Specific Soluble Oligomers of Amyloid-β Peptide Undergo Replication, And Form Non-Fibrillar Aggregates in Interfacial Environments.

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Running Title: Replicating strain of off-pathway Aβ42 oligomers.

Background: Oligomers of amyloid-β peptides are implicated in the etiology of Alzheimer’s disease. Results: Specific ‘off-pathway’ oligomers of Aβ42 show unique replication properties upon interacting with monomers. Conclusions: The results indicate that oligomers that are formed along pathways outside the fibril formation pathway may undergo replication. Significance: Mechanistic details of Aβ soluble oligomers will enable better understanding of Alzheimer’s disease pathology.

SUMMARY
Aggregates of amyloid-β (Aβ) peptides have been implicated in the etiology of Alzheimer’s disease (AD). Among the different forms of Aβ aggregates, low-molecular weight (LMW) species ranging between ~2 and 50mers, also called ‘soluble oligomers,’ have emerged as the species responsible for early synaptic dysfunction and neuronal loss. Emerging evidence suggests that the neurotoxic oligomers need not be formed along the obligatory nucleation-dependant fibril formation pathway. In our earlier work, we reported the isolation of one such ‘off-pathway’ 12-18mer species of Aβ42 generated from fatty acids, called LFAOs (large fatty acid-derived oligomers) (1). Here, we report the physiochemical aspects of LFAO-monomer interactions as well as LFAO-LFAO associations in the presence of interfaces. We discovered that LFAOs are a replicating strain of oligomers that recruit Aβ42 monomers and quantitatively convert them into...
LFAO assemblies at the expense of fibrils, a mechanism similar to prion propagation. We also found that in the presence of hexane-buffer or chloroform-buffer interfaces, LFAOs are able to associate with themselves to form larger, yet non-fibrillar, aggregates. These results further support the hypothesis that LMW oligomers can be generated via non-fibril formation pathways. Furthermore, the unique replicating property of off-pathway oligomers can hold profound significance for AD pathology.

INTRODUCTION

Formation of assemblies by a protein called amyloid-β (Aβ) peptide in the cortical and hippocampal regions of the brain is mainly responsible for the cognitive decline and memory loss that occurs in Alzheimer’s disease (AD). The brains of AD patients contain a large number of insoluble proteinacious assemblies or aggregates that are deposited as senile plaques. These aggregates are mainly composed of 40- and 42-amino acid long peptides called amyloid-β (Aβ; Aβ40 and Aβ42, respectively)(2). An overwhelming body of evidence has emerged in recent years that disputes the classic ‘amyloid cascade theory’ and implicates the smaller, soluble aggregates to be the primary neurotoxins responsible for neuronal loss, as opposed to the fibrils (3-8). Studies have indicated that soluble oligomers and not the plaque load, in both cell culture and in transgenic animal models are not only toxic but also correlate better with the level of cognitive disability (8-10). Several such observations suggest low molecular weight (LMW) ‘soluble oligomers’ ranging between ~2-60mers are involved in the early synaptic dysfunction in AD (3,5,7,11). Needless to say, this has led to an unprecedented focus on identifying and characterizing smaller, low-molecular weight (LMW) aggregates of Aβ both in vitro and in vivo.

The process of Aβ aggregation is a nucleation dependent phenomenon in which the formation of a critical mass of self-assembled aggregates called the ‘nucleus,’ along with concomitant conformational change, is a prerequisite for the emergence of mature fibrils. Such a mechanism generates a classic sigmoidal growth curve containing a lag-phase prior to the growth phase. In such a process, it is clear that smaller oligomers, which are the intermediates of fibril formation, are formed transiently along the pathway. However, it is becoming evident from many recent reports that there are alternative pathways of aggregation (12-14), and that neurotoxic oligomers can also be populated via alternate pathways (15-17). Recently, a 12-16mer oligomeric species of Aβ42 called ‘globulomers’ was reported to be formed independently of the fibril formation pathway (18), and a similar observation was reported for a 9-15mer species generated in the presence of SDS (19). Moreover, annular protofibrils (APF) formed from prefibrillar oligomers (PFOs) failed to convert to fibrils even after an extended period of time, suggesting these pathogenic oligomers are formed outside the nucleation-dependant fibril formation pathway (20). Reports such as these have shown that there are multiple pathways of aggregation that compete with fibril formation, especially during the pre-nucleation state, giving rise to several kinds of oligomers. Therefore, it is important to understand the physiological consequence of such a process; one apparent property of ‘off-pathway’ oligomers is that they convert to fibrils, if at all, at a much slower rate than the fibril formation ‘on-pathway’ counterparts and hence possess longer half-lives. This in
turn can result in prolonged toxicity to the neuronal cells and therefore has profound significance in AD pathology. Unfortunately, there is paucity of structural, mechanistic and pathological understanding of LMW oligomers in general, and more so with the oligomers that are not formed along the fibril formation pathway. In our previous work, we reported that non-esterified fatty acid (NEFA) interfaces can induce multiple pathways of Aβ aggregation in carefully controlled conditions of NEFA concentrations and Aβ-to-NEFA molar ratios (1). More importantly we reported that two distinct ‘off-pathway’ oligomers of Aβ42 can be generated: 4-5mers (small fatty acid-derived oligomers; SFAOs) and 12-18mers (large fatty acid-derived oligomers; LFAOs). Here, we report the isolation and characterization of LFAOs along with two important physiochemical properties: a) LFAOs seem to be a replicating species that quantitatively convert Aβ42 monomers into LFAOs at the expense of fibrils, a mechanism similar to prion protein propagation, and b) in the presence of interfaces, LFAOs are able to form larger but non-fibrillar diffuse aggregates, further supporting our hypothesis on alternate aggregation pathways.

EXPERIMENTAL PROCEDURES

Materials. Aβ42 was synthesized by the Peptide Synthesis Facility at the Mayo Clinic (Rochester, MN) using routine Fmoc chemistry. MALDI-ToF mass spectrometry revealed > 90% purity of the peptide. Sodium dodecylsulphate (SDS) and thioflavin-T (ThT) were procured from Sigma (St. Louis, MO). Lauric acid (C12) fatty acid was purchased as a sodium salt from NuCheck Prep Inc (Elysian, MN). All other chemicals were obtained from VWR Inc.

Preparation of Aβ42 monomers. Lyophilized stocks of synthetic Aβ42 were stored at - 20 °C, desiccated. Briefly, 1.5-2 mg of peptide was dissolved in 0.5 ml of 30 mM NaOH and stored for 15 min at room temperature prior to size exclusion chromatography (SEC) onto a 1 × 30 cm Superdex-75 HR 10/30 column (GE Life Sciences) attached to an AKTA FPLC system (GE Healthcare, Buckinghamshire) to remove any preformed aggregates as previously reported (1). The column was pre-equilibrated in 20 mM Tris-HCl (pH 8.0) at 25 °C and run at a flow rate of 0.5 ml/min. One minute fractions were collected. Concentrations of Aβ were determined by UV-Vis spectrometry on a Cary 50 spectrophotometer (Varian Inc) using a molar extinction coefficient of 1450 cm⁻¹M⁻¹ at 276 nm (www.expasy.org) corresponding to the single tyrosine residue in Aβ42. Peptide integrity after SEC was again confirmed by MALDI-ToF mass spectrometry, which shows a monoisotopic molecular mass of 4516.31 Da in good agreement with a calculated mass of 4513.13 Da. Monomeric Aβ42 fractions were stored at 4 °C and used within 2 to 5 days of SEC purification in all experiments to avoid any preformed aggregates in our reactions.

Aβ aggregation reactions. Preparation and isolation of LFAOs. LFAOs were prepared and isolated as described previously (1). Briefly, the freshly purified Aβ42 (50 μM) was incubated with 50 mM NaCl and 5 mM C12 fatty acid at 37 °C for 48 h. After 48 h the sample was subjected to SEC and the fractions corresponding to the peak near the void volume (Vv) were collected. Concentrations of collected fractions were determined by UV absorbance. All isolated LFAO fractions
were stored at 4 °C and used within 2 days after SEC isolation in all experiments.

**Agitation experiments with hexane-buffer and chloroform-buffer interfaces.** To a freshly purified 2 μM LFAO or Aβ42 monomer (control) in 20 mM Tris, pH 8.0, 5% (v/v) hexane (ρ = 0.6548 g/mL) and chloroform (ρ = 1.483 g/mL) were added independently, and mixed vigorously using a vortex mixer for one minute agitation followed by five minutes of rest. After eight cycles of agitation (approximately 1 h), the samples were then dialyzed against 20 mM Tris-HCl (pH 8.0) at 25 °C using a 2 kDa MWCO Slide-A-Lyzer G2 dialysis cassette (Thermo Scientific) for 23 h. Afterwards, the dialyzed samples were subjected to immunoblotting, dynamic light scattering (DLS) and atomic force microscopy analyses (AFM).

**Suspension experiment with chloroform-buffer interface.** The suspension method reported previously was followed in our experiments (21). The freshly purified 2 μM LFAO or Aβ42 monomer (control) (0.3 mL) was suspended on top of 100% chloroform solution (0.3 mL) in a 1.5 mL siliconized Eppendorf tube without mixing. The samples were kept at 25 °C for 24 h without any disturbance. After 24 h, the samples were removed from just above the interface without disturbing the interface. The samples from both reactions were subjected to immunoblotting, DLS and AFM analysis.

**LFAO replication experiment.** Monomeric Aβ42 (20 μM) was incubated alone or with 2% (0.4 μM) LFAO seed in 20 mM Tris pH 8.0 at 25 °C for 72 h. Aliquots of samples were removed at 0, 24, 48 & 72 h and subjected to SEC on a Superdex-75 HR 10/30 column after spinning at 19000g for 20 min to remove fibrils. Fraction 17 from each SEC fractionation at 0, 24, 48 & 72 h was subjected to immunoblotting and circular dichroism (CD).

**LFAO replication experiment with Dansyl-Aβ42 monomer.** The above experiment was repeated with Dansyl-Aβ42 (Dan-Aβ42) monomer, which was purified similar to the wild-type Aβ42 as mentioned above. A freshly purified 50 μM Dan-Aβ42 monomer sample was incubated with 1 μM LFAO under the same conditions as mentioned above. Aliquots of samples were removed after 72 h and subjected to SEC onto a Superdex-75 HR 10/30 column after spinning at 19000g for 20 min to remove fibrils. Fractions 17 & 18 from SEC fractionation were subjected to immunoblotting and emission fluorescence spectroscopy using a Cary Eclipse spectrometer (Varian Inc) in scan mode. The dansyl emission was monitored at 450 nm after exciting at 350 nm using 10 nm slits.

**Fluorescence spectroscopy.** ThT fluorescence (F) was monitored in a microcuvette with a Cary Eclipse spectrometer (Varian Inc) after 15-fold dilution of Aβ42 samples into 5 mM Tris-HCl (pH 8.0) containing 10 μM ThT. Continuous measurements of F were taken for 1 min with the excitation and emission wavelengths fixed at 450 and 482 nm respectively, and the excitation and emission slits set at 10 nm.

**Dynamic light scattering (DLS).** The DLS was performed on a Zetasizer nano S DLS instrument (Malvern Inc., Worcestershire, UK). Each sample measurement consisted of...
6 runs of 10 sec each with a pre-equilibration time of 40 sec. After the measurement, the number (%) was exported and plotted against size using Origin 7.0 software.

**Polyacrylamide gel electrophoreses (PAGE) and immunoblotting.** Samples were dissolved in loading buffer (1x Laemmli buffer) containing 1% SDS, applied without heating to 4-12% NuPage gels (Invitrogen) containing bis-Tris, and resolved in 2-(N-morpholino)ethanesulphonic acid (MES) running buffer with 0.1% SDS. Dye-linked MW markers (Blue Plus2 Prestained Standards, Invitrogen) were run in parallel for calibration. Gels were electroblotted onto 0.45 μm immobilon nitrocellulose membranes (BioTrace™ NT, Life Sciences Inc). Blots were boiled in a microwave oven in PBS for 2 min and were blocked overnight with 1X PBS containing 5% nonfat dry milk and 0.1% tween-20 before probing (1-2 h) with 1:1000-1:2500 dilutions of Ab9 monoclonal antibody, which detects amino acid residues of Aβ (1-16). Blots were then incubated with anti-mouse horseradish peroxide (HRP) conjugate and developed with ECL reagent (Thermo Scientific).

**Atomic force microscopy (AFM).** Mica was cleaved using a razor blade and taped to a magnetic sample holder. The mica stub was then covered with a 3-aminopropyltriethoxy silane (APTES) solution (500 μL APTES in 50 mL 1mM acetic acid) for 20 minutes. The APTES solution was then decanted and the mica was rinsed with 150 μL of deionized water, four times. After rinsing, the mica stub was dried with compressed N₂ gas and stored in a desiccator for an hour. Next, 150 μL of 0.25-1 μM Aβ sample was added to the mica and allowed to adsorb for 30 minutes. The sample was then decanted and the mica stub was rinsed with 150 μL of deionized water, four times. Finally, the mica stub was dried with compressed N₂ gas and stored in a desiccator until imaging. The surface topography of each sample was explored by imaging the peptide after it had been adsorbed onto APTES-treated freshly cleaved mica. These images were obtained via a Dimension 3000 atomic force microscope (Digital Instruments) in tapping mode using RTESP etched silicon probes (length: 125 mm, nominal force constant: 40 N/m, and resonance frequency: 350 kHz) (Veeco Instruments). While under ambient environmental conditions, the scan rate was held constant at 1 Hz. All standard image-processing techniques were performed on Nanoscope version 5.30 r2 image analysis software. Nanoscope as well as Gwyddion version 2.7 softwares were used to calculate feature heights by two methods; 1) Section analysis, to extract height profiles and 2) particle analysis, to determine the statistical distribution of pixel heights for individual aggregates. Representative height profiles can be found in Figure 3 as well as the supplemental information. Multiple areas were imaged for each sample and while height, phase and amplitude data were collected simultaneously, amplitude images are used most often in the text to discuss morphology variation between samples.

**Analytical ultracentrifugation (AUC).**

*Sample preparation.* FITC-labeled Aβ42 (FITC-Aβ42) was purchased in a lyophilized form (Bachem Inc) and stored at –20 °C prior to use. A stock containing 5 mM FITC-Aβ42 in DMSO was prepared as described as above. The stock was then diluted to 100 μM in 20 mM Tris pH 8.0 and used for generating FITC-labeled LFAO (FITC-LFAO). Briefly, for the generation and isolation of FITC-LFAO, 40 μM wild-
type A\textbeta42 and 10 \textmu M FITC-A\textbeta42 were incubated with 5 mM C12 fatty acid under the same conditions as described above for LFAO preparation, and LFAOs were isolated using a similar protocol. For the replication experiments, monomeric A\textbeta42 (20 \textmu M) was incubated with 2\% (0.4 \textmu M) FITC-LFAO seed in 20 mM Tris pH 8.0 at 25 \degree C for 72 h. After 72 h the sample was subjected to sedimentation velocity analysis. The 0.4 \mu M FITC-LFAO seeds alone were used as a control. Similarly, FITC-fibrils were prepared by mixing 45 \mu M WT- A\textbeta42 with 5 \mu M FITC-A\textbeta42 and incubating with 150 mM NaCl in 20 mM Tris pH 8.0 at 37 \degree C for 2-3 days. The ThT fluorescence was monitored daily until it reached a plateau. The sample was then centrifuged at 19000g for 20 min. The pellet was resuspended in 20 mM Tris pH 8.0 and used for AUC analysis.

Sedimentation velocity. Samples were mixed by brief vortexing and then spun in a tabletop centrifuge for approximately five seconds to ensure that no sample was lost on the walls of the tube. Samples were then loaded into 1.2 cm path length sedimentation velocity cells (Sedvel60) and placed in an XLA Analytical Ultracentrifuge equipped with a fluorescence detection system (Aviv FDS). The temperature on the centrifuge was equilibrated until it remained constant at 20 \degree C for at least five minutes. The centrifuge was then accelerated to 5,000 rpm, where the focus depth and gain for the fluorescence detection system were adjusted to maximize the signal collected. The centrifuge was then accelerated to 60,000 rpm and data collection began immediately after final velocity was reached. Each scan was averaged over five consecutive scans to increase the signal to noise ratio. The run was stopped when the fluorescence intensity vs. radial distance profiles remained constant between scans, indicating that the boundary had pelleted. The samples were then re-run for approximately 30 minutes at 60K using absorbance optics to collect pseudo absorbance data. All data was transferred to a separate computer for analysis; the pseudo-absorbance data was used to calculate the meniscus position for each sample using the meniscus wizard in the software program DCDT2+ (version 2.3.2) (22). The software program Sedfit (Sedfit89) (23) was used to generate c(s) distributions for the FDS data with 0.1S resolution. The c(s) distribution for each sample was integrated and divided by the area, and all data are presented as normalized c(s) distributions. The software program, Sedfit was also used to generate c(M) distributions after generating the c(s) distribution by assuming a constant diffusion coefficient for all samples and f/f0 = 1.2. The c(M) distributions were normalized using the same method as described for the c(s) distributions.

Circular dichroism (CD). CD spectra were obtained in the far UV region with a Jasco J-815 spectropolarimeter (Jasco Inc, Easton, MD). Samples were placed in a 0.1 cm path-length quartz cuvette (Hellma) and were monitored in continuous scan mode (260-190 nm). The acquisition parameters were 50 nm/min with 8 s response time, 1 nm bandwidth and 0.1 nm data pitch, and data sets averaged over two scans. Spectra of appropriate blanks were subtracted from each data set as indicated. The corrected, average spectra were smoothed using a ‘mean-movement’ algorithm with a convolution width of 25 using the Jasco spectra analysis program.

RESULTS
LFAOs are not discrete but a disperse mixture of oligomeric species. In our
previous work, we showed that Aβ42 upon incubation with varying concentration of non-esterified fatty acids (NEFAs) generates two distinct types of oligomers; LFAOs (12-18mers) and SFAOs (4-5mers) at near and above critical micelle concentrations of NEFA, respectively (1). In the same report, we also indicated that LFAOs can be isolated using Superdex-75 size exclusion chromatography (SEC). In the current report, first we quantified the amount of NEFA (lauric acid), if any, associated with the isolated LFAOs, and estimated the molecular size distribution of LFAOs by SEC and AUC. An Aβ42 sample, incubated with buffered 5 mM lauric acid at 37°C for 48 h, was subjected to fractionation on a Superdex-75 SEC column (Figure 1A; smooth line). The sample eluted in two major peaks: one between fractions 17 and 20 and the other between fractions 22 and 25. In addition, a small peak near the void volume of the column (fraction 16) was also observed. Immunoblots of the inclusive volume fractions 22-25 suggested they were Aβ42 monomers consistent with our previous observation (1) (data not shown). LFAOs eluted in the partially included volume (fractions 17-18) as previously observed (Figure 1A). The presence of NEFA was quantified using free fatty acid assay kit (Biovision Inc, CA) as previously shown (24). Such an examination indicated only a negligible amount (< 0.1%) of NEFA present in these fractions containing isolated LFAOs (Supplementary Figure, S1).

In order to ascertain the molecular weight of LFAOs based on retention volumes, the SEC profile was compared with those of globular protein standards (Gel filtration standard, catalog # 151-1901; BioRad Inc) (data not shown). Based on this analysis, the molecular weight of LFAOs was estimated to be between 60 and 200 kDa. In addition, protofibrils (PFs) of Aβ42, which are high molecular weight species (> 200000 Da; > 500mers), were also generated following a previously established protocol (25,26) and subjected to fractionation under similar conditions. The PFs sample exhibited two elution peaks, the PFs at the void volume (Vo; black arrow) at fraction 15 and monomers at fractions 22-25 (Figure 1A; dashed line). The PF fractionation also supported our conclusion with globular protein standards that the molecular weight of LFAOs is between 60 and 200 kDa. Subjecting the LFAO fractions (fractions 17 and 18) to another cycle of fractionation through SEC yielded 7.3 % monomers, which is comparable to the 10.6 % obtained in the first SEC fractionation. This suggests that LFAOs undergo partial dissociation upon dilution (Supplementary Figure S2). The AFM image of the isolated LFAOs showed largely a bimodal distribution of non-fibrillar punctuate dots ranging between 7-10 and 16-19 nm in height (Figure 1A; inset).

The molecular size distribution of LFAOs was also estimated by sedimentation velocity experiments in an analytical ultracentrifuge (AUC). As described in Experimental Procedures, FITC-labeled Aβ42 was introduced into the oligomeric assembly as a reporter to allow monitoring via a fluorescence detection system (AUC-FDS). More importantly, labeling also reduces the amount of sample needed while increasing the sensitivity at low concentrations. The c(s) sedimentation coefficient distribution profiles of the obtained from these experiments are shown in Figure 1B. The sizes of the LFAOs were analyzed both before and after fractionation to observe size difference, if any. The Aβ42 sample in the presence of 5 mM lauric acid
prior to SEC showed a distribution of peaks with a sharp peak centered around 4 S and a more disperse peak between 6 and 12 S along with a peak at ~1 S (Figure 1B; dotted lines). The 1 S peak corresponds to monomeric Aβ, as determined by the control sample (Supplementary Figure S3A). An immunoblot of the Aβ42 sample incubated in 5 mM lauric acid prior to SEC indicated a disperse band between 56 and 110 kDa (Figure 1B; inset, lane B). The AUC analyses of the SEC fractionated LFAOs (fraction 17) showed an overall distribution similar to the one prior to SEC fractionation containing predominantly two species; a main peak centered at 5 S and a second, disperse peak between 7 and 12 S, along with some monomers (1 S) (Figure 1B; smooth line). As a comparison, we analyzed Aβ42 fibrils using AUC that showed a heterogeneous mixture of species between 40 and 200 S (Supplementary Figure S3B). It is noteworthy that a considerable decrease in the relative amount of monomers was also observed after fractionation based on the signal; 52.3% and 14.8% monomers before and after fractionation, respectively. The LFAOs showed a similar distribution of oligomers even after 100 times dilution in AUC (data not shown). It is also noteworthy that recently a similar sedimentation distribution profile was obtained for amyloid-derived diffusible ligands (ADDLs) of Aβ42 (27). In contrast to the LFAOs, the Aβ42 fibril control showed widely disperse sedimentation coefficient values between 50 S and 200 S (Supplementary Figure S3B). The immunoblots of both the SEC fractionated and unfractionated LFAOs samples showed largely a disperse band between 56 and 110 kDa with two distinct band distributions centered around 56-70 and 80-110 kDa (Figure 1B; inset). The band distributions in the immunoblots complement the bimodal distributions observed both in AFM as well as AUC data. Together, the data suggest that the LFAOs are not discrete oligomeric species but are a disperse distribution of oligomers ranging between 56-110 kDa (12-24mers).

**LFAOs are replicating strains.** Typically, ‘seeding’ is a process in which a small amount of preformed aggregate when added to monomeric Aβ enhances the latter’s aggregation rate towards fibril formation. Since the aggregate morphology is dependent on the physiochemical property of the seed itself, we wanted to explore whether LFAOs can ‘replicate’, or in other words, seed their own formation, by the addition of monomeric Aβ, a mechanism analogous to prion protein propagation. This property has been recently demonstrated by Kayed and coworkers, who reported that specific soluble oligomers called prefibrillar oligomers (PFOs) were able to seed their own replication upon interacting with monomeric Aβ (28). The property of replication could be a manifestation of the unique structure of the oligomeric assembly, and more importantly, may complement our previously reported hypothesis that such oligomers are formed along a pathway different from fibril formation (1,19). In order to explore this, a 20 μM sample of freshly purified, ‘seed-free’ Aβ42 was incubated with 0.4 μM isolated LFAOs as seeds (2% molar ratio) at room temperature. Aliquots from this sample were subjected to immunoblotting after 24, 48 & 72 h of incubation to monitor the reaction progress (Figure 2A). The amount of LFAOs loaded in each lane of the SDS-PAGE gel was kept constant at 28 ng based on the initial seed concentration, in order to make quantitative estimates of the blots. The immunoblot in Figure 2A, within
24 h of incubation with monomers, showed an LFAO band that was clearly more intense compared to the 0 h incubation sample (lane 3). Importantly, these bands are centered at 100 kDa, which is the higher band among the two observed for the isolated LFAOs (lane C), while the lower molecular weight (~56-70 kDa) band is absent (Figure 2A; 24 h). These bands became increasingly more intense after 48 h and 72 h, while the intensity of the monomer bands diminished, suggesting the conversion of monomers to LFAOs. Also, after 72 h, a high molecular weight species that failed to enter the gel was observed (lane T; 72 h) that was not present in the supernatant after the sample was centrifuged at 19,000xg for 20 min. This suggests that with time, fibrils started to emerge, which is possibly due to monomers progressing towards on-pathway fibrils independently. This observation is not surprising considering on-pathway fibril formation reaction is expected to proceed in parallel to monomer-LFAO interactions.

Concomitantly, the replication process was monitored by ThT fluorescence (Figure 2C). Aβ42 seeded with 2% LFAO (○ in Figure 2C) showed only a negligible amount of seeding with a lag-time of ~72 h versus ~84 h for the control (■ in Figure 2C). It is important to point out that upon seeding with 20% seeds, a significant reduction in lag-times was observed in our previous report (1). The immunoblots recorded during the lag phase (24, 48 and 72 h) showed the presence of a substantial amount of LFAOs in the seeded samples that was clearly absent in the control, suggesting that LFAOs had undergone replication during this time period. More importantly, the immunoblots also seem to indicate that the LFAO seeds are able to induce the formation of the 80-110 kDa oligomers at the expense of 56-70 kDa species observed within the unseeded LFAOs.

We then reasoned that since replication leads to an increase in the amount of LFAOs formed, a quantitative analysis would provide further proof of this process. To do so, a higher concentration of Aβ42 sample, 50 μM, was seeded with 1 μM LFAOs (2% molar seeds) to facilitate fractionation by SEC, while maintaining a constant ratio of Aβ42: LFAO seed. Immunoblots of aliquots at 24, 48 & 72 h time resulted in a banding pattern similar to that seen in Figure 2A (data not shown). The same aliquots were also subjected to fractionation by SEC. Prior to loading onto the Superdex-75 column, the samples were centrifuged to remove any fibrils present in the sample, and the supernatant was subjected to fractionation. As observed before in Figure 1a, the samples eluted near the void volume (Figure 2D; fractions 16-18), and immunoblotting confirmed that these species were predominantly LFAOs (lane 24 h; Figure 2B). The amount of LFAOs eluted in these fractions increased progressively with time (as inferred by the increase in absorbance), suggesting a quantitative increase in the amount of LFAOs generated. Overall, the sample showed a four-fold increase in the amount of LFAOs as compared to a non-seeded control after 72 h (Figure 2D). Importantly, immunoblots for fraction 17 from SEC also indicated a progressive increase in the amount of 80-100 kDa LFAOs (Figure 2B). The far-UV CD of the fractionated sample (fraction 17) also showed a progressive increase in β-sheet conformation, further supporting the increase in LFAO content upon seeding (Figure 2E). Finally, in order to verify the formation of 80-110 kDa species at the expense of 56-70 kDa species, the LFAO sample was monitored both before and after replication by sedimentation velocity analysis after 72 h of incubation (Figure 2F). Prior to seeding, a major species was
observed that was centered ~60 kDa along with some monomers as observed previously (Figure 2F; dotted lines). After replication, the data clearly showed a shift towards higher molecular weights (80-110 kDa), complementing the shift in banding pattern towards higher molecular weight observed in immunoblots (Figure 2F; smooth line). A small decrease in the relative amount of monomer present after seeding was also observed in AUC data (Figure 2F), which further supports the immunoblot data that indicated that quantitative amounts of monomers are converted to LFAOs upon initiating the seeding reaction.

AFM images of the supernatants of the seeded samples shown in Figure 2A are presented in Figure 3. Immediately after incubation (Figure 3A & D), samples exhibit only a few punctuate spherical particles of ~8 nm in height that are attributed to LFAOs. After 48 h of incubation (Figure 3B & E), samples exhibit a significant increase in the number of spherical LFAO particles with a slight increase in the height (~12 nm) that seem to align along a longitudinal axis (inset). Samples imaged after 72 h of incubation (Figure 3C and F) show a further increase in the number of particles with similar height (~14 nm), with simultaneous presentation of individual punctuate spherical particles aligned linearly (inset), and a small number of smooth fibrillar structures. Importantly, many of these linearly-aligned globular particles present an overall appearance of ‘nascent’ PFs. It is noteworthy that a similar ‘ordering’ of spherical particles was observed previously when Aβ40 was treated in the presence of aqueous – organic interface (21). These images support the AUC and immunoblotting data and indicate that the seeded LFAOs replicate.

Finally, in order to ensure that monomers are incorporated into the replicated oligomers, N-terminal Dansyl-labeled Aβ42 peptide (Dan-Aβ42) was used as a fluorescent probe. We expected that seeding of Dan-Aβ42 with LFAOs would lead to oligomers containing the labeled peptide, which can be detected by intrinsic fluorescence as shown in the schematic (Figure 4A). A seeding experiment was performed as described above, using 50 μM Dan-Aβ42 with 1 μM LFAOs at room temperature, and subjecting the seeded sample to SEC fractionation and immunoblotting after 72 h (Figure 4B). The immunoblot clearly showed an increase in LFAO formation after 72 h compared to 0 h (Figure 4C; lanes 4, 5 & 6). The immunoblot of the fractionated sample, fractions 17 & 18 also showed the presence of replicated LFAOs (Figure 4C; lanes 8 & 9). Clearly, the unreacted monomers fractionated in the inclusion volume at fraction # 24 (Figure 4C; lane 10). The fluorescence spectra of fractions 17 & 18 obtained by exciting the sample at 350 nm while monitoring dansyl emission at 450 nm are shown in Figure 4D. Both fractions clearly exhibited dansyl emission at 450 nm, indicating that Dan-Aβ42 monomers have been incorporated into the LFAO assembly during the seeding reaction, providing unambiguous evidence for such a mechanism. Together, the collective data suggest that LFAOs are a replicating strain that can induce the formation of similar oligomeric species upon interacting with Aβ42 monomers.

LFAOs form non-fibrillar, diffuse aggregates in aqueous-organic phase interfaces.

Buffer-hexane & buffer-chloroform agitation experiments. Aqueous-organic phase interfaces have been known to
influence Aβ aggregation tremendously (20). Specifically, prefibrillar oligomers formed distinct ring-/pore-like structures called, ‘annular protofibrils’ (APF) promoted by water-hexane interfaces and failed to form on-pathway fibrils upon long term incubation (20). We wanted to explore whether LFAOs were able to form similar non-fibrillar aggregates upon exposure to aqueous-organic phase interfaces. For these experiments, two interfacial systems were employed; hexane-water (as previously reported) and chloroform-water. First, the freshly isolated LFAOs (2 μM) were mixed and agitated with 5% (v/v) hexane in water following a protocol described by Kayed and co-workers (20) (see Experimental Procedures). The agitated sample was dialyzed for 24 h at room temperature, after which the samples were analyzed by dynamic light scattering (DLS). LFAOs in buffer alone showed a monodisperse peak with a diameter of ~10 nm (Figure 5A; black peak), in agreement with the 7-10 nm range of heights observed in AFM images (Figure 1A; inset). In contrast, LFAOs treated in hexane-water showed the formation of a mixture of large species with hydrodynamic diameter ~ 700 nm (Figure 5A; grey peak). The corresponding immunoblot of the sample did not show a high molecular weight species, however, a sharp decrease in the intensity of the LFAO band was observed (Figure 5B; lane 6). It is interesting to note that the control Aβ42 monomers (in the absence of LFAOs) showed a high molecular weight band that failed to enter the gel upon similar hexane treatment (Figure 5B; lane 3).

In order to see whether the formation of large amorphous aggregates in hexane can be replicated in other organic solvents, the effect of chloroform was investigated. Chloroform was used rather than hexane because it is denser than water and buffer can be readily suspended on top of the organic layer. The LFAO sample (2 μM) was incubated with 5% chloroform, agitated, and dialyzed in a similar fashion as the hexane sample. A polydisperse distribution of species containing two major peaks (~700 and 1000 nm), similar to that observed in the hexane-treated sample, was obtained by DLS (Figure 5A; light grey). The sample treated with chloroform showed a disperse band between 200 and 260 kDa in the immunoblot (Figure 5B; lane 5). The immunoblot of the control monomer treated similarly also showed a high molecular weight band that failed to enter the gel (Figure 5B; lane 2).

AFM images of the LFAO and control Aβ42 monomers subjected to aqueous/organic solvent mixtures are shown in Figure 6. Large spheroidal aggregates with heights of ~33 nm are observed in LFAO samples treated with hexane (Figure 6A and Supplementary Figure S4A). These aggregates are at least four times larger in apparent diameter than the individual LFAO molecules, and appear to result from LFAO association (Figure 6A; inset). Fibrillar structures are observed in Aβ42 control monomer samples treated with hexane (Figure 6B). It is noteworthy to mention that LFAOs failed to form the ring-like structures seen with APFs (20), suggesting that different molecular organization processes occur for different soluble oligomers. LFAO samples treated with chloroform show large clusters of aggregates with ~21 nm in height (Figure 6C and Supplementary Figure S4C). However, no fibrillar material was observed in Aβ42 monomers treated with chloroform (Figure 6D) despite the fact that a high molecular weight band that failed to enter the gel was observed for the same sample in
the immunoblot (Figure 5B; lane 2). It is likely that dilution of the sample during the preparation procedure for AFM analysis dissolved the unstable aggregates formed in chloroform interfaces. This observation is similar to the one reported by Nichols and co-workers in which Aβ40 generated unstable aggregates in a chloroform-buffer interface, which were completely disaggregated upon dilution (29). The collective data shows that when LFAOs are exposed to aqueous-organic interfaces, large, non-fibrillar, disperse aggregates are formed. This suggests that LFAOs assemble into aggregates that may not lie along the fibril formation pathway.

**Chloroform-buffer suspension experiment.** The agitation experiments mentioned above cause rapid exchange of phases, leading to increased surface area of interfaces, which in turn affects association of LFAOs. Since agitation is in some respects a harsh method of aggregation, we wanted to see if similar changes occurred in a milder form of interfacial treatment by simply suspending the aqueous layer containing Aβ on top of a more dense chloroform layer. One such aqueous-organic phase suspension experiment has been reported in which the buffer containing Aβ40 was gently suspended on top of a chloroform layer and aggregation was monitored (21). These conditions led to a rapid aggregation of Aβ40 peptide to form both globular as well as short, protofibril-like structures. A similar procedure was followed for LFAOs, and within two hours of incubation, a monodisperse peak centered ~80 nm in diameter emerged in DLS, which increased to ~150 nm peak in the next 24 h (Figure 7A). A similar trend was observed in the immunoblot of the sample, in which a band at ~150 kDa was seen within 2 h of incubation, and after 24 h a much larger molecular weight band was observed that failed to enter the gel (Figure 7B; lanes 4 and 5).

Numerous punctuate dots of globular species with widely varying heights (7-30 nm) were seen in the AFM image of the same sample after 24 h of incubation (Figure 7C and Supplementary Figure S5A). The species resembled the LFAO aggregates observed in the agitation experiments, but they were much larger in size. In sharp contrast, control Aβ42 monomers neither showed any significant increase in size, as witnessed by immunoblots (Figure 7B; lane 2), nor showed morphology similar to that of the LFAOs (Figure 7D). In fact the height of the particle seen in AFM image was smaller (~6 nm) than that of pure LFAOs (Supplementary Figure S5B). Interestingly, the globular particles observed with monomer control were neither seen on a western blot nor did DLS indicated the presence of such a species. Again, one possible explanation for this observation is that the unstable aggregates formed in chloroform-buffer interfaces disaggregate upon dilution, generating monomeric Aβ42 as previously observed (29). Nevertheless, the data clearly show the absence of fibrillar material with LFAOs, which complements our previous observations, as well as our hypothesis that LFAOs are formed along an alternative pathway of aggregation similar to APFs and fibrillar oligomers observed by Kayed and co-workers.

**DISCUSSION**

In this report, we have shown that LFAOs are not discrete but diffuse oligomeric species with a broad molecular weight range of 56-110 kDa. Within this molecular weight range, two populations of LFAO species exist, at 56-70 kDa and 80-110 kDa, which approximately translate to 12-15mers and 18-24mers. It is
fundamentally important to understand if there are any residual NEFAs associated with LFAOs as they may affect the latter’s physiochemical properties. Our analysis indicated that NEFAs are essentially absent in the isolated LFAO samples (Supplementary Figure S1). It is also important to note that any residual amount of NEFA, if present, may not influence Aβ aggregation properties as their concentration would be well below its critical micelle concentration (CMC), as reported previously (1). AUC sedimentation velocity data revealed that LFAOs are mixtures of oligomers, with sedimentation coefficients of 5S and 7-12S and some monomers. This observation is similar to that reported for ADDLs, which were also found to be a heterogeneous mixture containing species larger than 90 kDa that displayed a disperse c(S) distribution between 10S and 25S in sedimentation velocity experiments (27). A sequential back-to-back SEC fractionation of LFAO samples showed no significant reduction in the concentration of monomers present in the sample, which suggests that LFAOs undergo dissociation upon dilution (Supplementary Figure S2). This further supports other findings that LFAOs are disperse, and not discrete, aggregates.

A significant feature of LFAOs is their replicating property, which has potentially far-reaching implications for AD pathology. First, the observation of LFAOs acting as ‘seeds’ to produce more of the non-fibrillar aggregates implies the conversion of non-pathogenic species to potentially more toxic strains, similar to a mechanism observed with prion diseases (Figure 8A) (30-34). The direct implication of this property in AD is the possibility that the LFAOs can act as a ‘molecular sink’ to recruit more Aβ monomers away from fibrils. Although qualitative analysis has revealed this important property of LFAOs, a rigorous quantitative increase in the amount of LFAOs converted could also be of profound practical application. Briefly, by following a cyclic amplification method similar to the one introduced by Soto and co-workers for amplifying prion aggregates (35,36), with a low concentration seed from a physiological source, one will be able to ‘scale-up’ the amount of the seed using exogenous monomers to the levels amenable for in vitro biophysical analyses while preserving the structural integrity of the source. We are currently exploring this aspect and will report it later.

Comparison of LFAOs with other oligomers indicates similarities and differences. There are only a handful of reports on the replication property of Aβ oligomers among which those reported by Glabe and co-workers are the most recent ones. They identified three specific soluble oligomer sub-types classified mainly on the basis of their immunoreactivity towards conformation-specific antibodies (20,28,37). These are, PFOs, fibrillar oligomers (FO), and APFs, and they are recognized by the conformation specific antibodies, A11, OC, and αPF, respectively (28). Among these, both PFOs and FOs showed self-replication property by converting monomers into oligomers of the same type (28,38). The main distinction between PFOs and FOs is that while the former is recognized by the polyclonal antibody A11, the latter is detected by the fibril specific antibody, OC (39). Our attempts to detect LFAOs with the oligomer specific antibody A11 did not yield conclusive results. Although we could not get a positive signal with A11 for LFAOs, it would be inconclusive to say LFAOs may be structurally different from the epitope recognized by A11, as we could not generate appropriate positive controls for A11-
specific oligomers, even after several attempts. Nevertheless, a biochemical
difference between the two oligomers is that 
while FOs resemble fibril structure and seed fibril formation, PFOs do not. The LFAOs
reported here seem to resemble FOs rather than PFOs based on morphological heights, 
which range between 5 and 15 nm for FOs (38) as compared to 8-13 nm reported here
for LFAOs. However, further comparative analyses of LFAOs with other self-
replicating oligomers such as FOs could not be made, largely due to the paucity of 
stringent biophysical investigation of the replication process. The APFs on the other
hand that are generated from hexane-water interfaces, are characterized by a unique 
pore-like structure that leads to membrane permeabilization and disruption of ion
homeostasis causing cell death (40). As shown in Figure 6, similar treatment of 
LFAOs with hexane-water and chloroform-water interfaces failed to generate ring-like 
structures similar to APFs. Perhaps this difference is due to the fact that LFAOs are
more similar to FOs than PFOs, and APFs are generated from PFOs.

**Physiochemical properties of LFAOs showcase reactions along the off-fibril formation pathway.** This report also
reiterates the fact previously reported by us and many others that oligomers need not be
formed along the obligatory fibril formation

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FIGURE LEGENDS.

Figure 1. Isolation and characterization of LFAOs. A) The SEC fractionation of LFAOs using Superdex-75 column in 20 mM Tris pH 8.0 at room temperature with a flow rate of 0.5 mL/min and a fraction size of 1 mL. As a comparison, a sample of protofibrils (PF) of Aβ42 was also fractionated. Vo indicates the void volume based on globular protein standards. (inset): AFM image of fractionated LFAOs reproduced from (Kumar et al, 2011). Scale bar represents 2.5 μm. B) Normalized c(S) distribution profile generated from a sedimentation velocity experiment performed at 50,000 rpm. (inset): Immunoblot of LFAOs before (B) and after fractionation (fractions 17 and 18). Single arrow indicates 50-70 kDa band while double arrow indicates 80-110 kDa band. The results shown are a single data set among the three independent acquisitions.

Figure 2. LFAOs replicate upon interacting with monomers. A) Immunoblot of 20 μM Aβ42 incubated with 0.4 μM LFAO (2 % seed) for 72 h at room temperature before and after SEC. Lane C represents LFAO control (338 ng). Lanes 1 and 2 are Aβ42 control after 72 h in the absence of LFAO seed and LFAO alone (28 ng) respectively. Lane 3 is aliquot of the 20 μM Aβ42 seeded with 0.4 μM LFAO immediately after incubation (0 h). Lanes T and S represent the total and supernatant of the sample after centrifugation at 19000xg for 20 min, at the indicated times of incubation. The volume of sample loaded was kept constant to ensure 28 ng of the parent LFAO was maintained. B) Immunoblot of fraction 17, fractionated by SEC after 24, 48 & 72 h of incubation of the samples in A (lanes S). C) ThT fluorescence data of the seeded reaction. The arrow indicates the 72 h point to which seeding reaction was monitored. D) Fraction 17 of the seeded samples that were fractioned on a Superdex-75 column after 24, 48 & 72 h along with a control sample (no seed) after 72 h. E) The corresponding far-UV CD spectra of the fraction 17 and, F) Normalized relative molecular weight distributions, c(M) profiles obtained from sedimentation velocity data for control LFAOs (dotted line) and seeded oligomers (smooth line). (Inset) Expanded region of the c(M) plot indicated in the figure. The results shown are representative among at least three reproducible results.

Figure 3. Morphological changes during LFAO replication. Aliquots of the samples from Figure 2 A (before SEC) were probed at different time points to see the replication of oligomers after incubating 20 μM Aβ42 with 0.4 μM LFAO (2 % seed) for 72 h at room temperature. A, B & C, represent the amplitude AFM images of seeded sample after 0, 48 & 72 h, respectively (z-scale 0-0.8 V). D, E & F represent the corresponding height data from A, B & C, respectively, with a z-scale of 0-40 nm. The white scale bar represents 2.5 μm and the inset shows a field of dimensions 1 x 1 μm surrounding the particles indicated with arrows. The white boxes in D-F indicate the particles for which height analyses were conducted. Height profiles were extracted from the flattened height data and can be seen from the z-direction in the height images and from the x,y-direction below each image. These height profiles demonstrate how approximate feature heights were determined for each sample. The determined feature heights were confirmed via particle analysis (Nanoscope version 5.30 r2 image analysis software) shown at the bottom as a secondary technique to increase confidence in reported values.
Figure 4. Incorporation of Dan-Aβ42 monomer into replicated oligomers after seeding. A) Schematic for incorporation of Dan-Aβ42 into replicated LFAOs after seeding for 72 h. B) SEC fractionation of 50 μM Dan-Aβ42 seeded with 1 μM LFAO (2 % seed) for 72 h at room temperature. C) Immunoblot showing the comparison of seeded sample before and after SEC: Lane 1-50 μM Dan-Aβ42 monomer (1.8 μg); lane 2-LFAO seed (36 ng); lane 3- Dan-Aβ42 control in the absence of seeds after 72 h; lane 4 – aliquot of the seeding reaction immediately after incubation at 0 h; lanes 5 & 6 – the total and supernatant of the reaction respectively; lanes 7-9 – SEC fractions 16,17 and 18 shows replicated oligomers (arrows), and lane 10 – monomer fractionated in the included volume (fraction # 24). LFAO seed amount of 36 ng was kept constant in the immunoblot.D) Fractionated sample, fractions 16, 17 & 18 shows the Dan-Aβ42 emission at 450 nm upon exciting at 350 nm with a band width of 20 nm (10/10). The results are one among three reproducible data sets.

Figure 5. LFAO agitation in interfacial environments. A) The increase in size of LFAO after agitation with 5% (v/v) hexane or chloroform in buffer followed by dialysis for 24 h was monitored by DLS. B) Immunoblot of the agitation experiments along with monomer controls. Lane 1 shows control monomer Aβ42; lanes 2 and 3 dialyzed 24 h Aβ42 sample treated with chloroform & hexane, respectively. Lane 4 shows control LFAO; lanes 5 and 6, dialyzed 24 h Aβ42 sample treated with chloroform & hexane, respectively.

Figure 6. Morphology of aggregates formed after treatment with chloroform and hexane. A & B) AFM images of LFAO and monomer Aβ42 control, respectively, agitated with hexane. A) LFAOs show the presence of small globular aggregates and large clumps of aggregates. (Inset) shows a 1 μm expansion of the aggregate indicated in white arrow. B) Control monomers treated with hexane show the presence of fibrils. (Inset) shows a 1 μm expansion of the aggregate indicated in white arrow. C & D) AFM images of LFAO and monomer Aβ42 control, respectively, agitated with chloroform. C) LFAOs show the presence of several large clump of aggregates. (Inset) shows a 1 μm expansion of the aggregates indicated in white arrows. D) Control monomers treated with chloroform failed to show the presence of any aggregates. The white scale bar represents 1 μm and the square represent 5 x 5 μm field.

Figure 7. Chloroform Suspension experiment. A) LFAO was suspended over 100% chloroform solution and sample was removed from above the interface after 2 and 24 h to monitor the increase in size by DLS. DLS data indicate that the size of LFAO (black peak) was shifted from 11.03 to ~80 nm in 2 h (light gray peak) and to ~160 nm in 24 h (dark gray peak), indicating the formation of large aggregates. B) Immunoblot showing Aβ42 monomer and LFAO sample removed from above the interface after 24 h represented by M and LFAO. Lanes 1 & 2 show control Aβ42 monomer and monomer sample removed from above the interface (in the aqueous buffer side), respectively, after 24 h. Lanes 3 shows control LFAO; 4 & 5, LFAO sample removed from above the interface after 2 & 24 h, respectively. C) AFM image of chloroform interface sample after 24 hr, showing the presence of large clumps of aggregates.
(white arrows). D) AFM image of control monomer treated with chloroform interface shows no appreciable aggregate structure. The white scale bar represents 2.5 μm.

Figure 8. Schematic diagram depicting the generation of ‘off-pathway’ oligomers and replication. A) The classic ‘on-pathway’ fibril formation process is depicted along with an alternative ‘off-pathway’. The process of replication undergone by off-pathway oligomers is shown in dotted lines. B) The aggregation process is shown as a thermodynamic funnel for intrinsically disordered proteins such as Aβ, towards fibril formation. This path is considered to be the nucleation-dependant ‘on-pathway’ species. The spherical balls represent LMW oligomers. However, the oligomers may adopt a different pathway such that they are trapped in local energy minima as ‘off-pathway’ species (oval ball; grey area of the funnel). Such off-pathway species would have a significant energy barrier to overcome in order to proceed towards fibrils. Also, the off-pathway oligomers can recruit more monomers and dictate them to adopt such alternate pathways (replication process shown in A). This may further increase the energy barrier for replicated off-pathway oligomers to overcome while significantly increasing their half-lives.
FIGURE 3
FIGURE 4

A

Dan-Aβ42

LFAOs

Replicated LFAOs

B

Absorbance (a.u.)

Fraction number

C

kDa

D

F (a.u.)

Fraction number

fraction # 17

fraction # 18

fraction # 16

Wavelength (nm)
FIGURE 5
FIGURE 7
Specific soluble oligomers of amyloid-β peptide undergo replication, and form non-fibrillar aggregates in interfacial environments.

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