of A β 42 by binding to existing A β 42 aggregates [12]. Jan et al. [13] showed increasing A β 40/A β 42 ratio reduced fibril formation and led to reduced toxicity. The fibrils of A β 40 can seed the aggregation of A β 42, and vice versa [14,15]. Our previous EPR studies show that A β 42 and A β 40 form interlaced amyloid fibrils [16]. A recent NMR study found that mixing of A β 40 and A β 42 led to the formation of a fibril whose structure is different from that of either pure A β 40 or A β 42 fibrils [17].

In addition to amyloid fibrils, A β aggregation also generates soluble oligomers, which are considered to be the more toxic and pathogenic form of A β aggregates [18]. Using an environment-sensitive fluorophore, Frost et al. [19] showed that A β 40 and A β 42 formed mixed pre-fibrillar aggregates. Iljina et al. [20] used single-molecule two-color coincidence detection to show that

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A β 42 and A β 40 co-oligomerize at physiologically relevant concentrations. More recently, Baldassarre [21] used Fourier transform infrared spectroscopy to show that A β 42 and A β 40 form mixed oligomers with randomly distributed A β 42 and A β 40 strands in the β -sheet. In all three studies, the oligomers were prepared by lowering the sample pH from basic to neutral. Specific oligomer protocols such as A β -derived diffusible ligands [22] and globulomers [23] are used to prepare oligomers for *in vitro* [24,25] and *in vivo* studies [18,26,27]. But no studies have been reported on A β 40–A β 42 interactions in these specific oligomer preparations.

In this work, we aim to study whether A β 42 and A β 40 directly interact with each other in A β globulomers, which are prepared in the presence of low concentrations of SDS [23,28]. The continuous-wave EPR spectrum is sensitive to spin-spin interactions when spin labels are within a range of approximately 20 Å. In a previous study of A β 42 globulomers [29], we showed that the spin labels introduced at the same residue position are approximately 10–15 Å apart, consistent with an antiparallel β -sheet arrangement in globulomers. The EPR spectra of A β 42 oligomers formed by 100% spin-labeled A β 42 are broadened by spin-spin interactions, which are diminished in globulomers formed by a mixture of spin-labeled and wild-type A β 42. This provides us an approach to study the interactions between A β 42 and A β 40 in the globulomers (Fig. 1). Globulomers prepared with a mixture of spin-labeled A β 42 and wild-type A β 40 will show reduced spin-spin interactions if A β 42 and A β 40 interact at molecular level, and unchanged spin-spin interactions if A β 42 and A β 40 do not interact at molecular level.

2. Material and methods

Preparation of wild-type A β proteins. Detailed procedure of A β preparation has been described previously [29]. Briefly, A β 40 and A β 42 proteins were expressed in *E. coli* C41 (DE3) cells (Lucigen) using a fusion protein construct, GroES-ubiquitin-A β [30]. Following purification using a nickel column under denaturing conditions, the fusion protein partners were cleaved off using a deubiquitylating enzyme, Usp 2-cc [31]. This leads to the purification of full-length A β 40 or A β 42 proteins without any extra residues. Finally, A β proteins were buffer exchanged to 30 mM

ammonium acetate, pH 10, lyophilized, and stored at -80 °C. Throughout the purification process, A β proteins were kept in buffers of pH 10 and never exposed to acidic conditions. The identity of the protein was confirmed using mass spectrometry.

Preparation of spin-labeled A β 42. A cysteine mutant of A β 42, G37C, was constructed using site-directed mutagenesis approach with QuikChange mutagenesis kit (Agilent). Protein purification was performed similarly as wild-type A β . For spin labeling, A β 42 G37C protein was first treated with 10 mM TCEP at room temperature for 20 min to break any disulfide bonds. Then the protein was buffer exchanged to labeling buffer (20 mM MOPS, 7 M guanidine hydrochloride, pH 6.8). Spin labeling reagent MTSSL, 1-oxyl-2,2,5,5-tetramethylpyrrolidine-3-methyl methanethiosulfonate (Adipogen), was added at 10-fold molar excess and incubated at room temperature for 1 h. Then the labeling mixture was buffered exchanged to 30 mM ammonium acetate (pH 10), lyophilized, and stored at -80 °C. The extent of labeling was evaluated with mass spectrometry. Only samples with labeling efficiency of >95% were used in the subsequent experiments. The spin label side chain is named R1 and the spin-labeled A β 42 mutant is thus named G37R1.

Preparation of A β globulomers. Lyophilized powder of wild-type A β 40, wild-type A β 42, and spin-labeled A β 42 (G37R1) was first dissolved in 100% 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) at 1 mM, bath-sonicated for 5 min, and then incubated at room temperature for 30 min. HFIP was allowed to evaporate overnight in a fume hood. HFIP treated samples were solubilized in dimethyl sulfoxide (DMSO) at 5 mM and bath-sonicated for 5 min. The A β concentration was determined using a fluorescamine method [32]. We prepared four samples of A β globulomers. Sample 1 is 100% spin-labeled A β 42. Sample 2 is a mixture 25% spin-labeled and 75% wild-type A β 42. Sample 3 is a mixture of 25% spin-labeled A β 42, 25% wild-type A β 42, and 50% wild-type A β 40. Sample 4 is a mixture of 25% spin-labeled A β 42 and 75% wild-type A β 40. To prepare globulomers, these four A β samples in DMSO were diluted using phosphate-buffered saline (PBS) (20 mM phosphate, 140 mM NaCl, pH 7.4) to 400 μ M containing 0.2% (final concentration) SDS. After incubation for 6 h at 37 °C, these four A β samples were further diluted with 3 vol of deionized water to a final concentration of 100 μ M and incubated for another 18 h at 37 °C. For collecting the

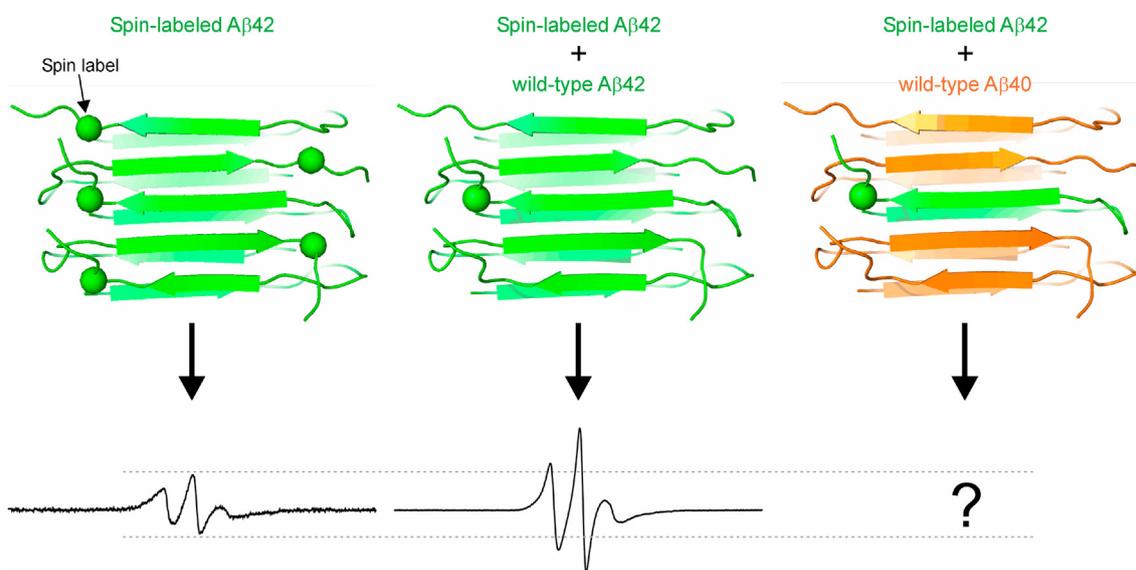


Fig. 1. Rationale of study design. Due to spin-spin interactions, A β 42 oligomers formed by only spin-labeled A β 42 have an EPR spectrum with broadened lines and reduced amplitude. When the oligomers are formed by spin-labeled and wild-type A β 42, the spin-spin interaction is weakened, resulting in an EPR spectrum of sharper lines and higher amplitude. The EPR spectrum of oligomers formed by spin-labeled A β 42 and wild-type A β 40 would provide information on whether A β 42 and A β 40 interact at molecular level in these oligomers. Dashed lines are drawn to aid for comparison.

globulomers, the samples were centrifuged at 14,000 g for 20 min to remove insoluble aggregates, and the supernatant was concentrated using an ultrafiltration filter with 30-kD molecular mass cut-off. Samples in the retentate contained the globulomers.

Transmission electron microscopy. A β globulomers (5 μ L) was placed on glow-discharged copper grids covered with 400-mesh Formvar/carbon film (Ted Pella). Then the samples were negatively stained using 2% uranyl acetate and examined under a JEOL JEM-1200EX transmission electron microscope at 80 kV.

EPR spectroscopy. A β globulomer samples were loaded into glass capillaries (VitroCom) sealed at one end. A Bruker EMX EPR spectrometer equipped with the SR4102ST cavity was used for X-band continuous-wave EPR measurements at room temperature. A microwave power of 20 mW and a modulation frequency of 100 kHz were used. Modulation amplitude was optimized for each individual sample. Scan width was 200 G. All EPR spectra were normalized to the same number of spins.

3. Results and discussion

To investigate whether A β 42 and A β 40 form mixed oligomers with molecular interactions, we took advantage of the dipolar interaction between spin labels when they are in a range of approximately 20 Å. The dipolar interaction broadens the EPR spectrum. As shown in Fig. 1, when A β 42 globulomers are prepared with 100% spin-labeled A β 42, the resulting EPR spectrum is broader and has lower amplitude than globulomers prepared with a mixture of spin-labeled and wild-type A β 42. This is because interdigitation between spin-labeled and wild-type A β 42 in the oligomer increases the distance between spin labels, and thus reduces the dipolar spin-spin interaction. If A β 40 and A β 42 interact with each other at molecular level, then a mixture of spin-labeled A β 42 and wild-type A β 40 will lead to a similar level of reduction in the dipolar interaction between spin-labeled A β 42.

Using spin-labeled A β 42, G37R1, where R1 represents the spin label, wild-type A β 42 and A β 40, we prepared four globulomer samples with different ratios of the three A β proteins. The first sample is prepared with 100% spin-labeled A β 42. Our previous studies show that the spin labels at position 37 are approximately 12 Å apart in the globulomer [29]. This sample gives strong dipolar interaction between spin labels. The second sample is a mixture of 25% spin-labeled A β 42 and 75% wild-type A β 42. This sample provides a control for reduced dipolar interaction. The third sample is a mixture of 25% spin-labeled A β 42, 25% wild-type A β 42, and 50% wild-type A β 40. If A β 40 does not form molecular interactions with A β 42, we will observe a reduction of dipolar interaction but less than the mixture of 25% labeled and 75% wild-type A β 42 sample. If A β 40 interacts with A β 42 in the oligomers, then this sample will give an EPR spectrum that is similar to the 75% wild-type A β 42 sample. The fourth sample is a mixture of 25% spin-labeled A β 42 and 75% wild-type A β 40. In the absence of A β 40-A β 42 interaction, this sample will give an EPR spectrum that is the same as the 100% spin-labeled sample. In the presence of molecular interactions between A β 40 and A β 42, this sample will resemble the mixture of 25% labeled and 75% wild-type A β 42 sample.

We examined the morphology of the four globulomer preparations using transmission electron microscopy (Fig. 2). In addition to stand-alone oligomers (open arrowheads in Fig. 2), we found that the A β globulomers have strong tendency to bind to each other, forming elongated and networked structures. Importantly, the four samples show similar morphology under electron microscope, suggesting that mixing of spin-labeled A β 42 with either wild-type A β 42 or A β 40 had little effect on the globulomer morphology.

Next we performed EPR measurements on these four globulomer samples. As shown in Fig. 3A, globulomers of 100% spin-

labeled A β 42, G37R1, show dipolar-broadened spectrum with smaller amplitude than the EPR spectrum of mixed globulomers with 25% labeled and 75% wild-type A β 42. When globulomers are prepared with A β 42 and A β 40, either in a mixture of 25% spin-labeled A β 42, 25% wild-type A β 42, and 50% wild-type A β 40, or a mixture of 25% spin-labeled A β 42 and 75% wild-type A β 40, the resulting EPR spectra are extremely similar to the mixture of 25% labeled A β 42 and 75% wild-type A β 42, suggesting that A β 40 can substitute A β 42 in forming mixed globulomers.

Although we observed abundant elongated and networked structures formed by A β globulomers, the EPR spectra consist of sharp lines, suggesting highly mobile structure in the oligomers (Fig. 3A). These spectral features are distinct from the EPR spectra of amyloid fibrils formed by the same spin-labeled A β 42 mutant (Fig. 3B). In the fibrils, the EPR spectral lines are much broader, resulting in greatly reduced spectral amplitude. Another characteristic of the fibril spectrum is that the three EPR resonance lines collapse toward the center line as a result of the spin exchange interaction in the parallel in-register β -sheet structure of A β fibrils [33,34]. Therefore, even though the A β globulomers have a strong tendency to bind to each other, the structures remain unchanged and are very different from the structures in fibrils.

The findings that A β 42 and A β 40 have molecular interactions in globulomers are consistent with previous reports that A β 42 and A β 40 form mixed prefibrillar aggregates. Previously, Barth and coworkers [21] used Fourier transform infrared spectroscopy to show that A β 42 and A β 40 form mixed oligomers with randomly distributed A β 42 and A β 40 strands in the β -sheet. In their study, they prepared A β oligomers by rapidly changing the solution pH from >11 to 7.4. In addition to showing the mixed nature of A β oligomers formed by A β 42 and A β 40, the infrared spectroscopy also provided evidence for the antiparallel signature of β -sheets. Another study by Frost et al. [19] used an environment-sensitive fluorescent label to show that A β 42 and A β 40 formed mixed prefibrillar oligomers when the buffer pH was adjusted from 10 to 7. Iljina et al. [20] used two-color coincidence detection method to show that A β 42 and A β 40 formed mixed oligomers at physiologically relevant concentrations. What distinguish this work from previous ones is that we prepared oligomers using a well-established protocol for globulomers [23,28]. A similar protocol was used by Paravastu and coworkers to prepare “150 kDa oligomers”, which were shown to consist of both out-of-register parallel β -sheets and antiparallel β -sheets in NMR studies [24]. EPR data in our previous work are consistent with antiparallel β -sheet structure [29]. Together with the reports on mixed A β 42-A β 40 amyloid fibrils [16,17], the evidence is strong for molecular interactions between A β 42 and A β 40 in the aggregation process and the aggregated products.

Our findings also have *in vivo* implications. The composition of amyloid fibrils in the brain has been extensively studied. A β 42 has been found to be the major component of parenchymal plaques [6–8], even though A β 40 is several-fold more abundant than A β 42 in the brain [3–5]. Several studies found that soluble A β aggregates isolated from brains of Alzheimer's disease patients contains both A β 42 and A β 40 [35–37]. Wildburger et al. [36] used mass spectrometry to study the A β isoforms in soluble A β oligomers and found that both A β 42 and A β 40 are present in the soluble aggregates, with A β 42 clearly more abundant than A β 40. Using end-specific antibodies, Yang et al. [37] also found that the high molecular weight A β oligomers consist of both A β 42 and A β 40. Mass spectrometry revealed the presence of heterogeneous A β 42-A β 40 dimers in the brain extracts, suggesting that A β 42 and A β 40 indeed form mixed oligomers in the brain [35]. In mouse models of Alzheimer's disease, A β 40 has been shown to have strong anti-amyloid effect [10]. Using BRI-A β 40 construct to overexpress A β 40, Kim

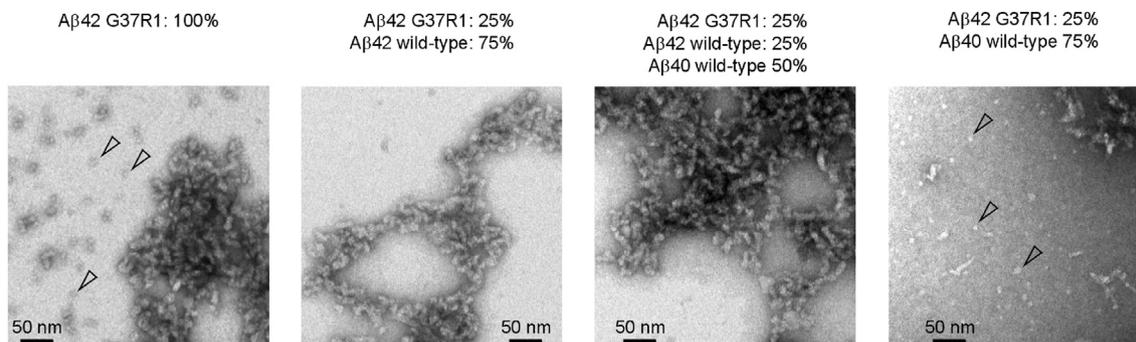


Fig. 2. Transmission electron micrographs of A β globulomers prepared with different mixtures of spin-labeled and wild-type A β proteins. Some stand-alone globular oligomers are present (open arrowheads), but most of the oligomers stick together to form elongated and networked structures. Overall, oligomers prepared from different mixtures of spin-labeled A β 42, G37R1 (where R1 represents the spin label), with either wild-type A β 42 or A β 40 show similar morphology.

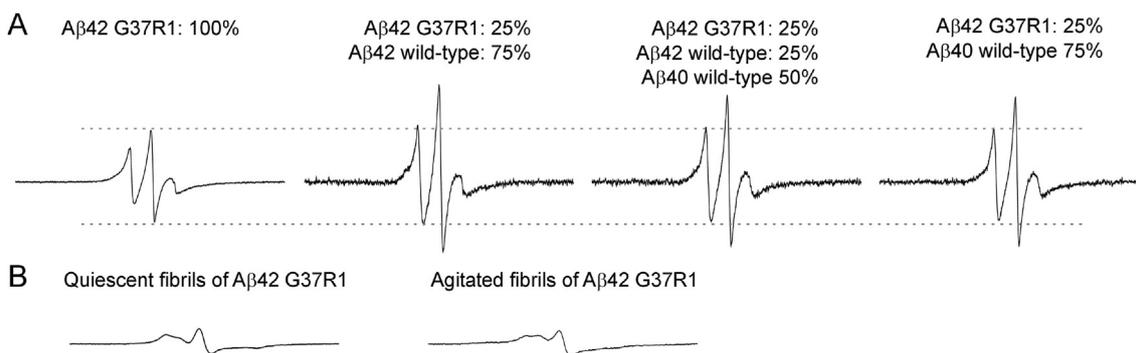


Fig. 3. EPR spectra of A β globulomers and fibrils. (A) EPR spectra of A β globulomers prepared with different mixtures of spin-labeled and wild-type A β proteins. R1 represents the spin label. Note that the EPR spectra of oligomers prepared with a mixture of spin-labeled A β 42 and wild-type A β 40 are very similar to those of oligomers prepared with a mixture of spin-labeled A β 42 and wild-type A β 42, suggesting A β 42 and A β 40 are interchangeable in the formation of A β globulomers. (B) EPR spectra of A β fibrils prepared with 100% spin-labeled A β 42. Note that the EPR spectra of A β fibrils are dramatically different from those of oligomers, suggesting different underlying structures. Dashed lines are drawn to aid for comparison. Scan width for all EPR spectra is 200 G.

et al. [10] showed that two fold increase of A β 40 levels in Tg2576 mice reduced A β deposition by ~80% at 11 months and ~50% at 20 months. Selective overexpression of A β 42 had the exact opposite effect of plaque pathology [38]. There have yet to be studies on the correlation between A β 42/A β 40 ratio in oligomers and the severity of Alzheimer's symptom. Such information would provide invaluable insights into the role of A β 42-A β 40 interaction in the pathogenesis of Alzheimer's disease and lay foundations for new avenues of therapeutic intervention.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbrc.2020.11.092>.

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