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Conformational Dynamics of Specific A β Oligomers Govern Their Ability to Replicate and Induce Neuronal Apoptosis

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Abstract

Oligomers of amyloid- β (A β) have emerged as the primary toxic agents responsible for early synaptic dysfunction and neuronal death in Alzheimer disease (AD). Characterization of oligomers is an important step in the progress towards delineating the complex molecular mechanisms involved in AD pathogenesis. In our previous reports, we established that a distinct 12–24mer neurotoxic oligomer of A β 42, called Large Fatty Acid derived Oligomers (LFAOs), exhibits a unique property of replication in which LFAOs directly duplicate to quantitatively larger amounts upon interacting with monomers. This self-propagative process of replication is somewhat reminiscent of prion propagation. In this report, we sought to investigate the concentration-dependent conformational dynamics LFAOs undergo and how such transitions manifest in their ability to replicate and induce neuronal apoptosis. The results indicate that LFAOs undergo a concentration-dependent transition between 12mers and disperse 12–24mers with a dissociation constant (K_d) of 0.1 μ M. The two species differ in their respective tertiary structures but not their secondary structures. This conformational dynamics of LFAOs correlates with their ability to replicate and to induce apoptosis in SH-SY5Y human neuroblastoma cells, with 12mers being more neurotoxic and prone to replication than 12–24mers. The latter result implicates the self-propagating replication process to dominate at low physiological concentrations. The observations made in this report may have profound significance in deciphering the elusive roles of A β oligomer phenotypes and in determining their prion-type behavior in AD pathology.

TOC image

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SUPPORTING INFORMATION AVAILABLE

Supplementary Figure S1 is available online.

AUTHOR CONTRIBUTIONS

VR conceptualized the research while VR and MAM planned and executed it. DND and KMP contributed equally in terms of experimental efforts. DND was also involved in parts of conceptualization and manuscript writing. MAM and KMP conceptualized and planned cell culture experiments and were involved in parts of manuscript writing.

Keywords

Amyloid; Oligomer; LFAO; Prion; Replication; and Propagation

Alzheimer's disease (AD) is a fatal neurodegenerative disorder that results in acute loss of memory and cognitive functions among elderly patients. One of the pathological hallmark of an AD brain is the proteinaceous extracellular plaque deposits comprised mainly of amyloid- β (A β) aggregates.¹ Aggregation of A β , which precedes amyloid plaque deposition, is responsible for acute loss of neurons in AD brains. Neuronal loss initially occurs within the hippocampal region of the brain and becomes progressive and widespread with the onset of the disease, affecting the entire cortex at advanced stages of pathology.² Among the various aggregates formed during A β aggregation, low molecular-weight soluble oligomers broadly ranging between 2 and 50mers have emerged as the primary neurotoxic agents responsible for synaptic dysfunction and neuronal loss.^{3–5} Although the mechanisms of oligomer toxicity and synaptic dysfunction have received substantial attention over the last decade, precise molecular mechanisms underlying the progression and dissemination of oligomer toxicity remain unclear. In this context, recent advances from animal studies suggest a prion-like propagation mechanism for the observed dissemination of toxicity and neuronal loss.^{6–11} Intracerebral injection of endogenous and synthetic A β seeds was observed to induce widespread deposition of A β in transgenic animals. Importantly, such depositions also occurred at sites distant from the point of injection, implicating propagation as a plausible mechanism for dissemination of toxicity. In prion diseases, a pathogenic strain (conformationally-unique seed) is faithfully propagated by a template-assisted mechanism without structural and functional distortions.¹² Seeding of A β fibril formation is also a template-assisted process by which monomers associate with a fibril seed, leading to the formation of large aggregates with varying high molecular masses. In light of these mechanisms, the role of low molecular-weight soluble oligomers in prion-type propagation and dissemination of toxicity remains unclear.

Generation and isolation of homogenous A β oligomers *in vitro* has been challenging, which has impeded investigations into their biophysical and pathological behavior. We recently reported the generation of a distinct 12–24mer A β 42 oligomer in the presence of non-esterified medium-chain saturated fatty acids, called Large Fatty Acid-derived Oligomers (LFAOs), that could be isolated free of fatty acid association.¹³ These oligomers were later identified to be kinetically trapped, off-pathway species with unique biochemical properties.¹⁴ One of the intriguing properties of LFAOs is that within a narrow window of incubation time, in the presence of A β monomers, they undergo self-propagative replication to form quantitatively more LFAOs.^{14,15} Replication differs from both prototypical prion-like propagation and A β fibril formation in that replication occurs by LFAOs interacting with monomers to generate quantitatively more of similar oligomers as opposed to larger

aggregates. Replication provides a unique perspective on propagation that may help explain the progressive dissemination of toxic A β aggregates in cerebral parenchyma. So far, such monomer-oligomer and oligomer-oligomer reactions leading to non-fibrillar products have seldom been investigated in detail. Probing the details of such a process could provide unique insights into the mechanisms of oligomer amplification and spread of toxicity, in addition to aiding the understanding of fundamental A β self-assembly mechanisms.

In the context of exploring prion-like mechanisms among A β oligomers, our laboratory has demonstrated that LFAO replication follows an inverse correlation with the molar percentage of LFAO seeds (% seeds).¹⁵ In the same report, the propensity of LFAOs to form oligomers of two disperse molecular weight ranges upon replication was established. These results lead to the question of whether concentration-dependent conformational and aggregation dynamics of LFAOs could be responsible for the observed inverse correlation between concentration and replication efficiency. The data also raises the question of whether the concentration-dependent changes are also responsible for correlations with neuronal apoptosis in cell culture.

The present work determines the molecular reasons for the inverse proportionality between replication efficiency and LFAO concentration using biophysical methods. In addition, the ability of LFAOs to induce apoptosis in a concentration-dependent manner in human neuroblastoma cells was investigated. The data suggest that LFAOs display concentration-dependent dynamics between the formation of 12mer and 12–24mer oligomer assemblies, which involves conformational changes restricted to tertiary structure. This dynamic manifests in replication efficiency and cellular activity. This report underscores a potential mechanism by which conformer-specific propagation of A β aggregates could be achieved as observed for prion proteins¹² and opens the door for further investigations into the structures of replicating A β oligomers in AD brains.

MATERIALS AND METHODS

Materials

Wild-type A β 42 (henceforth termed as A β) stocks synthesized from the Peptide Synthesis Facility at the Mayo Clinic (Rochester, MN) and from AnaSpec Inc. (Fremont, CA) were used in this study. Monoclonal Ab5 antibody was generously supplied by the University of Florida (Gainesville, FL). The Superdex-75 HR 10/30 size exclusion chromatography (SEC) column was purchased from GE Life Sciences (Marlborough, MA). Gel electrophoresis and immunoblotting materials were purchased from either Bio-Rad Laboratories Inc. (Hercules, CA) or Thermo Scientific (Waltham, MA). Sodium salt of Lauric acid (C12:0) was procured from NuCheck Prep, Inc. (Elysian, MN). Super Signal West Pico Chemiluminescent Substrate was procured from Thermo Scientific (Waltham, MA). Trizma base, trizma hydrochloride, DCVJ, BSA, SDS, and Triton X-100 were purchased from Sigma-Aldrich (St. Louis, MO). TNF- α as well as all other chemicals were procured from VWR, Inc. (Radnor, PA). All cell culture media and reagents were obtained from Sigma-Aldrich.

Preparation of A β Monomers

Lyophilized stocks of synthetic A β 42 were stored at -20°C . A β monomer purification was performed as described previously.¹⁴ Briefly, 0.8–1.5 mg of peptide was dissolved in 0.5 mL of 35 mM NaOH and allowed to stand at 25°C for 30 min prior to fractionation on a Superdex-75 HR 10/30 SEC column attached to an AKTA FPLC system (GE Healthcare, Buckinghamshire). The SEC column was pre-equilibrated in 20 mM Tris-HCl (pH 8.0) at 25°C , and monomer was purified at a flow rate of 0.5 mL/min. One-minute fractions were collected, and concentrations of A β fractions were determined by UV-Vis spectrometry on a Cary 50 spectrophotometer (Varian Inc.) using a molar extinction coefficient of $1450\text{ cm}^{-1}\text{ M}^{-1}$ at 276 nm (expasy.org), corresponding to the single tyrosine residue in A β 42. Periodically, the peptide purity after SEC fractionation was confirmed by MALDI-ToF mass spectrometry, which revealed a monoisotopic molecular mass of 4515 Da. A β monomers were stored at 4°C and used within 48 h of purification.

Preparation of LFAOs and Fibrils

LFAOs were purified as described previously.¹⁴ Briefly, freshly purified A β monomer (50 μM) was incubated with 50 mM NaCl and 5 mM C12:0 fatty acid at 37°C in quiescent conditions. After 48 h of incubation, the sample was subjected to centrifugation at $18,000g$ for 20 min prior to SEC purification. Fractions corresponding to the peak near the void volume were collected. LFAOs were stored at 4°C and used within 72 h of purification. A β fibrils were generated by incubating A β monomer (100–150 μM) in 150 mM NaCl at 37°C in quiescent conditions for 4–5 days. After incubation, the sample was subjected to centrifugation at $18,000g$ for 20 min, and the supernatant was then carefully removed and discarded. A β fibrils were then resuspended in fresh 20 mM Tris-HCl (pH 8.0) with 0.01% NaN₃. A β fibrils were kept at 4°C and used within three weeks. ThT fluorescence and centrifugation was used to determine percentage conversion to A β fibrils, which was typically 80 – 90%.

LFAO Replication Reactions

Freshly purified LFAOs (0.5, 1, 3, 10, and 14 μM) were co-incubated with freshly purified A β monomer (50 μM) at 25°C in quiescent conditions. After 72 h, aliquots of each sample were taken for immunoblotting analysis, followed by centrifugation at $18,000g$ for 20 min and quantification using SEC. Quantitative fold increase of LFAOs upon replication was calculated by normalizing the SEC chromatogram for the seeded reactions against the corresponding chromatograms for the seeds alone.

SDS and native PAGE with Immunoblotting

For native PAGE, samples were diluted into 1X Laemmli loading buffer, then loaded without heating onto BioRad 4–20% precast gels resolved in 1X Laemmli running buffer. For SDS-PAGE, samples were diluted into 1X Laemmli loading buffer containing 1% SDS, then loaded without heating onto NuPAGE 4–12% Bis-Tris gels resolved in 1X MES running buffer containing 0.1% SDS (Life Technologies). Pre-stained MW markers (Novex Sharp Protein Standard, Life Technologies) were run in parallel for MW determination. For immunoblotting, proteins were transferred to 0.2 μm nitrocellulose membrane (BioRad) and

boiled for 1 min in a microwave oven in 1X PBS, followed by blocking for 1.5 h in 1X PBS containing 5% nonfat dry milk. Blots were then probed overnight at 4°C with a 1:6000 dilution of Ab5 monoclonal antibody, which detects amino acids 1–16 of Aβ. Blots were then incubated with a 1:6000 dilution of anti-mouse, horseradish peroxidase conjugated secondary antibody and developed with ECL reagent (Thermo Scientific).

ANS and DCVJ Fluorescence Spectroscopy

8-anilinonaphthalene-1-sulfonic acid (ANS) and 9-(2-dicyanovinyl) julolidine (DCVJ) fluorescence were measured using a Cary Eclipse spectrometer (Varian Inc.) in scan mode. For ANS, Aβ samples (6 μM) were equilibrated for 1 min in ANS (100 μM) before measuring the fluorescence using excitation and emission wavelengths of 388 nm and 400–650 nm, respectively. For DCVJ, Aβ samples (5 μM) were equilibrated for 1 min in DCVJ (10 μM) before measuring the fluorescence using excitation and emission wavelengths of 433 nm and 400–650 nm, respectively. For ANS titration experiments, LFAO samples were diluted to the desired concentration by adding 100 μM ANS directly to the cuvette, followed by a 1 min equilibration time before measuring fluorescence using the parameters described above. The area under each curve (405–650 nm) was normalized for each respective Aβ monomer concentrations. The data obtained for ANS binding was fit to the following equation for a monomer-dimer model using Origin 8.5 software.

$$F - \frac{2}{M} * \left\{ F_0 - (F_0 - F_f) * \left(\frac{(4M + K_d) \pm \sqrt{(4M + K_d)^2 - 16M^2}}{8} \right) \right\} \quad \text{Eq 1}$$

Here, F is the fraction of dimer, M is the total LFAO concentration, F_0 is the initial fluorescence value, F_f is the final fluorescence value, and K_d is the apparent dissociation constant.

Fourier Transform Infrared (FTIR) Spectroscopy

Immediately before collecting spectra, Aβ LFAOs and fibrils were lyophilized using vacuum evaporation and resuspended at a concentration of 1 mM with D₂O. FTIR was then immediately performed in grazing angle attenuated total reflectance mode (GATR-FTIR) using a Nicolet 8700 (Thermo Scientific) instrument equipped with a VariGATR™ accessory (grazing angle 65, germanium crystal; Harrick Scientific). Spectra were collected by accumulating 4096 scans between 1750–1550 cm⁻¹ with a resolution of 2 cm⁻¹. All spectra were collected while purging the VariGATR™ attachment and FTIR instrument with N₂ gas along the infrared beam path to minimize the peaks corresponding to atmospheric moisture and CO₂. Spectra were processed by blank subtraction (using D₂O as blank) and baseline correction using OriginLab 8.0 software.

Circular Dichroism (CD)

CD spectra were obtained in the far-UV region with a Jasco J-815 spectropolarimeter using either a 0.1 or 1.0 cm path length quartz cuvette (Hellma) and monitored in continuous scan mode (260–190 nm). Acquisition parameters were 50 nm/min with 8 sec response time, 1 nm bandwidth, and 0.1 nm data pitch. Each spectra represents an average of 16 scans per

sample. Averaged spectra were smoothed using the savitzky-golay algorithm with a convolution width of 15 using the Jasco spectrum analysis program.

Cell Culture

Human neuroblastoma SH-SY5Y cells (American Type Culture Collection, Manassas, VA) were maintained in a 1:1 mixture of Ham's F12K medium and DMEM (F12K/DMEM) containing 10% FBS. Medium was supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin. All cultures were maintained at 37 °C in a humid atmosphere of 5% CO₂ and 95% air.

Cellular Apoptosis

To assess the ability of LFAOs to induce apoptosis, a DeadEnd fluorometric TdT-mediated dUTP Nick-End Labeling (TUNEL) system (Promega, Madison, WI) was used. Prior to experimentation, SH-SY5Y cells were seeded at a density of 1×10^6 cells/well onto 22 × 22 mm glass coverslips and maintained for 24 h in F12K/DMEM media containing 10% FBS. For cell treatment, 10 µM LFAOs were diluted in F12K/DMEM containing 1% FBS to final concentrations of 0.01, 0.1, and 1 µM and added to cells for 24 h. Cells treated with buffer equivalent or 25 µM H₂O₂ served as negative and positive controls, respectively. Following 24 h incubation (37 °C, 5% CO₂), treatment was removed, and cells were fixed with 4% paraformaldehyde in PBS (10 min, 25 °C). Cells were then permeabilized with 0.1% Triton X-100, 0.01 M glycine in PBS (10 min, 25 °C) and washed twice with PBS (10 min, 25 °C). Cells were then placed in 100 µL equilibration buffer (10 min, 25 °C) before incubation in 50 µL of rTdT incubation buffer (1 h, 37 °C), which labels the DNA strand breaks with fluorescein-12-dUTP. After incubation, labeling was halted by adding 17 mM salinesodium citrate (pH 7.0) containing 150 mM NaCl (15 min, 25 °C), and cells were washed 3 times with PBS (5 min, 25 °C). Labeled cells were mounted onto glass slides using fluoroshield containing DAPI for nuclear staining. Cells were imaged under a Nikon Eclipse 80i fluorescent microscope using a 40x objective. For each slide, three or four different fields were captured for analysis. For each field, both a DAPI image and TUNEL image were acquired. Custom MATLAB (MathWorks, Natick, MA) functions were developed to determine the fraction of apoptotic cells present for each sample. From DAPI images, the total number of cells was assessed using MATLAB to identify pixel intensity regions. The boundary of these regions was then increased in a stepwise manner until the pixel intensity of the boundary fell below the threshold, indicating that the boundary of the nuclei had been reached. Once all nuclei boundaries were determined, the total number of nuclei present was recorded by MATLAB. Using the nuclei boundaries determined from the DAPI images, the average TUNEL pixel intensity within each nucleus was determined from the corresponding TUNEL image. Cells exhibiting an average TUNEL pixel intensity above 20 were deemed apoptotic. The fraction of apoptotic cells was defined as the number of apoptotic cells divided by the total number of cells. For each treatment 300 – 600 cells were analyzed.

Caspase Activation

An Image-iT LIVE Green Poly Caspases detection kit (Life Technologies, Carlsbad, CA) was used to determine if caspase activation was the driving force behind LFAO-induced cellular apoptosis. SH-SY5Y cells were prepared and treated for 24 h with 0.01, 0.1, and 1

μM LFAOs, as described above. Cells treated with buffer equivalent or $2 \text{ U}/\mu\text{L}$ $\text{TNF-}\alpha$ served as negative and positive controls, respectively. Following 24 h incubation (37°C , 5% CO_2), treatment was removed, and cells were stained using a fluorescent inhibitor of caspases (FLICA) reagent for detection of a wide range of active caspase enzymes (caspase-1, -3, -4, -5, -6, -7, -8 and -9) and using Hoechst 33342 for labeling of nuclei. Cells were rinsed with phenol red free F12K/DMEM containing 1% FBS (PRF F12K/DMEM, 1% FBS) and incubated (37°C , 5% CO_2) for 1 h with 1X FLICA stain diluted in PRF F12K/DMEM, 1% FBS. After incubation, the stain was removed and cells were washed twice with PRF F12K/DMEM, 1% FBS before 10 min incubation (37°C , 5% CO_2) with Hoechst 33342 stain (diluted 1:1000 in PRF F12K/DMEM, 1% FBS). The cells were then washed twice and mounted to slides using a fixative solution. Slides were imaged 2 h after staining to avoid signal loss using a Nikon Eclipse 80i fluorescent microscope with a 40x objective. For each slide, three to five different fields were captured for analysis. For each field, both a Hoechst image and a FLICA image were acquired. Custom MATLAB functions were developed to determine the fraction of caspase active cells. From Hoechst images, the total number of cells was assessed as described above for DAPI images. From corresponding FLICA images and using the nuclei boundaries determined from the Hoechst images as a reference, MATLAB was used to determine the average FLICA pixel intensity within a region that included both the nucleus and a specified region outside the nucleus, which accounts for caspase staining that can reside within both the nucleus and cytoplasm. Cells exhibiting an average FLICA pixel intensity above 5 were deemed caspase active. For each treatment, 200 – 600 cells were analyzed.

RESULTS

Dependence of LFAO Seed Concentration on Replication

In an effort to determine the dependence of LFAO seed concentration on replication, the replication efficiency was assessed as a function of varying LFAO seed concentrations. To initiate replication, freshly purified $\text{A}\beta$ monomers ($50 \mu\text{M}$) were seeded with 0.5, 1, 3, 10, or $14 \mu\text{M}$ LFAOs and the samples were incubated at 25°C under quiescent conditions. After 72 h of incubation, the efficiency of replication was evaluated by quantifying the increase in LFAOs using a Superdex-75 size exclusion column (Figure 1A–E). The quantitative fold increase was then obtained by normalizing the SEC chromatogram for the seeded reactions (smooth line) against the corresponding chromatograms of the seeds alone (dashed line), yielding an exponentially inverse correlation with seed concentration (Figure 1F). This observation is in good agreement with our previous report on LFAO replication.¹⁵ As LFAO seed concentration increased from 0.5 to $3 \mu\text{M}$, the fold increase (replication efficiency) decreased from 6.1 to 2.6 (Table 1). However, increasing the LFAO seed concentration to 10 and $14 \mu\text{M}$ resulted in insignificant replication efficiency of 1.7 and 1.5, respectively (Figure 1F, Table 1). Immunoblotting of the replicated samples and their corresponding seeds (Figure 1F, *inset*) revealed that the replicated LFAOs (lanes 2, 4, 6, 8, and 10) showed diffuse bands containing roughly 56–60 (single arrow) and 80–110 (double arrow) kDa molecular weight (MW) distributions, which corresponds to $\text{A}\beta$ 12–24mer species. However, the immunoblots of corresponding LFAO seeds prior to incubation with monomers (lanes 1, 3, 5, 7, and 9) showed a concentration-dependent banding pattern. At low concentrations

(0.5 μ M), predominantly a 12mer band was observed. However, as the concentration of LFAOs increased, a disperse 12–24mer band was evident in the immunoblots (Figure 1F, *inset*). The bimodal distribution of LFAOs at high concentrations corresponding to 56–110 kDa has been previously confirmed by analytical ultracentrifugation.¹⁴ These observations indicate that the unusual inverse correlation between seed concentration and replication efficiency could be attributed to the dynamics involved between the two oligomer distributions of LFAOs namely, 12mer and 12–24mer.

Quantitative analysis of LFAO replication

In light of inverse correlation between the efficiency of LFAO replication and seed concentration, a quantitative analysis was performed based on a simple two pathway model at equilibrium conditions. Since it has been previously established that LFAO replication occurs via an alternative pathway (off-pathway) that competes with the on-pathway fibril formation, the following model was considered.

In this model, all aggregates formed along the on-pathway were considered as one species (F) for simplicity purposes. One could argue that the depletion of monomers during incubation with LFAO seeds (replication) is a function of on- and off-pathway reactions. Hence, accounting for quantitative amounts of LFAOs, monomers (M), and fibrils (F) at equilibrium conditions would provide insights into the dynamics of replication. After time t of incubation, if M_o is the initial monomer concentration, M_c is the concentration of the monomer consumed, and M_f is the concentration of free monomers, then;

$$M_o = M_c + M_f - M_d \quad \text{Eq 2}$$

where M_d is the concentration of monomers obtained due to partial dissociation of LFAOs. In evaluating the amount of monomer consumed (M_c), the following condition was considered;

$$M_c = \sum (LFAO, F) = M' + M'' \quad \text{Eq 3}$$

where M' and M'' are monomers consumed along on- and off-pathways, respectively. In addition, monomers obtained via dissociation of LFAOs occurring during zonal SEC fractionation is linearly proportional to the initial LFAO concentration. Hence,

$$M_d = f([LFAO]) = (s)[LFAO] + b \quad \text{Eq 4}$$

where s is the slope and b is the y intercept. Combining the above Eq 2–4 and rearranging yields,

$$(M_f)_t = (M_o - M') + (M_o - M'') + (s)[LFAO] + b \quad \text{Eq 5}$$

In this equation, M_o is a known quantity while M' (fractions 18–20, smooth line; Figure 2A), M_f (fractions 23–27, dashed line; Figure 2B) are experimentally measurable quantities in equilibrium conditions by SEC. Similarly, the dissociation of LFAOs during SEC as a function of its concentration can be determined experimentally to yield the values of s and b in Eq 5 (Figure S11). Dissociation of aggregates formed along the on-pathway may also occur during fractionation, which is difficult to measure experimentally. Nevertheless, our model will be able to predict the total amount of monomers committed towards on-pathway (M'), which can be correlated with the experimentally derived M' value for unseeded reaction (control; Figure 2C). Such validation in turn, will provide a quantitative basis for the LFAO replication occurring along the off-pathway.

Upon analyses, the net increase in the concentrations of replicated LFAOs ($LFAO$) showed an exponential increase with increasing LFAO seed concentration (●; Figure 2D). Similarly, the net change in free monomer concentration (M_f) displayed an exponential decrease within the same LFAO seed concentration range (□; Figure 2D). The extent of change in M_f concentrations for 0.5–14 μ M LFAO seeded reaction was calculated to fall within the range of 42–47 μ M, which is higher than the experimentally derived values of 37–41 μ M (Table 1). The difference between the calculated and experimental values (~6 μ M) provides the amount of monomers that are committed towards on-pathway (M' , Table 1). This M' value derived from our model is in good agreement with the experimental value of 6 μ M (Figure 2C; Table 1), which suggests that roughly 10% of initial monomers are committed to the on-pathway aggregation within 72 hours of incubation. It is interesting to observe that the net increase in replicated LFAO concentrations and the net decrease in free monomer concentrations display near identical exponential phases (Figure 2D). This observation, in conjunction with the inference that a constant amount of monomer is directed towards on-pathway irrespective of LFAO seed concentration, suggests that more monomer is expended to off-pathway replication as LFAO seed concentration is increased. However, it is intriguing to observe that although the amounts of replicated LFAOs showed an expected increasing trend with increasing LFAO seed concentration (Figure 2D), the efficiency of replication (fold-increase) showed an inverse correlation with LFAO seed concentration as described previously (Figure 1F). The following experiments were designed to sort this potential anomaly, which could be due to the conformational dynamics between 12 and 12–24mers.

Conformational Insights into LFAO Assembly

It is imperative to gain insights into the conformation of LFAOs in order to better understand the reasons behind replication behavior. To do so, LFAOs were investigated using the extrinsic fluorescent dyes ANS and DCVJ. ANS is well known to bind to solvent-exposed hydrophobic surfaces within proteins, which leads to an increased fluorescence intensity and a blue shift in the emission spectrum upon binding.¹⁶ DCVJ has been reported to bind amyloid aggregates leading to increased fluorescence, the extent of which can discriminate between the nature of aggregates.¹⁷ The binding efficiency of the two dyes to A β monomers, LFAOs, and fibrils was evaluated by incubating the samples in the presence of 17 or 2 molar excess of ANS and DCVJ dyes, respectively (Figure 3 A and B). As expected, both ANS and DCVJ showed no significant increase in their fluorescence intensities in the presence of A β monomers (□; Figure 3A and B). On the other hand, ANS and DCVJ bound to fibrils

effectively to generate significant intensities, suggesting the presence of hydrophobic pockets within the fibril structure (; Figure 3A and B). However, LFAOs indicated highest degree of ANS and DCVJ binding (●; Figure 3 A and B). ANS binding to LFAOs was marginally higher than fibrils, indicating that the LFAO assembly may involve more solvent exposed hydrophobic surfaces (Figure 3A). However, LFAOs displayed a roughly 2-fold increase as compared to fibrils when probed with DCVJ (Figure 3B). DCVJ has been reported to bind preferentially to oligomeric states of amyloidogenic proteins,¹⁷ which is in agreement with our observation. Together, the data reveals that LFAO structure is dissimilar to that of fibrils and could be conformationally distinct. We have previously reported that LFAOs have a β -sheet secondary structure similar to fibrils.¹³ However, the characteristic of the β -sheets (parallel or anti-parallel) that comprise the LFAO assembly has not been investigated. Therefore, the conformation of LFAOs was further probed using Fourier transform infrared spectroscopy (FTIR), which is an effective tool in distinguishing parallel and anti-parallel β -sheets of polypeptides on the basis of amide bond stretching frequencies.¹⁸ Upon investigating the control sample of bovine serum albumin (BSA), a known α -helical protein, an absorbance peak at 1655 cm^{-1} corresponding to α -helix was observed (dash-dot line; Figure 3C). Inspection of FTIR spectrum for fibrils revealed an intense absorbance band at 1626 cm^{-1} , indicative of parallel β -sheets (dashed line; Figure 3C), in agreement with previously established reports.^{19, 20} The spectrum for LFAOs also showed predominantly parallel β -sheets with an absorbance peak corresponding to 1626 cm^{-1} (smooth line; Figure 3C). No absorbance peak corresponding to anti-parallel β -sheet ($\sim 1690\text{ cm}^{-1}$) was observed for LFAOs or fibrils. This characteristic of LFAOs is surprising, as several reports indicate that soluble oligomers are comprised of predominantly anti-parallel β -sheets.^{21–23} However, this data does support our previous observation that LFAOs could be immuno-detected by the conformation-specific OC antibody, indicative of the fibril-like nature of LFAOs.¹⁵

Concentration-dependent Structural Dynamics of LFAOs

In light of the observed inverse correlation between LFAO concentration and replication efficiency (Figure 1) and potentially unique conformational characteristics of LFAOs (Figure 3), the concentration-dependent dynamics of LFAOs was investigated. Immunoblots of increasing concentrations of LFAOs revealed that the larger molecular weight distribution (80–110 kDa) increases as LFAO concentration increases from 0.5 to 10 μM (Figure 4A), as observed previously.^{14,15} At 0.5 μM , LFAOs are primarily 12mers (single arrow), with negligible 24mer band (double arrow). However, increasing the LFAO concentration to 10 μM reveals a more disperse oligomer, comprised of 12 and 24mer LFAOs. Identical banding patterns were obtained with a silver staining method (data not shown). Our previous investigation on molecular size of LFAO by sedimentation velocity revealed two molecular weight distributions centered around 56 and 110 kDa suggesting 12–24mers as the predominant species, which is in agreement with the immunoblot data presented here.¹⁴ Considering the possibility of dissociation of LFAOs upon treatment with SDS during PAGE, LFAO (10 μM) was also subjected to PAGE under non-denaturing conditions (Lane N; Figure 4B). The immunoblot showed predominantly two molecular weight distributions, further supporting the conclusion that LFAOs are predominantly 12–24mers. The difference between the molecular weights of 12 and 24mers was further elucidated by SEC on a

Superdex-75 column. A sample of 10 μM LFAOs fractionated at an elution volume of 8.67 mL, while 0.5 μM LFAOs eluted at a more inclusive volume of 8.79 mL, indicating a smaller size of LFAOs at low concentrations (Figure 4B and 4B *inset*).

To investigate whether LFAOs undergo conformational dynamics dependent upon concentration, ANS binding was performed at various LFAO concentrations by serial dilutions of a stock LFAO sample (Figure 5A and 5B). A decrease in LFAO concentration resulted in a corresponding decrease in ANS fluorescence and a concomitant red shift in the emission spectra (Figure 5A). Normalized titration curves for monomer, fibrils, and LFAOs are shown in Figure 5B. A β monomers showed insignificant ANS binding as observed previously (Figure 3A) and failed to show any concentration-dependent changes ($\square$; Figure 5B). Fibrils displayed a consistent amount of ANS binding along the entire concentration range between 0.5 and 8 μM ($\square$; Figure 5B). However, LFAOs displayed a transition in ANS binding between 0.5 and 8 μM ($\bullet$; Figure 5B). At lower concentrations ($< 1 \mu\text{M}$), LFAOs bind ANS to a lesser degree than fibrils. However, at higher concentrations ($> 1 \mu\text{M}$) LFAOs show greater ANS binding than fibrils. This transition in ANS binding indicates changes in the conformation of LFAOs upon dilution, which does not occur for monomers or fibrils. The data was fit with a monomer-dimer model (Eq 1), which revealed a dissociation constant (K_d) of 0.104 μM . To further investigate whether the changes in conformation resulted in differences in LFAO secondary structure, far-UV circular dichroism (CD) spectroscopy was used. The spectra obtained for low (0.5 μM) and high (8 μM) LFAO concentrations showed identical β -sheet structure with negative minima at 217 nm (Figure 5C). These spectra were similar to those obtained for fibrils at the corresponding concentrations (Figure 5D), suggesting that no secondary structure changes are involved during the conversion of 12mer to 12–24mers. Together, the data suggest that LFAO conformational changes may predominantly involve tertiary structure changes without any change to the secondary structure of LFAOs.

LFAO-induced Cellular Apoptosis Displays an Inverse Concentration Dependence

Cellular neurotoxicity of LFAOs was analyzed by investigating their ability to induce apoptosis to human neuroblastoma SH-SY5Y cells. The TUNEL assay, which labels nicks in the DNA strand to indicate apoptosis, was selected due to its wide use in evaluating the effect of A β toxicity.^{24–26} Cells treated with buffer equivalent (CONT, Figure 6B) displayed negligible TUNEL fluorescence, indicating minimal apoptosis. However, cells treated with 0.01 μM LFAOs for 24 h exhibited significant apoptosis compared to the control (Figure 6A and 6C). Additionally, the level of apoptosis induced by 0.01 μM LFAOs was comparable to that of cells treated with 25 μM H₂O₂, the positive control (Figure 6B and 6C). The toxicity of LFAOs decreased with increasing LFAO concentration, as 0.1 μM and 1 μM LFAOs failed to induce significant levels of cellular apoptosis (Figure 6A and 6C). In order to better reflect physiological concentrations of A β , the concentrations of LFAOs used in this assay are lower as compared to biophysical characterization experiments. Nevertheless, cellular results correlate with the dynamics of LFAOs observed from ANS binding as well as replication efficiency.

LFAOs Induce Caspase Activation Over a Wide range of LFAO Concentrations

The ability of LFAOs to activate caspase enzymes was assessed in SH-SY5Y cells using a FLICA-based assay capable of detecting a wide range of caspase activity. When SH-SY5Y cells were treated with buffer equivalent (CONT, Figure 7B and 7C), negligible FLICA-based fluorescence was observed, indicating minimal caspase activation. In contrast, cells treated with LFAOs at concentrations of 0.01, 0.1, and 1 μ M all exhibited a significant increase in caspase activation compared to the control (Figure 7A and 7C). Additionally, caspase activation in cells treated with 1 μ M and 0.1 μ M LFAOs was comparable to that of cells treated with TNF- α , the positive control (Figures 7B and 7C). However, caspase activation in cells treated with 0.01 μ M LFAOs was significantly less than that observed following 0.1 μ M and 1 μ M LFAO treatment. These results demonstrate that LFAOs strongly induce caspase activation over a 100-fold range of concentration and that LFAO-induced caspase activation varies with the concentration of LFAOs.

DISCUSSION

The data presented here showcase the ability of LFAOs to undergo conformational reorganization in a concentration-dependent manner that manifests as an increase in molecular weight of the oligomers from 12mers (56 kDa) to diffuse 12–24mers (56–110 kDa). Although this increase in molecular weight at high concentrations was reported in our earlier reports,¹⁴¹⁵ the current study provides further insights into the biophysical nature of LFAOs and structural transitions they undergo. It is intriguing to note that based on ANS analyses, LFAOs 12mers, which predominate at low concentrations, contain less solvent-exposed hydrophobic surfaces than fibrils. Whereas within LFAOs that exist as diffuse 12–24mers at higher concentrations, the solventexposed hydrophobic surfaces are more prevalent. This observation reinforces the fact that 12–24mer LFAOs are disperse as compared to the 12mers. In addition, this also suggests that the conversion of 12 to 12–24mers is concentration dependent and involves conformational reorganization. A closer investigation into the conformation of LFAOs revealed that the oligomers are composed of parallel β -sheets similar to fibrils, an observation that is in agreement with our previous conclusion based on conformation-specific antibody detection.¹⁵ However, parallel β -sheet conformation observed for LFAOs is different from predominantly anti-parallel β -sheets reported for certain oligomers.^{21–2327} Perhaps this difference is due to the off-pathway nature of LFAOs, making them conformationally unique. Furthermore, the ability of LFAOs to undergo replication could be attributed to their distinct conformational traits. It has to be borne in mind that a conformationally heterogeneous ensemble of oligomers could be present during the initial stages of aggregation in the AD brain, especially along the altered aggregation landscape. Hence, the existence of oligomers with parallel β -sheets is likely. Moreover, in our experiments, the nature of the β -sheet conformation of LFAOs was determined at high concentrations ($\sim$ 1 mM) by FTIR. It remains to be understood whether LFAOs show differences in the nature of β -sheet at low concentrations, as detection by FTIR becomes prohibitive at these concentration ranges. Nevertheless, it is interesting to observe that although 12 and 12–24mer LFAOs show differences in the degree of exposed hydrophobic surfaces at low and high concentrations, the overall β -sheet secondary structure is maintained. This suggests that the structural differences between 12 and 12–24mer LFAOs

mainly exist in their respective tertiary structure and not within secondary structures, implicating a reorganization of oligomer assembly to be the likely source of structural differences between the two forms.

To explore the physiological relevance of this structural difference observed in LFAOs, SH-SY5Y human neuroblastoma cells were treated with a 100-fold range of LFAO concentrations and evaluated for apoptosis via TUNEL staining. In addition, LFAO-induced caspase activation was assessed to explore one possible mechanism by which these aggregates may induce apoptosis. The lowest treatment concentration of LFAOs (0.01 μM) effectively induced apoptosis, as indicated by TUNEL staining, while higher concentrations failed to elicit any significant apoptotic response (Figure 6). Interestingly, the same trend was not observed for caspase activation. Instead, all LFAO treatment concentrations provoked a significant increase in caspase activity (Figure 7). This result may suggest that caspase activation is not the only driving force for LFAO-induced apoptosis. Apoptosis may also be induced via the upregulation of apoptosis-inducing factors (AIF), which are normally located in the intermembrane mitochondrial space. However, either in response to apoptotic stimuli or following mitochondrial damage, AIF are released into the cytosol, which translocate to the nucleus and cause substantial DNA fragmentation.²⁸ The release of AIF and the triggering of apoptosis via AIF has been reported to occur independent of the caspase signaling cascade.^{28,29} While the correlation between AIF and $\text{A}\beta$ -induced apoptosis has not yet been established, $\text{A}\beta$ aggregates, particularly oligomers, can induce mitochondrial damage and dysfunction that is believed to play a key role in the pathogenesis of AD.^{30,31} Furthermore, it has been suggested that mitochondria may in part induce AD-associated apoptosis by the opening of the mitochondrial permeability transition pore, which leads to activation of both AIF and caspases.³¹

LFAO-induced caspase activity was observed in the absence of apoptotic TUNEL staining at higher LFAO concentrations (0.1 and 1 μM). Thus, induction of caspase activity by LFAOs may elicit cellular responses other than apoptosis. In fact, LFAOs may trigger inflammatory caspases, rather than apoptotic caspases. In particular, three of the caspases detected in the current study (-1, -4, and -5) regulate innate inflammatory immune responses, rather than apoptotic factors.³² While $\text{A}\beta$ can induce apoptotic caspases, $\text{A}\beta$ and AD have also been linked to activation of caspase-1, the most common inflammatory caspase, by several mechanisms. NLRP3 inflammasome, a caspase-1 promotor, is activated by $\text{A}\beta$ aggregates.³³ IL-1 β and IL-18, which lead to caspase-1 activation, are significantly upregulated in AD patients and transgenic AD mice,³⁴ and $\text{A}\beta$ oligomers can disrupt potassium channels, leading to caspase-regulated inflammatory responses.³⁴ It is important to note that while inflammatory responses can present neuroprotective effects, chronic activation of inflammatory caspases is not without consequence in AD. Caspase-1 activation can induce tau cleavage, can activate apoptotic caspase-6, and may impair glycolysis.³⁴

A comparison of cellular studies with biophysical data establishes that LFAO dynamics correlate with their physiological behavior. LFAO-induced cellular apoptosis was observed exclusively at the lowest treatment concentration (0.01 μM), while LFAO-induced caspase activation was more pronounced at higher treatment concentrations (0.1 μM and 1 μM). These phenomena parallel the concentration-dependent size and conformation dynamics of

LFAOs. As concentration increases, LFAOs transition from predominantly 12mers to 12–24mers (Figure 1), with a dissociation constant of $\sim 0.1 \mu\text{M}$. Moreover, this increased presence of 12–24mers is accompanied by a conformational change, as evidenced by ANS binding (Figure 5B). Oligomer size and conformation have been shown to heavily impact the toxicity of A β aggregates.^{35–37} The data presented here suggest that the size and conformation of LFAOs found at lower concentrations are capable of eliciting a more potent apoptotic response than those found at higher concentrations. Meanwhile, the higher treatment concentrations eliciting a greater caspase response suggest the 12–24mer conformation triggers primarily inflammatory caspases. While further studies are required to determine the dominant mechanism by which LFAOs induce apoptosis and caspase activation, the qualitative similarities between the biophysical data and LFAO-induced cellular responses underscore the role of LFAO conformational dynamics in their physiological activity.

In addition to the physiological response in cells, perhaps a significant effect of LFAO conformational dynamics is reflected in their ability to replicate in the presence of A β monomers. Although the concentration of replicated LFAOs seems to increase with the increase in LFAO seed concentrations, the efficiency of LFAO replication (fold-increase) exponentially decreases for the same seed concentrations, which seems to violate first order kinetics and presents a potential anomaly. However, this report has established that the observed inverse correlation is due to conformational differences between 12 and 12–24mer LFAOs. An overlay of replication efficiency and extent of 12 or 12–24mers, as shown in Figure 8, brings this aspect to perspective. Near identical concentration-dependent responses are observed for the two reactions. Figure 9 schematically summarizes the important results from this report. It is clear that 12–24mer LFAOs resist replication, while the 12mers seem to replicate more efficiently. Furthermore, cell culture results establish that 12mers are more toxic than 12–24mer LFAOs. Collectively, a picture emerges that suggests under physiological concentrations (nM), LFAO-type oligomers could undergo replication preferentially as opposed to seeding towards fibril formation. The modest quantitative amplification resulting from a numerically large number of replication reactions (in the presence of multiple seeds) occurring in the AD brain would yield an exponentially large number of ‘replicons’, which in turn could lead to effective propagation and dissemination of toxic oligomers. Contextually, such propagation could be associated with prion-type mechanisms; however, mechanistically, LFAO replication differs from prion propagation as replication results in the generation of a quantitatively large amount of similar oligomers upon LFAO interaction with monomers, as opposed to larger aggregates. However, since 12–24mer LFAOs seem to resist replication, it is intriguing to know if higher concentrations of LFAOs may undergo a more conventional prion-type propagation, where LFAOs grow as distinct repeating units leading to morphologically and physiologically unique aggregates. These aspects of LFAOs, along with details of their structure are currently under investigation and will be reported at a later date.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

LFAO replication efficiency. A–E) SEC chromatograms for LFAO replication with LFAO seed concentrations of 0.5, 1, 3, 10, and 14 μM , respectively. Dashed and smooth lines represent LFAO seed and seeded reactions after 72 h of incubation, respectively. F) Quantitative LFAO increase at varying concentrations. *Inset*) Immunoblot of LFAO replication at various concentrations. For each LFAO concentration, the left and right lanes represent LFAO seed and seeded reactions, respectively. Monomer control at 72 h is listed as lane C. The amount loaded was kept constant at 34 ng for LFAO seed and seeded samples (based on initial LFAO concentration), while 3.4 μg was loaded for the monomer control (lane C).

Figure 2.

Quantitative analysis of LFAO replication. A–C) Representative SEC chromatograms of reactions that were used to derive the parameters in Table 1. A) Seed (dashed) and seeded (smooth) reactions containing 14 μM LFAO and 50 μM monomers after 72 h of incubation. B) Reactions of 50 μM monomer alone at 0 h (smooth) and that with 14 μM LFAO (dashed) at 72 h of incubation. C) A reaction of 50 μM monomer without seed at 0 h (smooth) and 72 h (dashed) of incubation. D) Net increase in LFAO concentration upon replication ($\Delta LFAO$; ●) and net change in free monomer concentration (ΔM_f ; □) as a function of initial LFAO seed concentration. $\Delta LFAO$ and ΔM_f are also shown in panels A and B, respectively. Data represent an average of three independent trials.

Figure 3.

Biophysical characterization of LFAOs. A–B) ANS and DCVJ binding fluorescence, respectively, for A β monomers ($\square$), LFAOs ($\bullet$), and fibrils ($\triangle$). ANS and A β concentrations were kept constant at 100 μM and 6 μM , respectively. DCVJ and A β concentrations were kept constant at 10 μM and 5 μM , respectively. C) FTIR of A β LFAOs (smooth), A β fibrils (dashed), and BSA (dash-dot).

Figure 4.

Molecular size of LFAOs. A) PAGE immunoblot of 0.5, 1, 2, 4, 6, and 10 μ M LFAOs. The amount loaded onto each lane was kept constant at 45 ng. Lane N is an immunoblot of 10 μ M (113 ng) LFAO sample electrophoresed under non-denaturing conditions (without SDS). Single and double arrows represent LFAO 12mer and 24mer band, respectively. B) SEC elution profiles for 0.5 (smooth) and 10 (dashed) μ M LFAOs. *Inset*) Elution volumes for 0.5, 1, 3, 10, and 14 μ M LFAOs, respectively.

Figure 5.

Conformational changes during LFAO dynamics. A) ANS scans of 0.5, 1, 2, 4, 6 and 8 μM LFAOs along with 100 μM ANS blank (dashed). B) ANS fluorescence scans from (A) were integrated and normalized against $A\beta$ concentrations, which were then plotted for monomer ($\square$), LFAOs ($\bullet$), and fibrils ($\triangle$). The data were fit with Eq 1. C and D) Circular dichroism spectra for 0.5 (smooth) and 8.0 (dashed) μM LFAO (C) and fibril (D) samples, respectively. The perpendicular dotted line indicates 217 nm.

Figure 6.

LFAO-induced cellular apoptosis. A) Nuclear DAPI (blue) and TUNEL (green) stained images of SH-SY5Y cells following 24 h treatment with 0.01, 0.1, and 1 μM LFAOs along with B) negative (CONT, buffer equivalent) and positive (25 μM H₂O₂) controls. Images are representative of 3–7 independent experiments. C) Cellular apoptosis quantified using MATLAB functions applied to single-channel fluorescence microscope images. Results are reported as the fraction (TUNEL/DAPI) of apoptotic cells. ***p<0.001 vs. negative control. Error bars represent SEM, n = 3–7.

Figure 7.

LFAO-induced caspase activation. A) Nuclear Hoechst (blue) and FLICA-labeled active caspase (green) stained images of SH-SY5Y cells following 24 h treatment with 0.01, 0.1, and 1 μ M LFAOs along with B) negative (CONT, buffer equivalent) and positive (2 U/ μ L TNF- α) controls. Images are representative of 3–5 independent experiments. C) Cellular caspase activation quantified using MATLAB functions applied to single-channel fluorescence microscope images. Results are reported as the fraction (FLICA/Hoechst) of caspase activated cells. *** p <0.001 and ** p <0.01 vs. negative control; + p <0.05 vs. 0.01 μ M LFAO. Error bars represent SEM, n = 3–5.

Figure 8. Comparison of LFAO conformational dynamics and replication efficiency. Overlay of LFAO replication efficiency () (from Figure 1F) with ANS binding fluorescence (●) (from Figure 3D).

Figure 9.

Schematic diagram of the summary of LFAO dynamics and its consequence. The 12mer and 12-24mer LFAO structure is sketched based on biophysical data (FTIR, ANS, and CD).

Table 1

Quantitative analysis of LFAO replication. Data derived from three individual SEC experiments (denoted as *) shown in Figures 1, 2 and S1 are summarized along with their standard deviations in parentheses. The results calculated from the experimental results are denoted as ‘◆’, while the known parameters are denoted as ‘●’. The symbols M_0 , M' , M'' , M_r and M are defined within Eqs 2–6, and are described in the text.

Monomer (μM)	LFAO Seed (μM)	Replication Efficiency	Replicated LFAO (μM)	LFAO (μM)	Calculated Monomer (μM)	Actual Monomer (μM)	Monomer (μM)	Monomer On Pathway (μM)
M_0			M''			M_r		M'
50	0	—	—	—	—	44	—	6*
50	0.5	6.1 (±0.3)	3.0 (±0.66)	2.5	47	41 (±1.8)	-9	6
50	1.0	3.8 (±0.81)	3.8 (±0.81)	2.8	47	42 (±1.9)	-8	5
50	3.0	2.6 (±0.47)	7.7 (±1.4)	4.7	45	40 (±1.8)	-10	5
50	10	1.7 (±0.10)	17 (±1.0)	7.0	43	37 (±2.7)	-13	6
50	14	1.5 (±0.15)	22 (±2.1)	7.6	42	37 (±1.7)	-13	5
●	●	*	*	◆	◆	*	◆	◆