Structural Basis for Increased Toxicity of Pathological \(A\beta_{42}:A\beta_{40}\) Ratios in Alzheimer Disease

Kris Pauwels, Kyle L. Morris, Wim Jonckheere, Annelies Vandersteen, Geoff Kelly, Joost Schymkowitz, Frederic Rousseau, Annalisa Pastore, Louise C. Serpell, and Kerensa Broersen

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The \(\beta\)-amyloid peptide (\(A\beta\)) is directly related to neurotoxicity in Alzheimer disease (AD). The two most abundant alloforms of the peptide co-exist under normal physiological conditions in the brain in an \(A\beta_{42}:A\beta_{40}\) ratio of \(\sim 1:9\). This ratio is often shifted to a higher percentage of \(A\beta_{42}\) in brains of patients with familial AD and this has recently been shown to lead to increased synaptotoxicity. The molecular basis for this phenomenon is unclear. Although the aggregation characteristics of \(A\beta_{40}\) and \(A\beta_{42}\) individually are well established, little is known about the properties of mixtures. We have explored the biophysical and structural properties of physiologically relevant \(A\beta_{42}:A\beta_{40}\) ratios by several techniques. We show that \(A\beta_{40}\) and \(A\beta_{42}\) directly interact as well as modify the behavior of the other. The structures of monomeric and fibrillar assemblies formed from \(A\beta_{40}\) and \(A\beta_{42}\) mixtures do not differ from those formed from either of these peptides alone. Instead, the co-assembly of \(A\beta_{40}\) and \(A\beta_{42}\) influences the aggregation kinetics by altering the pattern of oligomer formation as evidenced by a unique combination of solution nuclear magnetic resonance spectroscopy, high molecular weight mass spectrometry, and cross-seeding experiments. We relate these observations to the observed enhanced toxicity of relative ratios of \(A\beta_{42}:A\beta_{40}\) in synaptotoxicity assays and in AD patients.

Alzheimer disease (AD) is a multifactorial neurodegenerative disease that mainly affects the growing population of the elderly. The primary agents of AD, the \(\beta\)-amyloid peptides (\(A\beta\)), are produced from the amyloid precursor protein by sequential endoproteolytic cleavages. The severity of dementia correlates with soluble assemblies of \(A\beta\) peptides rather than with the final fibrillar \(A\beta\) deposits observed in the brain (1) and a plethora of different toxic oligomers have been identified (2–5).

Imprecise cleavage of the amyloid precursor protein substrate by \(\gamma\)-secretase or altered catabolism of the \(A\beta\) peptides affect the relative amounts of \(A\beta_{42}\) and \(A\beta_{40}\), the two main \(A\beta\) fragments (6–8). An increased \(A\beta_{42}:A\beta_{40}\) ratio seems to coincide with more aggressive forms of the disease compared with cases of sporadic AD (9) and affects synaptic activity, viability of neuronal cells, and memory formation in animals (7, 8, 10–12). Recently, minor shifts in the \(A\beta_{42}:A\beta_{40}\) ratio have been reported to drastically influence the formation of neurotoxic oligomers (13, 14). Despite the very similar chemical nature of the two peptides, they seem to have quite different structural and biophysical properties. \(A\beta_{42}\) is known to be highly fibrologenic and more prone than \(A\beta_{40}\) to form neurotoxic assemblies (13, 15–17). Different architectures of \textit{in vitro}-generated amyloid fibrils from pure \(A\beta_{40}\) and \(A\beta_{42}\) peptides have been revealed by nuclear magnetic resonance (NMR) (18), electron microscopy (EM) (19), and x-ray fiber diffraction methods (20–22). A limited number of studies have demonstrated that \(A\beta_{40}\) and \(A\beta_{42}\) each affect the aggregation rates of the other, and it is generally reported that \(A\beta_{40}\) inhibits the aggregation of \(A\beta_{42}\) (12, 14, 23–27).

To date, most structural and biophysical studies have been performed using \(A\beta_{40}\) or \(A\beta_{42}\) in isolation. However, the aberrant behavior of neurotoxic \(A\beta\) peptides directed by the \(A\beta_{42}:A\beta_{40}\) ratio requires the need to simultaneously investigate \(A\beta_{40}\).

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\(\ddagger\) Present address: Dept. of Physics 12-908, Drexel University, 3141 Chestnut St., Philadelphia, PA 19104.

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\(\S\) To whom correspondence should be addressed: Faculty of Science and Technology, Nanobiophysics, MIRA Institute for Biomedical Technology and Technical Medicine, University of Twente, Enschede (The Netherlands). Tel: 31-0534893655; Fax: 31-0534891105; E-mail: kbroersen@utwente.nl.

\* The abbreviations used are: AD, Alzheimer disease; SPR, surface plasmon resonance; HDX, hydrogen deuterium exchange; DMSO, dimethyl sulfoxide; TEM, transmission electron microscopy; ThT, thioflavin T; HSQC, heteronuclear single quantum coherence.
and Aβ_{42}. In the present study, we address how Aβ_{40} and Aβ_{42} influence and modulate assembly and consider how structural aspects of intermediates along the aggregation pathway can direct the cytotoxic response of Aβ_{42}:Aβ_{40} ratios. By combining transmission electron microscopy (TEM), x-ray fiber diffraction, surface plasmon resonance (SPR), solution NMR, and high molecular weight mass spectrometry, we have characterized the start and end states of different relevant Aβ_{42}:Aβ_{40} ratios. Using the unique combination of \( ^{15}N \)-edited and \( ^{15}N \)-filtered NMR experiments, we have been able to isolate the specific behavior of either Aβ_{40} or Aβ_{42} in mixtures. We show that Aβ_{40} and Aβ_{42} can interact and that they mutually influence their aggregation behavior. Interestingly, cross-seeding and mass spectrometry (MS) experiments reveal differences in the prefibrillar stage of aggregation, which are reflected by different aggregation kinetics.

### Experimental Procedures

**Preparation of Aβ Peptide Ratios**—The Aβ_{40} and Aβ_{42} peptides and their uniformly \( ^{15}N \)-labeled variants were purchased from rPeptide (rPeptide catalogue no. A-1112-1, A-1118-1, A-1101-2, and A-1102-2). The Aβ_{40} and Aβ_{42} peptides were combined in monomeric form in the desired ratios as described in detail elsewhere (28). In brief, Aβ peptides were dissolved in 1,1,1,3,3,3-hexafluor-2-propanol, Aβ_{42} and Aβ_{40} were then mixed in molar ratios of 1:9 and 3:7 together with pure Aβ_{42} and Aβ_{40} samples, and after evaporation of 1,1,1,3,3,3-hexafluor-2-propanol, they were redissolved in dimethyl sulfoxide (DMSO). The peptide was passed through a desalting column and eluted in a 50 mM Tris-HCl, 1 mM EDTA buffer, pH 7.5. Peptide concentrations were measured by the Bradford assay or UV absorbance at 280 nm \( (ε_{280} = 1490 \text{ M}^{-1} \cdot \text{cm}^{-1}) \). The samples were kept on ice until required, with a maximum lag time of 30 min.

**Stern Analysis**—N-terminally labeled biotin-linker chain Aβ_{40} (biotin-Aβ_{40}) and biotin-Aβ_{42} (rPeptide catalogue no. A-1112-1 and A-1118-1, respectively) and Aβ_{40}, Aβ_{42}, and a non-assembling peptide with the sequence KAAEAAAKKFFE (29) were treated as described above except using a 10 mM HEPES, 100 mM NaCl, 1 mM EDTA, and 0.05 mM NaN\(_3\), pH 7.4 buffer, and the peptide was eluted using a 2-ml Zeba spin column for buffer exchange. SPR measurements were carried out on a Biacore\(^{\text{TM}}\) 2000 system (GE Healthcare) using carboxymethylated dextran preimmobilized with streptavidin sensor chips (GE Healthcare). A volume of 150 \( μl \) of biotin-Aβ_{40} or biotin-Aβ_{42}, was immobilized to the sensor surface at a concentration of 10 \( μM \) at a flow rate of 30 \( μl/min \). Concentrations of 10 \( μM \) of Aβ_{40}, Aβ_{42}, or KAAEAAAKKFFE were injected at 3 \( μl/min \). Measurements were done in triplicate and analyzed with the built-in BIAevaluation software. Curve fitting relied on the Marquardt-Levenberg algorithm, and the change in response was fitted to the binding isotherm \( R_\text{eq} = R_\text{max}(A)/(k_\text{off}(k_\text{on})+|A|) \), where \( R_\text{eq} \) is the equilibrium response, \( R_\text{max} \) is the maximum signal response, \( |A| \) is the analyte concentration, \( k_\text{off} \) is the dissociation rate constant, and \( k_\text{on} \) is the association rate constant.

**Fiber Diffraction**—Samples of mature fibers were aligned by suspending a droplet of solution at 4 mg/ml between two wax-tipped capillaries positioned end-to-end. Fibers were mounted on a goniometer head, and x-ray diffraction data were collected using a Rigaku CuK\( _{\alpha} \) rotating anode with a wavelength of 1.5419 Å and RAXIS IV+ detector. Specimen to detector distances were 160 and 250 mm with an exposure time of 10 min. Diffraction patterns were examined in CLEARER (30). For additional inspection, meridional, and equatorial axes signals were sampled through an angular search width of 60\(^\circ\), exported as a function of distance (pixels), and plotted using Bragg-Law.

**TEM Analysis**—Aliquots of 4 \( μl \) Aβ were adsorbed for 30 s onto freshly prepared carbon-coated and glow-discharged copper grids, washed briefly with milli-Q water, and subsequently stained with 1% \( (w/v) \) uranyl acetate for 30 s. Samples were examined with a JEOL 1200 transmission electron microscope operating at 100 KV.

**Cross-seeding Monitored with ThT**—Aliquots (100 \( μl \)) of each Aβ ratio at 50 \( μM \) were incubated at 25 °C in 50 mM Tris, 1 mM EDTA, pH 7.5. After 24 h incubation these samples were sonicated at 4 °C for 10 min at maximum power and mixed with freshly and simultaneously prepared Aβ ratios to induce (cross-)-seeding of Aβ aggregation. Final concentrations in these mixtures were 0.5 \( μM \) of sonicated Aβ and 25 \( μM \) of monomeric Aβ. The seed preparations were examined by TEM.

**ThT Fluorescence**—Aβ peptide samples of 25 \( μM \) were incubated with 12 \( μM \) thioflavin T (ThT) in a total volume of 150 \( μl \) in a Greiner 96-well plate. Fibrillization kinetics were followed using a Fluostar OPTIMA fluorescence plate reader using 440 nm excitation wavelength and an emission wavelength of 480 nm. Readings were recorded in triplicate every 10 min for a period of 24 h.

**High Molecular Weight MS**—High mass measurements were performed at CovalX AG (Schlieren, Switzerland) using an ABI 4800 MALDI TOF mass spectrometer retrofitted with CovalX HM2 TUVOS high mass system. A phosphate-buffered saline buffer was used to prepare the Aβ ratios, which were subjected to cross-linking with glutaraldehyde at specific time points. Each sample was mixed with sinapinic acid matrix (10 mg/ml) in acetonitrile/water (1:1, \( v/v \)), TFA 0.1% and spotted on the MALDI plate (SCOUT 384, AchorChip). High-mass MALDI TOF MS analysis was performed using standard nitrogen laser and focusing on different mass ranges from 8 to 1000 kDa in linear and positive mode and at a gain voltage of 3.14 kV and an acceleration voltage of 20 kV for HM2 High-Mass detection. The instrument was calibrated using insulin, BSA, and IgG. The analysis was repeated in triplicate.

**Solution NMR**—Aβ samples for NMR studies varied between 20 and 200 \( μM \) (monomer concentration) in 50 mM Tris-HCl, 1 mM EDTA at pH 7.5, supplemented with 10% \( (v/v) \) D\( _2\)O (>99.96%, Sigma Aldrich). The experiments were performed at 25 °C either on a Bruker Avance (equipped with cryoprobe) or on a Varian Inova spectrometer both operating at 14.1 Tesla (600 MHz). \( ^{15}N \) sofar heteronuclear single quantum coherence (HSQC) spectra were each collected over 30 min to monitor aggregation. \( ^{15}N \) NOESY-HSQC and \( ^{1}H\)-\(^{1}H\) TOCSY experiments were recorded at 5 °C to obtain sequence specific \( ^{1}H_{\alpha} \), \( ^{15}N \) assignments to identify the HSQC peaks. A combination of \( ^{15}N \)-edited and \( ^{15}N \)-filtered experiments (31) acquired on samples containing uniformly \( ^{15}N \)-labeled and unlabeled.
Aβ peptides at different ratios was used to selectively monitor Aβ\textsubscript{42} and Aβ\textsubscript{40} in solution.

Protection factors of mature Aβ fibers were measured by comparing the amide peak intensities obtained for Aβ samples incubated in H\textsubscript{2}O or in D\textsubscript{2}O after for a period of 672 h to allow amide exchange. The fibers were collected by centrifugation, washed, and incubated in D\textsubscript{2}O at 25 °C for 48 h, flash-frozen to quench the hydrogen-deuterium exchange (HDX), lyophilized, and redissolved in 100% DMSO-d\textsubscript{6} (99.9%, Cambridge Isotope Laboratories) acidified with 0.1% (v/v) trifluoroacetic acid (Fluka) for 30 s, followed by a 10-fold dilution with perdeuterated DMSO (32). Amide exchange was measured by collecting cross-peak assignment was confirmed with a 15N-resolved NOESY experiment.

Cross-seeding Monitored via NMR—Seeds were prepared of pure Aβ\textsubscript{40} or pure Aβ\textsubscript{42} as described above. An aliquot of 30 µl seeds (50 µM equivalent monomer concentration) was mixed with 330 µl of the corresponding Aβ samples that were preincubated in an NMR tube (Shigemi), whereas the non-seeded signals were monitored. 15N-edited and 15N-filtered spectra (31) were acquired as a function of time to simultaneously monitor both Aβ alloforms in the 1:9 and 3:7 ratios, whereby Aβ\textsubscript{42} was 15N-labeled and Aβ\textsubscript{40} was unlabeled. Only one-dimensional proton spectra were recorded as a function of time for the pure Aβ\textsubscript{40} or pure Aβ\textsubscript{42} unlabeled samples.

RESULTS

**Direct Interactions between Aβ\textsubscript{40} and Aβ\textsubscript{42}—** SPR was used to explore whether Aβ\textsubscript{40} and Aβ\textsubscript{42} are able to directly associate. Either biotinylated Aβ\textsubscript{42} or Aβ\textsubscript{40} were tethered to a chip and measurements of interactions between Aβ\textsubscript{42}-Aβ\textsubscript{42} and Aβ\textsubscript{40}-Aβ\textsubscript{40} adsorption resulted in initial fast adsorption of the Aβ peptides, followed by a slower kinetics phase of peptide binding, characteristic for high affinity binding (Fig. 1). A similar binding profile has been reported for the aggregation and fibrillization of isolated Aβ\textsubscript{42}, where a high incidence of specific binding is observed between 11-mercaptoundecanoic acid tethered Aβ\textsubscript{42} and monomeric Aβ\textsubscript{42} in bulk solution (33). The interaction between tethered Aβ\textsubscript{42} with Aβ\textsubscript{40} monomers showed a similar binding profile but slightly weaker binding. Binding between the same Aβ alloform resulted in greater mass adsorption to the sensor surface compared with mixed Aβ oligomeric interactions. These data show that the strongest binding occurs between the same alloform such as Aβ\textsubscript{42}-Aβ\textsubscript{42} or Aβ\textsubscript{40}-Aβ\textsubscript{40}. However, strong specific binding was also observed between Aβ\textsubscript{42}-Aβ\textsubscript{40}.

**Different Molar Aβ\textsubscript{42}:Aβ\textsubscript{40} Ratios Are Structurally Similar at Beginning of Aggregation Process—** Because Aβ\textsubscript{42} and Aβ\textsubscript{40} were found to interact directly, we used NMR to explore whether the interactions could influence the conformation of Aβ at different Aβ\textsubscript{42}:Aβ\textsubscript{40} ratios immediately following their preparation. The peptides were treated according to a new protocol designed to yield completely solubilized samples without solvent contamination (13). 15N-labeling of one Aβ alloform at a time allowed us to monitor the individual structural behavior of each alloform within the context of different molar ratios. The structural fingerprints of pure Aβ\textsubscript{42} and Aβ\textsubscript{40} peptides by the 15N-1H HSQC spectra are in excellent agreement with data shown in the literature (Fig. 2) (27, 34). The spectra of 15N-labeled Aβ\textsubscript{42} at different molar ratios do not present chemical shift variations, not even for the resonances of the C terminus, which should be the most sensitive to even a small change of environment (Fig. 2B). This indicates that the co-presence of the two alloforms has no influence on the structure at an atomic level, as expected for the monomeric state. The same is observed for Aβ\textsubscript{40} (Fig. 2A). We conclude that samples with different Aβ ratios are structurally equivalent to samples of pure individual peptides prior to aggregation.

**Fibers Formed by Different Aβ Ratios Have Similar Morphology and Cross-β Structure—** Negative stain TEM was used for a morphological characterization of the end states of the Aβ aggregation reactions. Upon long term incubation, all Aβ
X-ray fiber diffraction showed that the samples of pure Aβ and mixed ratios all exhibit the classic cross-β fiber diffraction patterns described in the literature (38, 39), showing a strong meridional reflection at 4.7 Å and a major equatorial reflection at ~10 Å consistent with a cross-β architecture (Fig. 3B). The patterns arising from Aβ_{42} and Aβ_{40} fibrils both share the same 9.7–9.8 Å major equatorial reflection, which we attribute to the β-sheet spacing perpendicular to the fiber axis. The fiber diffraction pattern obtained from Aβ_{42} fibrils was distinguishable from Aβ_{40} fibrils only by the sharper signals likely to arise from a higher degree of order in the Aβ_{42} fibers, whereas the mixtures of the two peptides give rise to patterns that are virtually indistinguishable from that of Aβ_{40}. This could be due to the large amount of Aβ_{40} relative to Aβ_{42} such that the signal from Aβ_{40} dominates the pattern. Although subtle differences in the fiber diffraction patterns likely arise from differing degrees of order and composition of the samples, the equatorial signal positions and relative intensities of signals are largely comparable for all samples (Fig. 3C).

To confirm structural similarity of fiber architecture of various Aβ ratios at a higher resolution, we measured the protection factors of the Aβ peptides by acquiring 15N-1H HSQC spectra for monomeric Aβ_{40} and Aβ_{42} after resolubilizing amyloid fibers that were subjected to HDX (supplemental Fig. S1). Amide protection factors were measured by comparing the amide peak intensities obtained for the sample in H_2O and the sample in D_2O after the exchange period. The backbone amide chemical shift data are consistent with those previously reported in acidified DMSO-d_6 (32), although we observed (partial) overlaps in the cross-peaks of residues 11/23, 12/18, 13/27, 19/40, and 32/41 for Aβ_{42} (supplemental data). Only residues 11/23 and 13/27 have overlapping cross-peaks in the Aβ_{40} spectrum. The HDX pattern of 15N-labeled Aβ_{40} for the 1:9(15 N) and 3:7(15 N) ratios and for pure Aβ_{40} fibers shows that the stretches comprising residues 18–22 and 30–34 are more protected from solvent exchange than the N terminus (supplemental Fig. S2). The C-terminal residues 37–40 also appear more accessible to solvent exchange. This agrees with earlier observations and proposed models for Aβ_{40} fibrils (18, 40–42). The HDX patterns of 15N-labeled Aβ_{42}, Aβ_{40}, and the ratios showed only small differences. Extensive exchange times (up to 672 h) (supplemental Fig. S2) of Aβ_{42} showed no noticeable effects in agreement with the suggestion that Aβ_{42} fibrils are highly resistant to solvent exchange (32, 43). The pattern for pure Aβ_{42} and the 3:7(15 N) and 1:9(15 N) ratios is less distinct but indicates a clear distinction between the N- and the C-terminal halves with higher protection of the C-terminal half.

We conclude that the architecture of Aβ fibers in pure or mixed form is overall indistinguishable both at a macromolecular and high resolution level, although small differences at atomic level may be present. As these mature fibrils have been shown to have weak toxicity, we will focus on differences in the oligomeric regime.

Aβ_{42} and Aβ_{40} Affect Aggregation Kinetics of the Other—To address whether aggregation kinetics are affected by different ratios, we monitored the intensity of NMR spectra as a function of time, exploiting the disappearance of the resonances due to the increased molecular weight, typical of events in a slow
exchange regime. We exploited again the possibility of 15N-isotope labeling only of one of the Aβ alalloforms, in combination with 15N-edited filter experiments to monitor the aggregation of both the 15N-labeled and unlabeled peptides simultaneously. This experimental setup offers the advantage that the individual Aβ alalloforms are selectively observed in parallel and within the same sample preparation, thereby circumventing any uncertainty that might arise from different sample preparations or peptide batches. In all cases, we observed the concomitant disappearance of all peaks according to a cooperative behavior along the whole peptide or at least the region of it visible in the NMR spectrum. No sufficiently populated lower molecular weight assemblies were observed, indicating that Aβ aggregates directly into NMR invisible assemblies under these experimental conditions. Due to the long apparent lag phase, we did not detect a sigmoidal transition for Aβ40 and the 1:9 ratio over a time scale of 180 h (Fig. 4, A, E, and F). In contrast, pure Aβ42 aggregates very rapidly with a sigmoidal signal disappearance (Fig. 4B). Interestingly, the Aβ42 component of the 1:9 ratio remains in solution significantly longer in the presence of Aβ40. The kinetics recorded for Aβ40 and Aβ42 within the 3:7 ratios with either 15N-labeled Aβ42 or 15N-labeled Aβ40 (named 3(15 N):7 and 3:7(15 N), respectively) indicate that Aβ40 remains longer in solution compared with Aβ42, which aggregates more rapidly (Fig. 4, C and D). However, the complete loss of signal for Aβ42 in the presence of Aβ40 is delayed in comparison with pure Aβ42, suggesting that the shorter Aβ40 alalloform reduces the aggregation propensity of the longer Aβ42 alalloform. To make sure that these observations could not be explained as the average of populations containing only the same alalloforms, we analyzed a 5:5 ratio where 15N-labeled Aβ42 or Aβ40 are present in equimolar amounts of the unlabeled alalloform (Fig. 4, G and H). We observed a nearly simultaneous disappearance of the signals of Aβ42 and Aβ40, which strongly suggests co-aggregation of both peptides into mixed fibers. We conclude that Aβ40 and Aβ42 mutually influence the aggregation kinetics of the other.

Aβ40 and Aβ42 Ratios Both Form Complex but Different Ensembles of Oligomers—To investigate whether the observed alalloform influence on the aggregation arises from an impact on the formation of intermediates along the aggregation pathway, we followed aggregation of the Aβ mixtures using high molecular weight MS, a technique that uses high voltages to enable detection of high molecular weight species. Because non-cova lent complexes disassemble at these voltages, we incubated our samples prior to analysis with glutaraldehyde as cross-linking agent. The resulting masses reveal various interesting features (Fig. 5 and supplemental Table S1). First, the masses of Aβ42 and of the two mixtures are consistently larger than those of Aβ40 in support of the hypothesis that there are appreciable populations of oligomers that contain both alalloforms. Second, early aggregation proceeds through a monomer addition process during which oligomers gradually grow by the addition of one monomer at a time. Third, in all cases we observed that assemblies accumulate during aggregation, the maximum size of which depends on the Aβ42:Aβ40 ratio. At an incubation time of 1 h, Aβ40 samples contain oligomers with a range of sizes from dimers up to 13-mers. As the process continues, larger oligomers are formed and after 6 h of incubation, 25-mer assemblies are detected together with larger oligomers at apparent molecular weights of 186 up to 852 kDa. For 1:9 ratios, we observe formation of much smaller oligomers with a maximum of 8-mers and accumulation of larger sized oligomers at apparent molecular masses of 171 up to 515 kDa. The 3:7 ratios aggregate in a similar manner but share features closer to the pattern observed for Aβ42. No very large molecular weight oligomers are observed after 6 h of incubation, presumably because they become so large that either they cannot become mobile or are not efficiently cross-linked. These results reveal clear differences in the pattern of small oligomeric species formed under different ratio conditions, indicating a potential basis for the difference in toxic effect (13).

Differences between Aβ42:Aβ40 Ratios Reside along Aggregation Pathway—The influence of the Aβ40 and Aβ42 ratios on aggregation kinetics is also evident from cross-seeding experiments where sonicated protofibrils were added to monomeric solutions of different Aβ42:Aβ40 ratios. Seed preparations were verified by TEM (Fig. 6A) and added to freshly prepared monomeric Aβ solutions. The aggregation kinetics were followed by in situ ThT fluorescence (Fig. 6, B–E). Aβ40 aggregation was efficiently seeded by Aβ40 seeds and the 1:9 ratio seeds leading to elimination of the lag phase (Fig. 6B). The initial parts of the two curves overlap, indicating that the properties of Aβ40 are predominant, also in the 1:9 mixture. Addition of 3:7 seeds also induces aggregation, but some lag phase is retained. Addition of pure Aβ42 not only does not seed aggregation, but even lengthens the lag phase. Seeding of Aβ40, therefore appears to proceed in a highly specific manner with preference for the same alalloform. The 1:9 ratio is seeded by any seed but, as for pure Aβ40, Aβ40, and 1:9 seeds have a similar strong seeding effect, whereas Aβ42 and 3:7 seeds have a milder effect, which, however, still preferentially selects the same alalloform (Fig. 6C). In contrast, pure Aβ42 and the 3:7 ratio were equally effectively seeded by any Aβ seed, regardless of whether they were formed from Aβ40 or Aβ42 or a mixture (Fig. 6, D and E). Therefore, it appears that Aβ42 monomers have a higher degree of plasticity so that they may use a less specific surface as a template, whereas Aβ40 alalloforms have higher selectivity.

The effect of cross-seeding on the disappearance of the NMR signals of the different Aβ alalloforms was also studied. In these experiments, we limited ourselves to the addition of seeds of pure Aβ40 or pure Aβ42 to the preincubated samples of which the NMR signals were monitored. In all cases, adding seeds resulted in appreciable aggregation irrespectively of the time point at which the addition was made. The control experiments were performed without any addition and this ensured that the effect is the direct consequence of the addition. Pure Aβ42 monomers could be easily seeded by both peptides (Figs. 7, C and D). Induction of aggregation of Aβ40 with Aβ40 seeds was also highly efficient (Fig. 7B), whereas Aβ42 seeds induced some initial signal disappearance but with a delayed aggregation (Fig. 7A). In the 1:9 and 3:7 ratios, Aβ40 seeds could efficiently induce aggregation of the two Aβ alalloforms in the mixtures, while the Aβ42 seeds efficiently seeded the Aβ42 alalloform, whereas the Aβ40 alalloform lags behind in the aggregation. This implies that the presence of Aβ42 monomers in the ratios influences the behavior of the Aβ40 alalloform. Overall, it is observed that the Aβ40 seeds can efficiently induce aggregation of
FIGURE 4. **Aβ_{40} and Aβ_{42} show different aggregation behavior in different Aβ_{42}:Aβ_{40} ratios.**

- **a**, pure Aβ_{40} at a concentration of 180 μM does not aggregate within the timeframe of data collection.
- **b**, pure Aβ_{42} at a concentration of 20 μM displays a lag phase and a sigmoidal transition from monomeric species into NMR invisible aggregates.
- **c**, 3:7(15 N) ratio, whereby the Aβ sample is composed of 70% 15N-labeled Aβ_{40} which is monitored via HSQC (140 μM Aβ_{40} monomer concentration) and 30% unlabeled Aβ_{42} (at a monomer concentration of 60 μM), which is simultaneously monitored via the amide region 15N-filtered one-dimensional NMR spectrum.
- **d**, the 1:9(15 N) ratio with 10% 15N-labeled Aβ_{42} and 90% unlabeled Aβ_{40}.
- **e**, the 1:9:1 ratio whereby the 15N-labeled Aβ_{40} and unlabeled Aβ_{42} are present in equimolar amounts (60 μM of each alloform).
- **f**, the 5:5:5(15 N) ratio whereby equimolar amounts (60 μM) of unlabeled Aβ_{42} and 15N-labeled Aβ_{40} are present. The blue symbols represent Aβ_{40} and the black symbols correspond to Aβ_{42}.

**Structural Aspects of Aggregating Aβ_{42}:Aβ_{40} Ratios**
Aβ samples, whereas the Aβ₄₀ alloform responds less efficiently to the Aβ₄₂ seeds (Fig. 8 and Table 1). These data show a genuine difference between the two peptides at the level of the oligomeric state. They reveal that even a relatively small increase in Aβ₄₂ in the mixture confers aggregation properties to Aβ₄₀ that are markedly more similar to pure Aβ₄₂.

**DISCUSSION**

Although previous structural studies have mostly focused on pure Aβ alloforms and the identification of a single oligomeric species, the present work aims to understand the determinants of the toxicity of different Aβ₄₂:Aβ₄₀ ratios. We demonstrate by
FIGURE 6. Cross-seeding reveals that Aβ42 oligomers show plasticity, whereas Aβ40 oligomers display a higher selectivity. a, TEM of seed preparations. Seeds were prepared by incubation of 50 μM Aβ ratios for 24 h followed by sonication at maximum power for 10 min. From left to right: pure Aβ40, ratio 1:9, ratio 3:7, pure Aβ42. Bar, 0.2 μm. Freshly prepared seeds were added to monomeric solutions of Aβ42:Aβ40 ratios at final concentrations of 0.5 μM and 25 μM, respectively. b, ThT of Aβ40 monomers seeded with pure Aβ40 seeds (blue dotted trace); with seeds from ratio 1:9 (green dotted trace), with seeds from ratio 3:7 (red dotted trace), and with seeds from pure Aβ42 (black dotted trace). The blue dashed line represents the unseeded Aβ40 control. c, ThT of ratio 1:9 monomers seeded with Aβ ratios as compared with the non-seeded aggregation curve. The colors are as described in b. d, ThT of Aβ42 monomers seeded with Aβ ratios as compared with the non-seeded aggregation curve (red dashed line). Colors are as described in b. e, ThT of Aβ42 monomers seeded with Aβ ratios in comparison with the non-seeded sample (black dashed line). Colors are as described in b.

independent evidence from MS, NMR, and SPR that the two peptides interact, although recognition between the same alloforms is preferred over interactions between different ones. We therefore expect that the populations of the two peptides in the aggregates will be mixed. This explains and expands previous data (23, 25, 27) that indicate that Aβ40 and Aβ42 influence their respective aggregation properties.

To understand which step along the aggregation pathway is responsible for this effect, we compared the structures and morphologies of all the species formed. We show that the initial monomeric and final fibrillar states do not differ to a large extent. NMR analysis of freshly prepared samples at different Aβ42:Aβ40 ratios conclusively reveals the presence of predominant monomeric species that lack a regular and well defined structure. Therefore, at this stage, the peptides are not affected by the presence of the other alloform. Likewise, we do not observe appreciable differences between the mature fibrillar states by TEM, HDX, and fiber diffraction: fibers formed during long incubation times are virtually identical. The absence of significant differences in the start and end points of Aβ fibrillation directed our focus to the formation of transient oligomeric intermediates. Previous data had indicated differences in the protofibrillar morphologies and Fourier transform infrared spectroscopy data following short term incubation, which, together with the present data, underline the importance of the aggregation pathway and of the dynamics of the oligomeric state (13, 44, 45).

We observed subtle but clear differences between the different Aβ ratios along the aggregation pathway. NMR experiments visualizing the spontaneous aggregation (Fig. 4) showed that the presence of monomeric Aβ40 slows down the aggregation kinetics of Aβ42, increasing the time frame that soluble forms are found in solution for any peptide ratio. Vice versa Aβ42 stimulates Aβ40 aggregation as revealed by comparing the 3:7 and 1:9 ratios with pure Aβ40. This is compatible with the view that Aβ42 drives aggregation and acts as a template by lowering the kinetic barriers that prevent Aβ40 from aggregating (15, 25, 46). Aβ40 potentially delays Aβ42 aggregation through “non-productive” interactions. Although these conclusions are in agreement with previous reports (25, 27, 46), our cross-seeding data suggest that Aβ40 monomers specifically require Aβ40 oligomers to induce growth of mature fibrils, whereas Aβ42 monomers are less selective and are stimulated by all types of seeds.

It might be argued that there is an apparent discrepancy between the progressive Aβ aggregation as monitored by NMR (Fig. 4) and the cross-seeding data (Figs. 6–8). By NMR, we observe that Aβ42 stimulates Aβ40 to aggregate while Aβ40
simultaneously delays Aβ42 aggregation. Vice versa, the cross-seeding data reveal that monomeric Aβ40 is not efficiently seeded by sonicated Aβ42 protofibrils. It is reasonable to explain this discrepancy by assuming that the oligomers formed during the aggregation process have features that are distinct from the sonicated protofibrillar species (seeds) that may have undergone advanced structural maturation. For pure Aβ42, these seeds would not be the optimal templates to directly incorporate Aβ40 monomers and perhaps even entail a conformational restructuring to lead to productive aggregation. We cannot rule out the possibility that monomeric Aβ40 could be able to resolubilize Aβ42 seeds, thereby bringing Aβ42 into solution and promoting in this way productive aggregation, as hinted by Yan and Wang (27). Because our observations with high molecular weight MS underline that the aggregation process proceeds through a monomer addition mechanism, the dynamic interplay (productive and non-productive) of monomeric Aβ with soluble Aβ assemblies seems appropriate to explain toxicity of the Aβ42:Aβ40 ratios. This relevance of monomer addition processes for neurotoxicity was recently described by Jan and colleagues for pure Aβ aggregation (44).

In conclusion, our work indicates that the Aβ42:Aβ40 ratio behavior cannot be simply interpreted by stating that Aβ42 can...
induce \( A\beta_{42} \) aggregation while at the same time, \( A\beta_{40} \) can prevent or delay \( A\beta_{42} \) aggregation. Rather than the morphology of the amyloid fibrils, the \( A\beta_{42}:A\beta_{40} \) ratio modulates the \( A\beta \) oligomer formation. Our data indicate that neurotoxicity is more likely to be explained by the dynamic nature of the ongoing \( A\beta \) aggregation rather than by the prevailing view that \( A\beta \) toxicity is associated with a distinct assembly. A change in the \( A\beta_{42}:A\beta_{40} \) ratio induces differences in conformational plasticity of the oligomeric peptide mixtures and the pattern of detectable oligomeric species. That the oligomer formation along the amyloid assembly pathway is affected by the different \( A\beta \) ratios emphasizes the necessity to further expand our understanding of the exact compositional, temporal, and structural properties of the homo- and hetero-oligomers. The implications of this finding for AD therapy are fundamental: the results imply that it is less important to focus on lowering the total amyloid burden in patients, although it appears crucial to affect the relative ratios of the peptides.

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