**In Vitro** Characterization of Conditions for Amyloid-β Peptide Oligomerization and Fibrillogenesis*

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Extensive research causally links amyloid-β peptide (Aβ) to Alzheimer’s disease, although the pathologically relevant Aβ conformation remains unclear. Aβ spontaneously aggregates into the fibrils that deposit in senile plaques. However, recent *in vivo* and *in vitro* reports describe a potent biological activity for oligomeric assemblies of Aβ. To consistently prepare *in vitro* oligomeric and fibrillar forms of Aβ1–42, a detailed knowledge of how solution parameters influence structure is required. This manuscript represents the first study using a single chemically and structurally homogeneous unaggregated starting material to demonstrate that the formation of oligomers, fibrils, and fibrillar aggregates is determined by time, concentration, temperature, pH, ionic strength, and Aβ species. We recently reported that oligomers inhibit neuronal viability 10-fold more than fibrils and ~40-fold more than unaggregated peptide, with oligomeric Aβ1–42-induced neurotoxicity significant at 10 nM. In addition, we were able to differentiate by structure and neurotoxic activity wild-type Aβ1–42 from isoforms containing familial mutations (Dahlgren, K. N., Manelli, A. M., Stine, W. B., Jr., Baker, L. K., Krafft, G. A., and LaDu, M. J. (2002) *J. Biol. Chem.* 277, 32046–32053). Understanding the biological role of specific Aβ conformations may define the link between Aβ and Alzheimer’s disease, re-focusing therapeutic approaches by identifying the pernicious species of Aβ ultimately responsible for the cognitive dysfunction that defines the disease.

Amyloid plaques are one of the pathological hallmarks of Alzheimer’s disease (AD). Genetic findings over the past decade have further supported the pivotal and likely causal role that amyloid-β peptide (Aβ) plays in the etiology of AD (1–4). This research demonstrates that autosomal dominant mutations affecting the total amount or relative amount of the 42-versus 40-residue form of Aβ are sufficient to cause the disease (for review, see Ref. 5). However, a direct causal relationship between amyloid plaques and cognitive impairment remains unclear (6, 7). This apparent disconnect between plaque burden and neuronal dysfunction and loss has also been described in transgenic mouse models of AD (8, 9). Recent theories that reconcile these findings point to small soluble oligomeric or protofibrillar assemblies of Aβ that would likely escape the immunostaining and histopathological staining used to detect both diffuse amyloid deposits and senile plaques (10–13).

Soluble oligomers of Aβ have been isolated from brain, plasma, cerebrospinal fluid (14, 15), transfected cells (16), and cells derived from human brain (11, 17). *In vitro*, oligomeric and protofibrillar forms of Aβ have been shown to be directly neurotoxic and inhibit electrophysiologic activity that may be necessary for the formation and maintenance of memory (15, 17–21). Experimental *in vivo* and *in vitro* evidence linking oligomeric assemblies to neurodegeneration is reflected in a recent revision to the amyloid hypothesis of AD (22) and other diseases involving amyloidogenic proteins (for review, see Ref. 23). A complete study of Aβ, either *in vivo* or *in vitro*, requires an understanding of the conditions that drive peptide assembly toward one conformational state or another. Any change that affects the conformation of Aβ likely affects its biological activity.

In light of genetic and *in vivo* evidence, we have chosen to specifically focus on the longer, more hydrophobic Aβ1–42 species. We have determined that the critical initial step in the controlled assembly of Aβ1–42 is to remove preexisting structure. Using this chemically and structurally uniform unaggregated starting material, we demonstrate that changes in incubation time, concentration, temperature, ionic strength, and pH result in distinct structural species including soluble oligomers, protofibrils and short fibrils, extended fibrils, and insoluble fibrillar aggregates. We have reported recently (24) that oligomers formed using these methods inhibit neuronal viability 10-fold more than fibrils and ~40-fold more than unaggregated peptide, with oligomeric Aβ1–42-induced inhibition significant at 10 nM. In addition, we were able to differentiate by structure and neurotoxic activity wild-type Aβ1–42 from isoforms containing known familial mutations (24).

Considering the difficulties in detecting unaggregated and oligomeric Aβ using traditional light and electron microscopy, atomic force microscopy (AFM) was chosen for this study as the primary means of characterizing Aβ assembly state. AFM has proven uniquely well suited to the study of Aβ (15, 18, 25–28) and other amyloidogenic proteins (29–31), because it generates detailed three-dimensional information at a nanometer scale. This technique is capable of characterizing the wide range of structures present in aggregated Aβ mixtures with sufficient resolution to detect individual 0.9-nm structures in unaggre-
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scanning probe work station equipped with a MultiMode head using an E-series piezoelectric scanner (DRT Instruments, Santa Barbara, CA). AFM probes were single-crystal silicon microcantilevers with 300-kHz resonant frequency and 42 Newton/meter spring constant model OMCL-AC160TS-W2 (Olympus). Samples were imaged under dry helium. 10–50 µl of sample solution was spotted on freshly cleaved mica, incubated at room temperature for 5 min, rinsed with 0.02 µm of filtered (Whatman Anotop 10) deionized NANOpure water (Barnstead Thermoline), and blown dry with tetrafluoroethane (ClearTex Micro-Duster III). Image data were acquired at scan rates between 1 and 2 Hz with drive amplitude and contact force kept to a minimum.

**Western Blot Analysis of SDS-PAGE**—Gel electrophoresis and Western blot analysis were performed according to the manufacturer's protocols (Invitrogen) as described previously (24). Briefly, unheated samples in lithium dodecyl sulfate sample buffer were applied to 12% bis-Tris NuPAGE gels were electrophoresed using MES running buffer and transferred to 0.45-µm polyvinylidene difluoride membrane (Invitrogen). Membranes were blocked in 5% non-fat dry milk in Tris-buffered saline containing 0.025% Tween 20. Blots were incubated in the primary antibody 6E10 (mouse monoclonal against Aβ residues 1–16; Signet, Dedham, MA) or 4G8 (mouse monoclonal against Aβ residues 17–24; Signet, Dedham, MA). Immunoreactivity was detected using enhanced chemiluminescence (ECL; Amersham Biosciences) and imaged on an Eastman Kodak Co. Image Station 440CF. Molecular weight values were estimated using rainbow pre-stained molecular weight markers (Amersham Biosciences).

**Solubility Analysis**—Aβ42 preparations were centrifuged for 30 min at 4 °C at either 16,000 × g in an Eppendorf 5415D microcentrifuge (Beckman Coulter Instruments) or at 100,000 × g in a Beckman J2-21 ultracentrifuge using a TLA 120.1 rotor in 8 × 34-mm polycarbonate ultracentrifuge tubes (Beckman Coulter Instruments). Aβ supernatant and pellet fractions were extracted by dilution to 70% formic acid (EM Science), bath sonicated (model 2120; Branson) for 5 min, and incubated for 1 h at room temperature. Control samples were prepared in an identical manner using the same tubes and solutions in the absence of centrifugation. Values from these control samples were used to determine non-sedimenting background and were subtracted from test samples. For dot blot analysis, Aβ supernatant and pellet fractions were diluted to 23.3% formic acid in deionized water. Quantitative dot blot analysis was performed using a series 1055 96-well vacuum dot blot apparatus (Invitrogen) fitted with a 0.45-µm Immobilon-P membrane (Millipore). 40 µl of each diluted fraction was applied to the membrane and developed using the same methods described for Western blot analysis. Quantitation of immunoreactivity was determined using Kodak one-dimensional image analysis software (version 3.5.4).

**RESULTS**

**In vitro studies** necessary to define the structure and activity differences between small oligomeric and fibrillar assemblies of Aβ need to be based on procedures that consistently produce fully characterized, homogeneous structural populations. To achieve this goal, the first point to address is the intrinsic structural variability between different lots of Aβ peptide. This variability can exist between chemically identical peptide lots and often results in changes in biological activity in *vitro* (33, 34).

**HFIP Pretreatment Produces Uniform, Unaggregated Fields of Aβ42**—Removal of any preexisting structures in lyophilized stocks of Aβ1–42 peptide is required for controlled aggregation studies. In this study, lyophilized Aβ42 directly dissolved to 5 mM in 100% HFIP was characterized by AFM. This treatment resulted in a dense, homogeneous field of unaggregated peptide immediately after resuspension (Fig. 1A). After a 24-h incubation at room temperature, no aggregates, fibrils, or pro-totofibrils were detected in these HFIP Aβ42 solutions. The z-height value for the individual peptide structures as measured by AFM was 1.0 (± 0.3) nm, which agrees the expected size of a single Aβ monomer. However this technique is not capable of absolute molecular weight determination and therefore does not provide proof that these structures are monomeric. HFIP is a corrosive alcohol and is not compatible with *in vitro* and cell-based assays. For this reason, it was removed by evapora-
Detection of Aβ1–42 Fibrils after Direct Resuspension in 
MeSO—MeSO is a highly polar, water-soluble organic solv‐
ent commonly used to solubilize hydrophobic peptides, includ‐
ing Aβ (41), and is recommended by commercial suppliers 
(Bachem). Analytical ultracentrifugation studies have dem‐
onstrated that Aβ1–40 and Aβ1–42 are primarily monomeric in 
100% anhydrous MeSO (42, 43). In the current study, AFM 
analysis of different commercial lots of synthetic and recombi‐
nant Aβ1–42 directly resuspended to 5 mM in 100% anhydrous 
MeSO without HFIP pretreatment demonstrated that the 
peptide appears primarily unaggregated immediately after sol‐
ubilization, with structures measuring 1.1 (± 0.3) nm (Fig. 1A). 
However, in some cases small diameter (~2-nm) fibrils were 
observed (Fig. 1A, inset). After 24 h at room temperature, 
additional small Aβ fibrils were detected in Aβ1–42 MeSO 
solutions, even in preparations that were initially fibril-free. 
These small fibrillar structures were stable in MeSO for sev‐
eral weeks and formed even after HFIP pretreatment (data not 
shown). These results indicate that 100% anhydrous MeSO 
alone is not a sufficiently strong solvent to remove all struc‐
tural history present in lyophilized Aβ and maintain a concen‐
trated unaggregated peptide solution.

Detection of Aβ1–42 Fibrils, Protofibrils, Large Aggregates, 
and Oligomers after Direct Resuspension in Water—Re‐
suspension of lyophilized Aβ1–42 directly in dH2O or aqueous buffer 
is also used by investigators (44, 45) and recommended by 
commercial manufacturers (AnaSpec, Bachem, U.S. Peptide, 
Inc.). To determine the extent of structural heterogeneity pre‐
sent in lyophilized stocks of Aβ1–42, peptide was directly resus‐
pended to 5 mM in dH2O and characterized by AFM. Analysis of 
these solutions revealed a wide size range of peptide aggregates 
and extended fibrils immediately after resuspension (Fig. 1A). 
These structures included large (10–15-nm z-height) and small 
(2–5-nm z-height) amorphous peptide aggregates and interme‐
diate diameter fibrils (4–6 nm) greater than 1 μm in length. 
These structures persisted after 24 h at room temperature, in 
addition to an increase in the number of 2–5-nm globular 
aggregates. However, the extent of all the structures observed 
under these conditions is highly dependent on seeded aggrega‐
tion and thus dependent on the initial lyophilized peptide. 
These data indicate that direct resuspension of lyophilized 
Aβ1–42 in dH2O produces heterogeneous structural popula‐
tions incompatible with controlled aggregation studies. Note 
that the z-height range for this H2O preparation was main‐
tained at 5 nm, resulting in a greater contrast compared with 
images of HFIP- and MeSO-solubilized peptide.

Solubilization of HFIP-treated Peptide in MeSO Produces a 
Uniform, Unaggregated Field of Aβ1–42—To ensure that the 
HFIP-treated Aβ1–42 peptide film was fully solubilized, 
peptide stocks were resuspended to 5 mM in MeSO. The resulting 
solution contained unaggregated structures (Fig. 1B) that
measured 1.0 (± 0.3) nm by AFM (Fig. 1B, inset), comparable 
with the measurements for Aβ1–42 structures measured di‐
rectly in HFIP or MeSO (Fig. 1A). Solubilization of the HFIP- 
treated peptide directly into dH2O or aggregation buffer does

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not produce a fully resuspended unaggregated solution. The thin film resulting from the HFIP treatment is not easily resuspended directly in dH2O without the use of sonication or other methods. The peptide film is more soluble in MeSO facilitating complete resuspension of the HFIP-treated peptide (data not shown).

**Solution Conditions for the Formation of Oligomeric or Fibrillar Aβ1–42 Assemblies**—Once conditions were established that consistently produce unaggregated Aβ preparations, two different aggregation protocols were used with the HFIP-treated Aβ1–42 peptide stocks. Previous studies demonstrated the formation of small soluble oligomeric assemblies derived from Aβ1–42 either incubated in the presence of apolipoprotein J under physiologic conditions (18) or in cell culture medium where the oligomeric assemblies were referred to as ADDLs (amyloid-derived diffusible ligands) (19). For the studies presented herein, the cell culture medium method with a 24-h incubation at 4 °C was used for oligomer formation. For the fibril-forming conditions, previous studies have indicated that acidic pH favors fibrillogenesis for Aβ (46, 47) and other amyloid-forming proteins (48–50). The acidic pH method using 10 mM HCl with a 24-h incubation at 37 °C was chosen for fibril formation. The resulting oligomeric and fibrillar Aβ1–42 structures were characterized by AFM (Fig. 2A) and Western blot analysis of SDS-PAGE (Fig. 2B).

Immediately after resuspension under both the oligomer- and fibril-forming conditions, the majority of Aβ1–42 remained as unassembled structures measuring 1.0 nm (Fig. 2A, insets), although a few oligomers measuring between 2 and 4 nm were also detected (Fig. 2A). After a 24-h incubation under oligomer-forming conditions, Aβ1–42 assembled into predominately 2–4-nm oligomeric structures (Fig. 2A). No fibril formation was detected under these conditions. Within 24 h under fibril-forming conditions, Aβ1–42 converted to 4-nm amyloid fibrils that extended over several microns, some of which had a semi-periodic structure along the fibril axis. As previously reported, AFM measurements may underestimate the diameter of Aβ structures because of sample compression by the AFM probe (25, 51).

Western blot analysis of SDS-PAGE revealed Aβ1–42 monomer, trimer, and tetramer for unaggregated (0 h) samples incubated under both oligomer- and fibril-forming conditions (Fig. 2B, lanes 1 and 3, respectively). Larger oligomeric assemblies ranging from 30 to 60 kDa were detected after incubation for 24 h under oligomer-forming conditions. Additionally, there was an increase in the trimer and tetramer bands, as well as smearing between tetramer and monomer, possibly indicating interconversion between these assemblies during electrophoresis (Fig. 2B, lane 2). After 24 h, Aβ1–42 samples incubated under fibril-forming conditions contained high molecular weight immunoreactive Aβ that remained in the well, less abundant 30–60-kDa large oligomers, and a smear between tetramer and monomer (Fig. 2B, lane 4). The abundant tetramer detected after 24 h under oligomer-forming conditions was not present under fibril-forming conditions, although the increase in the trimer band and smearing between trimer and monomer were present in both preparations at 24 h.

Western analysis of SDS-PAGE for Aβ1–42 oligomers and fibrils was variable. In our previous report we observed more SDS-stable structures between monomer and tetramer, particularly dimer, in the 24-h fibril preparations than reported herein (24). Common trends observed in both studies for oligomeric preparations include detection of an abundant tetramer and the presence of >60-kDa large aggregates, including Aβ immunoreactive material that remains in the well of the gel. Unaggregated (0 h) Aβ1–42 samples typically contain some trimer and tetramer, in addition to monomer. Comparable results are obtained with the Aβ monoclonal antibodies 6E10, recognizing Aβ residues 1–16 (Fig. 2B), and 4G8, recognizing Aβ residues 17–24 (data not shown). The NuPAGE gel system used in these studies yields slightly different banding patterns with these two antibodies than Tris/Tricine and Tris/glycine gels where more dimer is detected (data not shown).
Thus, in contrast to the limited and variable information gained by the gel-dependent detection of SDS-stable Aβ structures, AFM analysis clearly differentiates between oligomeric and fibrillar Aβ1–42 structures that form under the conditions described for these experiments.

Aβ1–42 Assembly Is Dependent on Incubation Time and Peptide Concentration—Total peptide concentration is known to influence the fibrillogenesis of Aβ (47). To determine how Aβ peptide concentration affects assembly under oligomer- and fibril-forming conditions, Aβ1–42 concentrations ranging from 10 to 100 μM were prepared and analyzed immediately after dilution (0 h), after 24 h, and after 1 week.

Immediately after dilution, 2–4-nm Aβ1–42 oligomers formed rapidly under the oligomer-forming conditions at concentrations greater than 25 μM (Fig. 3A, 0 h). In general, a concentration-dependent increase in the number of 2–4-nm oligomers was observed between 10 and 100 μM for all three incubation periods (Fig. 3A). After 24 h, a slight increase in the number of 2–4-nm oligomers, but no fibril formation, was detected at each of the four concentrations (Fig. 3A, 24 h). After 1 week, a concentration-dependent increase in protofibrils (measuring ~200 nm) and some short fibrils both straight and curved (measuring <1 μm) were observed in the oligomer preparations at 50 and 100 μM, with very few protofibrils in 25 μM samples, and no protofibrils in the 10 μM Aβ1–42 solutions (Fig. 3A, 1 week). Of interest, no mature fibrils (measuring >1 μm) were detected at any of the time points or concentrations despite the detection of protofibrils and short fibrils, particularly abundant at 100 μM and 1-week incubation time. These results demonstrate that Aβ1–42 rapidly forms oligomers under these conditions and that only after extended incubation at Aβ1–42 concentrations ≥ 25 μM were significant numbers of protofibrils and slightly longer structures detected.

Under fibril-forming conditions, immediately after dilution Aβ1–42 forms 2–4-nm oligomers, in addition to some protofibrils at 50 and 100 μM (Fig. 3B, 0 h). After incubation for 24 h, no fibril formation could be detected at 10 μM, and mixed individual 4-nm fibrils, protofibrils, and oligomers were detected at 25 μM, 4-nm fibrils mixed with some 2–4-nm oli-
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Aβ1–42 assembly is dependent on ionic strength and pH. A, AFM. 5 mM Aβ1–42 in MeSO stocks were diluted to 100 μM in either 10 mM HCl (low ionic strength, acidic pH), 10 mM HCl + 150 mM NaCl (acidic pH, physiologic ionic strength), 10 mM Tris, pH 7.4 (neutral pH), or 10 mM Tris, pH 7.4, + 150 mM NaCl (neutral pH, physiologic ionic strength). Samples were prepared after a 24-h incubation at 37 °C. Representative 2 × 2-μm x-y, 10-nm total z-range AFM images are shown, except for the image of the acidic pH, physiologic ionic strength condition, which is scaled to 2 × 2-μm x-y, 25-nm total z-range. B, Western analysis of SDS-PAGE. Representative Western blots of samples prepared as described for A and analyzed as described in the legend for Fig. 2 are shown. The figure shows HCl (lane 1), HCl + NaCl (lane 2), Tris (lane 3), and Tris + NaCl (lane 4).

Oligomers were present at 50 μM, and dense networks of ~4-nm fibrils were present at 100 μM. After 1 week, only oligomeric and protofibrillar structures could be detected at 10 μM Aβ1–42. At 25 μM, mixed oligomeric, protofibrillar, and fibrillar Aβ1–42 structures were observed, with the fibrils appearing more rigid than at 24 h. At both 50 and 100 μM, dense fields fibrils were present. These results indicate that Aβ1–42 incubated under fibril-forming conditions results in concentration- and time-dependent fibril formation and that under these conditions, homogeneous fibril populations form within 24 h at 100 μM Aβ1–42 and are stable for at least 1 week.

Aβ1–42 Assembly Is Dependent on Temperature—Temperature has been shown previously to be an important factor in fibril assembly (52). Here we investigated the effects of temperature on oligomer- and fibril-forming conditions. Solutions of 100 μM Aβ1–42 incubated at 4 °C under the standard oligomer-forming conditions were also incubated at room temperature and 37 °C. Solutions of 100 μM Aβ1–42 incubated at 37 °C under the standard fibril-forming conditions were also incubated at room temperature and 4 °C. The resulting structures were characterized by AFM (Fig. 4A) and Western blot analysis of SDS-PAGE (Fig. 4B).

Under oligomer-forming conditions (4 °C), 100 μM Aβ1–42 incubated for 24 h produced homogeneous solutions of 2–4-nm oligomers comparable with Fig. 2A (Fig. 4A, 4 °C). At room temperature, the solution remained predominately oligomeric with a low concentration of short protofibrils (Fig. 4A, RT). At 37 °C, the solution contained both oligomers and an increased number of protofibrils (Fig. 4A, 37 °C). These results indicate that uniform populations of oligomers form at 4 °C, and increasing temperature produces limited protofibril formation within 24 h at 100 μM Aβ1–42. However, as with incubation time and peptide concentration, although protofibrils were present, no extended fibrils were observed with the oligomer-forming conditions at any of the temperatures.

Under fibril-forming conditions (37 °C), 100 μM Aβ1–42 incubated for 24 h produced extended ~4-nm fibrils, comparable with Fig. 2A (Fig. 4A, 37 °C). When the incubation temperature was lowered to room temperature, Aβ1–42 continued to form extended fibrils, although an increase in the oligomeric background was observed (Fig. 4A, RT). Solutions incubated at 4 °C contained predominately unassembled peptide and 2–4-nm oligomeric structures, in addition to a sparse population of 2–4-nm-diameter fibrils less than 2 μm in length. In summary, incubation at 37 °C produces the most uniform fibril populations, with fibril density decreasing significantly with temperature.

Western blot analysis of SDS-PAGE of Aβ1–42 incubated under oligomer-forming conditions detected SDS-stable tetramer, trimer, and monomer (Fig. 4B, lane 1). When the incubation temperature was raised to room temperature and 37 °C, there was a decrease in the amount of monomer and an increase in the presence of large oligomers (Fig. 4B, lanes 2 and 3). Under fibril-forming conditions, the majority of the large fibrillar assemblies were not SDS-stable as Aβ1 appeared as monomer, trimer, and tetramer, and decreasing temperature induced an increase in the amount of trimer and tetramer (Fig. 4B, lanes 4–6).

Aβ1–42 Assembly Is Dependent on Ionic Strength and pH—Two primary differences between the oligomer- and fibril-forming conditions are ionic strength and pH. For the oligomer-forming conditions, Aβ1–42 is incubated in cell culture medium at physiologic ionic strength and neutral pH. For fibril-forming conditions, Aβ1–42 is incubated in dilute HCl at low ionic strength and acidic pH. To determine how the combination of ionic strength and pH influence the assembly of Aβ1–42, 100 μM Aβ1–42 solutions were incubated at 37 °C at acidic pH and neutral pH and at both low and physiologic ionic strength. At acidic pH and low ionic strength (standard fibril-forming conditions), extended ~40-nm fibrils were detected by AFM, comparable with Fig. 2A (Fig. 5A). Adding 150 mM NaCl while maintaining the acidic pH resulted in the formation of dense fibrillar aggregates that vary in z-height from 5 to greater than 25 nm (Fig. 5A). Note that the z-height range
Fig. 6. AFM analysis of the solubility of Aβ1–42 assemblies.

Oligomeric and fibrillar Aβ1–42 samples were prepared as described in the legend for Fig. 2. Unaggregated solutions of Aβ1–42 were prepared by dilution of 5 mM Aβ1–42 Me₂SO stock solution to 10 µM in ice-cold dH₂O. Fibrillar aggregates of Aβ1–42 were prepared by incubation in 10 mM HCl + 150 mM NaCl for 24 h at 37 °C as described in the legend for Fig. 5. 100 µM samples were diluted 1:10 and either not centrifuged (total) or centrifuged for 30 min at 16,000 or 100,000 × g. Similar results were obtained if the preparations were diluted before or after centrifugation. Diluting the samples prior to centrifugation facilitated supernatant and pellet recovery. Total and supernatant fractions were prepared for AFM analysis. Representative 2 × 2-µm x-y, 10-nm total z-range AFM images are shown. Inset images, 200 × 200-nm x-y, 2-nm total z-range.

representation for this image was increased from 10 to 25 nm because of the size of the fibrillar aggregates. At neutral pH and low ionic strength, Aβ1–42 solutions remained primarily oligomeric comparable with Fig. 2A, in addition to several short fibrils (Fig. 5A). When the pH was neutral, and 150 mM NaCl was added, Aβ1–42 oligomers coalesced into primarily small oligomeric aggregates with z-height values measuring ~5–8 nm and protofibrils.

Oligomer formation was favored at neutral pH and physiologic ionic strength. However, some differences were present between Aβ1–42 assemblies formed in F-12 culture medium (Fig. 2A) and in a buffered salt solution (Fig. 5A). In F-12 culture medium, more evenly dispersed fields of 2–4-nm oligomers were observed by AFM, whereas Aβ1–42 incubated in buffered salt solutions yielded more aggregated clusters of oligomers and protofibrils. This may result from the differences in the inorganic salts or the presence of trace metals and/or amino acids in F-12 culture medium, initially selected based on oligomer-forming conditions reported in the literature (19). In an effort to simplify the oligomer-forming solution, several solutions of comparable pH and ionic strength were screened, including Dulbecco’s modified Eagle’s medium, artificial cerebral spinal fluid, and buffered salt solutions containing trace metals and N,N-dimethylglycine in place of individual amino acids. Although Aβ1–42 formed oligomeric assemblies in each of these solutions as determined by AFM (data not shown), globular structures were present in solution controls in the absence of Aβ, confounding the detection of Aβ oligomers prepared in these solutions. Ultimately, the most consistent preparations with the lowest background levels for AFM were obtained using F-12 cell culture medium.

Western blot analysis of SDS-PAGE revealed that samples prepared at acidic pH and low ionic strength contained Aβ1–42 tetramer, trimer, and monomer (Fig. 5B, lane 1). The addition of 150 mM NaCl at acidic pH corresponded to a slight decrease in trimer and a slight increase in large oligomers (Fig. 5B, lane 2). When Aβ1–42 was incubated at neutral pH and low ionic strength, a prominent smear of large oligomers was detected, in addition to Aβ tetramer, trimer, and monomer. The addition of 150 mM NaCl at neutral pH resulted in a decrease in the large oligomers (Fig. 5B, lane 4) relative to the same sample incubated without additional NaCl. The relative similarity between acidic pH samples analyzed by Western blot indicates that most of the dramatic conformational differences detected by AFM cannot be detected by Western analysis of SDS-PAGE.

Solubility of Aβ1–42 Assemblies—Sedimentation analysis has been used previously (53, 54) to characterize the aggregation state of Aβ under a variety of conditions. In the present study, the solubilities of unaggregated Aβ1–42, oligomers, and fibrils (Fig. 2) and the fibrillar aggregates (Fig. 5A, acidic pH and physiologic ionic strength) were estimated after centrifugation for 30 min at 16,000 and 100,000 × g. The soluble Aβ1–42 structures remaining in the supernatants were characterized by AFM. Unaggregated Aβ1–42 total and supernatant fractions (Fig. 6) contained structures that measured 1.0 (± 0.3) nm (Fig. 6, insets) and very few oligomeric assemblies. The total oligomeric and fibrillar Aβ1–42, as well as the 16,000 and 100,000 × g supernatants (Fig. 6), were comparable in size and morphology to the assemblies described for Fig. 2A. A decrease in the number of fibrils was observed in the 100,000 × g supernatant fraction, indicating that these fibrils may eventually sediment with prolonged ultracentrifugation. In contrast, fibrillar aggregates could not be detected by AFM in either 16,000 or 100,000 × g supernatants. No unaggregated or oligomeric structures were detected in the fibrillar aggregate 100,000 × g supernatant fraction at high resolution by AFM (Fig. 6, inset).

Approximately 90% of the total Aβ peptide remained in the supernatants when unaggregated, oligomeric, and fibrillar preparations were centrifuged at 16,000 × g for 30 min (Table II). Fibrillar aggregates were more insoluble yielding ~23% recovery from the 16,000 × g supernatants. Unaggregated and oligomeric preparations remained in the supernatant at 100,000 × g, whereas the fibrillar conformations began to sediment with ~51% remaining in the supernatant. Fibrillar aggregates completely sedimented at 100,000 × g. These percent-recovery results (Table II) agree with the presence of unaggregated, oligomeric, and fibrillar assemblies detected by AFM in supernatant fractions (Fig. 6). In addition, the absence of fibrillar aggregates in 16,000 and 100,000 × g supernatants (Fig. 6) coincides with low percent-recovery data (Table II). Taken together, these data indicate that unaggregated, oligomeric, and to some extent fibrillar, preparations of Aβ1–42 are soluble and that fibrillar aggregates formed at physiologic ionic strength and acidic pH are insoluble.

Aβ1–40 Requires Longer Incubation Times to Form Oligomers and Fibrils Comparable with Aβ1–42 Oligomers and Fibrils—Aβ1–40 is the predominant species of Aβ produced from the variable C-terminal cleavage of APP. The oligomer- and fibril-forming conditions defined for Aβ1–42 were applied to Aβ1–40. AFM characterization of Aβ1–40 immediately upon dilution reveals a fairly homogeneous field of unaggregated peptide structures (Fig. 7A). High resolution scans detect dense
fields peptide measuring 1.0 (±0.3) nm (Fig. 7A, insets for 0 h). No significant changes in Aβ1–40 assembly state could be detected under the Aβ1–42 oligomer- or fibril-forming conditions by AFM after 24 h (Fig. 7A), although some short, ~4-nm-diameter Aβ1–40 fibrils and 2–4-nm oligomers were detected under fibril-forming conditions. Both Aβ1–40 solutions were allowed to incubate for 6 weeks. At this time point, ~4-nm assemblies comparable in size to 24-h Aβ1–42 fibrils in Fig. 2A were detected under oligomer-forming conditions (Fig. 7A). At 6-weeks, solutions of Aβ1–40 incubated under fibril-forming conditions contained several 2–6-nm oligomeric assemblies and ~4–6-nm-diameter fibrils (Fig. 7A), slightly larger than 24-h Aβ1–42 fibrils (~4 nm) in Fig. 2A.

Western blot analysis of SDS-PAGE for Aβ1–40 incubated under oligomer (Fig. 7B, lanes 1 and 2)- and fibril-forming conditions (Fig. 7B, lanes 3 and 4)-forming conditions revealed Aβ tetramer and monomer in both preparations at 0 (Fig. 7B, lanes 1 and 3) and 24 (Fig. 7B, lanes 2 and 4)-h time points. Faint bands of larger oligomers between 30 and 60 kDa are present in both 24-h samples, and a faint smear centered at ~60 kDa can be detected in the 24-h sample incubated under fibril-forming conditions. Western blot analysis of long-aged Aβ1–40 under oligomer-forming conditions (Fig. 7B, lane 5) reveals monomer and a smear around ~60 kDa. Long-aged Aβ1–40 solutions incubated under fibril-forming conditions (Fig. 7B, lane 6) contain a faint monomer band, a smear around ~60 kDa, and large aggregates >60 kDa extending up to the well of the gel.

### DISCUSSION

The biological roles that Aβ plays in normal humans or in those that suffer from AD remain unclear. Definitive genetic studies, as well as a large number of in vitro and in vivo experiments, provide compelling evidence that Aβ1–42 is a direct causal agent for AD. What remains unclear is the relevant conformational assembly(s) of Aβ1–42 associated with the disease pathology. Early studies provided evidence that Aβ had to assemble into a fibrillar conformation to induce significant levels of neurotoxicity in vitro (55–57) or Aβ-induced tau phosphorylation (58). Recently, a number of studies have uncovered both in vitro and in vivo biological activities associated with soluble oligomeric and protofibrillar conformations of Aβ, challenging the idea that fibrillar amyloid is the causative pathogenic agent in AD. The first studies that characterized soluble oligomeric Aβ (18), a dimer-containing Aβ1–42 fraction (15), ADDLs (amyloid-derived diffusible ligands) (19), and protofibrils (51, 59) introduced the concept that amyloid fibrils are not the only biologically relevant Aβ conformation.

In the present study, Aβ1–42 pretreated in HFIP and solubilized in Me2SO produced uniform peptide solutions that were free from the structural history present in commercially available lyophilized stocks, laying the critical foundation for controlled aggregation studies. From this structurally and chemically identical starting material, conformationally distinct populations of oligomeric and fibrillar Aβ were produced by controlling the incubation time, concentration, temperature, pH, ionic strength, and Aβ species. Incubation time had a moderate effect on Aβ1–42 conformation in that the majority of the structures that formed within 24 h remained for up to 1 week. Concentration also had a moderate effect on conformation, particularly fibril formation, as the critical concentration for fibrillogenesis under these conditions appears to be between 10 and 50 μM, whereas oligomer formation can proceed at much lower concentrations (Fig. 3). Temperature also appeared to influence the rate of fibril formation to a greater extent than oligomer formation. Oligomer and protofibril formation was favored at neutral pH, and fibril formation was favored at acidic pH. Ionic strength had a significant effect on conformation, particularly the coalescence of soluble fibrillar Aβ1–42 into compact insoluble fibril aggregates at 150 mM NaCl. When these oligomer- and fibril-forming conditions for Aβ1–42 were applied to Aβ1–40, significantly longer periods of time were required for oligomeric and fibrillar structures to form that resembled those formed by Aβ1–42 within 24 h. Taken together, these results define specific conditions for reproducibly controlling the formation of unaggregated, oligomeric, fibrillar, and fibrillar aggregate conformations of Aβ1–42.

### In vitro biological activity studies

In vitro biological activity studies have demonstrated that oligomeric (17–19, 21) and protofibrillar (20, 60) conformations of Aβ are neurotoxic and impair electrophysiologic activity. Although experiments using a single conformation of Aβ can be informative, comparative experiments using multiple conformations of Aβ derived from a single starting material will further our understanding of how structure affects function. Using this approach, we demonstrated recently (24) a significant difference in neurotoxicity among unaggregated, oligomeric, and fibrillar Aβ1–42. Oligomers inhibited neuronal viability 10-fold more than fibrils and ~40-fold more than unaggregated peptide, with oligomer-induced toxicity significant at 10 nM. In addition, we were able to differentiate by structure and neurotoxic activity wild-type Aβ1–42 from isoforms containing known familial mutations (24). An in vitro comparative analysis using these preparations also demonstrated a significant increase in gial activation by Aβ1–42 oligomers compared with fibrils, as measured by morphological changes and increased expression of inflammatory markers including nitric oxide, inducible nitric-oxide synthase, and interleukin-1β.

The variability in Aβ activity reported in the literature is likely because of the structural heterogeneity present in com-

### TABLE II

**Quantitation of the solubility of Aβ1–42 assemblies**

<table>
<thead>
<tr>
<th>% Recovery Unaggregated</th>
<th>Oligomers</th>
<th>Fibrils</th>
<th>Fibrillar aggregates</th>
</tr>
</thead>
<tbody>
<tr>
<td>16,000 × g</td>
<td>89 (±4)</td>
<td>94 (±5)</td>
<td>85 (±1)</td>
</tr>
<tr>
<td>Supernatant</td>
<td>10 (±4)</td>
<td>5 (±4)</td>
<td>14 (±2)</td>
</tr>
<tr>
<td>Pellet</td>
<td>91 (±5)</td>
<td>91 (±7)</td>
<td>51 (±8)</td>
</tr>
<tr>
<td>100,000 × g</td>
<td>15 (±4)</td>
<td>6 (±5)</td>
<td>45 (±10)</td>
</tr>
</tbody>
</table>

*Significantly different from comparably treated unaggregated sample (p < 0.05).
Fig. 7. Aβ1–40 requires longer incubation times to form oligomers and fibrils comparable with Aβ1–42 oligomers and fibrils. A, AFM. Oligomeric and fibrillar Aβ1–40 samples prepared as described in the legend for Fig. 2 were mounted for AFM analysis immediately after dilution (0-h), 24 h, and 6 weeks. Representative 2 × 2-μm x-y, 10-nm total z-range AFM images are shown. Inset images, 200 × 200-nm x-y, 2-nm total z-range. B, Western analysis of SDS-PAGE. Representative Western blots of Aβ1–40 samples prepared as described in A were analyzed as described in the legend for Fig. 2. Oligomers and fibrils were sampled at 0 h (lanes 1 and 3), 24 h (lanes 2 and 4), and 6 weeks (lanes 5 and 6), respectively.

Commercial preparations that are chemically identical. Commercial sources of synthetic Aβ suggest resuspension in either H2O or Me2SO, and these solubilization procedures are reported throughout the literature. In this study, AFM analysis of lyophilized Aβ stocks directly resuspended in H2O detected a wide variety of structures including oligomers, fibrils, and globular aggregates. These structures could seed further aggregation, strongly influencing the formation and biological activity of the resulting solutions. Seed-free preparations of Aβ have been described using the polar solvent Me2SO, in conjunction with bath sonication and/or filtration (61, 62). Surprisingly, in this study AFM analysis of Aβ solutions in Me2SO that appeared initially uniform and unaggregated revealed fibril formation over time. Previous studies characterizing the formation of Aβ protofibrils (59) and oligomers (63) have emphasized the importance of removing preexisting seeds from Aβ stocks by size-exclusion chromatography. Although this approach appears to be successful, it is not always practical for the routine preparation of the large number of small peptide aliquots frequently required for biological assays or screening. Centrifugation is another method commonly used to separate soluble Aβ from insoluble fibrillar amyloid in both extracted tissue (64, 65) and in vitro aggregation preparations (66, 67). In this study, the fibrillar aggregates formed at acidic pH and physiologic ionic strength were insoluble and sedimented upon centrifugation at both 16,000 × g and 100,000 × g (see Fig. 6 and Table II). However, fibrillar preparations of Aβ1–42 remained in the supernatant at 16,000 × g and only partially sedimented at 100,000 × g (see Fig. 6 and Table II). Although these results confirm the solubility of unaggregated and oligomeric Aβ1–42 preparations, and the insolvibility of fibrillar aggregates, the importance of differentiating between preparations defined as “soluble” versus those that are truly “fibril-free” is emphasized by the solubility of fibril preparations. The Aβ structural conformations that formed under the conditions used in this study were readily discriminated by AFM, whereas Western blot analysis of SDS-PAGE provided only limited information. In light of the structural differences detected by AFM, it is clear that some of the Aβ1–42 and Aβ1–40 assemblies that form under the oligomer- and fibril-forming conditions used in this study dissociate during SDS-PAGE. In particular, the distinct structural morphologies in Aβ1–42 resulting from changes in pH and ionic strength detected by AFM (Fig. 5A) could not be differentiated clearly by Western blot analysis of SDS-PAGE (Fig. 5B). In addition, the electrophoretic process and the combination buffer, glycerol, and detergent present in SDS sample buffer may actually induce the formation of SDS-stable oligomers as reported previously (68). This may account for the SDS-stable oligomers, trimers, and tetramers detected in preparations of Aβ1–40 and Aβ1–42 that appeared unaggregated by AFM (see Figs. 2, 6, and 7). In addition, when distinct Aβ1–40 oligomers and fibrils were detected by AFM, no SDS-stable oligomeric bands were present. Despite these limitations, some general trends were observed between Western blot analysis of SDS-PAGE of Aβ1–42 preparations incubated under oligomer- and fibril-forming conditions. Oligomers produced slightly more SDS-stable tetramer and large oligomer bands than fibrils, and both conditions showed more SDS-stable trimer and tetramer than unaggregated peptide. Under fibril-forming conditions, the amount of SDS-stable trimer and tetramer appears to vary and may be in equilibrium with unstable assemblies that break down to monomer, the primary conformation observed in fibril preparations in our previous study (24). An indication of this molecular rearrangement between monomer and tetramer was indicated by the pronounced boundary and smear between these bands (Fig. 2B, lanes 2 and 4). These intermediates appear unstable during SDS-PAGE and form under both oligomer- and fibril-forming conditions. Interestingly, unaggregated preparations (Fig. 2B, lanes 1 and 3) that did contain some trimer and tetramer did not contain a smear between tetramer and monomer.

A more accurate representation of the molecular stoichiometry of the different assemblies as they exist in solution may require covalent cross-linking. This strategy was used recently (69) to demonstrate the complexity and dynamic nature of the prenucleation phases of Aβ1–40 assembly and specifically detected oligomeric intermediates that were not SDS-stable. Further evidence that SDS-PAGE alone cannot detect Aβ1–40 monomer-oligomer distribution as it exists in solution comes from studies using analytical ultracentrifugation (63) and mass spectrometry (70). These studies, like the covalent cross-linking study, revealed a more complex, dynamic equilibrium be-
tween monomer and oligomer. Thus, it does not appear possible to equate an aggregate conformation of Aβ1–40 or 1–42 as visualized by AFM to a particular molecular weight band from Western blot analysis of SDS-PAGE.

In general, ionic interactions are influenced by pH. In this study, solution pH significantly affected both oligomer and fibril assembly. Acidic pH has been shown previously (36, 71, 72) to favor fibril formation, possibly resulting from a partial denaturation state similar to that observed with other amyloid-β-precursor protein forms (73). This may be the result of protonation of the C-terminal carboxyl, N-terminal Asp3, Glu28, Asp34, His3, His24, or other Asp, Glu, or His residues. These charged amino acids may be involved in the stabilization of the local secondary structure that facilitates fibrillogenesis. Studies of modified Aβ1–40, in which Asp or His residues were replaced by Asn and Gln, demonstrated that a strong relationship exists between the protonation state at these positions and α-helical content (74). Fibrillogenesis is thought to proceed through an α-helical intermediate, and stabilization of local helical structure can accelerate this process (40). This may account for the very rapid conversion of unassembled Aβ1–40 to extended fibrils within 24 h of incubation at acidic pH as observed in this study.

In addition to ionic interactions influenced by solution pH, hydrophobic interactions also appear to have a significant effect, particularly on fibril formation. At low ionic strength, Aβ1–42 fibrils form but remain as individual extended structures for at least a week (Fig. 3B) and are resistant to sedimentation (see Fig. 6 and Table I). However, the salt-dependent coalescence of Aβ1–42 fibrils into supramolecular fibril aggregates (see Fig. 5A and Fig. 6) argues that exposed hydrophobic patches present on the surface of Aβ1–42 fibrils can drive the formation of dense, insoluble fibrillar aggregates. Alternatively, this process may be the result of polar interactions screened by the increased ionic strength, although it is more likely that the fibrillar aggregates resulted from non-polar surface interactions. Interestingly, the presence or absence of 150 mM NaCl did not have as significant of an effect on the coalescence of primarily oligomeric assemblies formed at neutral pH (Fig. 5A), indicating that these oligomeric assemblies may have already sequestered non-polar surfaces by adopting a favorable conformation.

Further evidence supporting the role of hydrophobic interactions comes from the dramatic differences between Aβ1–42 and Aβ1–40 conformations that were observed by AFM under the Aβ1–42 fibril- and oligomer-forming conditions. The additional hydrophobic Ile41-Ala5 residues found at the C termini of Aβ1–42 were necessary for the fibril and oligomer formation within 24 h as detected by AFM, as well as the different patterns of SDS-stable assemblies detected by Western blot analysis of SDS-PAGE. Therefore, these hydrophobic residues in Aβ1–42 appear to play an important role in vitro in the formation of stable oligomers fibrils. These findings provide evidence that hydrophobic forces play an important role in both differentiating Aβ1–40 and Aβ1–42 species and in the formation of oligomeric and fibrillar aggregates of Aβ1–42.

Polarity changes induced by protein oxidation may influence both ionic and hydrophobic interactions. In particular, oxidation of Aβ at methionine 35 alters the polarity at that position and may influence hydrophobic or ionic interactions necessary for fibril formation (28). In this recent study, AFM analysis differentiated Aβ1–42 solutions containing mixed short fibrils from Aβ1–42Met35–54 solutions containing only small, non-fibrillar aggregates. These results further emphasize how the distribution of oligomeric and fibrillar Aβ1–42 assemblies is affected by factors that influence molecular interactions.

The physical presence of amyloid plaques in vivo demonstrates that at some point, favorable conditions exist for their formation. However, in vitro fibril formation occurs in solution conditions and at Aβ concentrations that are decidedly non-physiologic. The trends observed in this study suggest that at lower concentrations and under physiologic solution conditions Aβ1–42 adopts a favorable, stable oligomeric conformation. At higher peptide concentrations and elevated temperature under oligomer-forming conditions, soluble oligomeric, protofibrillar, and short fibrillar conformations coexist, indicating that these structures may be in a conformational equilibrium. However, it is unlikely that these soluble assemblies represent on-pathway fibril intermediates under the oligomer-forming conditions used in this study. If this were the case, then increasing the peptide concentration, increasing temperature, or extending the incubation time would result in the rapid conversion of oligomeric, protofibrillar, and short fibrillar conformations to mature fibrillar conformations >1 μm in length. This result was not observed (see Fig. 3A and Fig. 4A). In vitro studies characterizing Aβ protofibrils as metastable fibril precursors demonstrated that in the absence of fibrillar seeds, the conversion of protofibrils to mature amyloid fibrils is a slow process (26). Additionally, the authors noted a disappearance of protofibrils that preceded the appearance of mature fibrils (26, 51), and in vitro dialysis experiments using radiolabeled Aβ indicate that protofibrils are in equilibrium with low molecular weight Aβ (60). Whether in vivo oligomeric and protofibrillar forms of Aβ1–42 represent stable end point assemblies that dissociate to monomer before proceeding to form mature fibrils via a nucleation-dependent polymerization mechanism remains to be determined. Amyloid deposits that form in vivo may result from microenvironments where nucleation-dependent polymerization can proceed and are only indirectly responsible for neurodegeneration and cognitive impairment in AD. However, a second and potentially more biologically significant soluble pool of amyloidogenic and/or protofibrillar Aβ favored by Aβ1–42 may play a more direct and deleterious role in AD pathology. These remaining mechanistic questions can be addressed by additional kinetic studies.

Evidence in the literature continues to build supporting a central causative role for Aβ in the neuropathology of AD. With increased significance comes an increased need for a better understanding of how Aβ structure affects function. The in vitro experiments reported in this study provide unique insights into the conditions that control the complex process of Aβ assembly by utilizing a single, homogenous starting material to produce multiple, distinct conformational species by varying the incubation conditions. Understanding the assembly and biological activity of both amyloidogenic and fibrillar forms of Aβ is important both mechanistically and because it influences whether therapeutic strategies in AD are best aimed at eliminating oligomeric assemblies of Aβ or plaque deposition. Furthermore, therapeutic approaches solely focused on fibril destabilization may have the undesirable side effect of increasing a soluble pool of neurotoxic Aβ oligomers and protofibrils. Experiments that take into account Aβ conformational differences will yield more consistent and interpretable results, providing the biological infrastructure for developing successful therapeutics for the treatment of AD.

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REFERENCES
