

Amyloid β -peptide polymerization studied using fluorescence correlation spectroscopy

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Background: The accumulation of fibrillar deposits of amyloid β -peptide (A β) in brain parenchyma and cerebrovasculature is a key step in the pathogenesis of Alzheimer's disease. In this report, polymerization of A β was studied using fluorescence correlation spectroscopy (FCS), a technique capable of detecting small molecules and large aggregates simultaneously in solution.

Results: The polymerization of A β dissolved in Tris-buffered saline, pH 7.4, occurred above a critical concentration of 50 μ M and proceeded from monomers/dimers into two discrete populations of large aggregates, without any detectable amount of oligomers. The aggregation showed very high cooperativity and reached a maximum after 40 min, followed by an increase in the amount of monomers/dimers and a decrease in the size of the large aggregates. Electron micrographs of samples prepared at the time for maximum aggregation showed a mixture of an amorphous network and short diffuse fibrils, whereas only mature amyloid fibrils were detected after one day of incubation. The aggregation was reduced when A β was incubated in the presence of A β ligands, oligopeptides previously shown to inhibit fibril formation, and aggregates were partly dissociated after the addition of the ligands.

Conclusions: The polymerization of A β is a highly cooperative process in which the formation of very large aggregates precedes the formation of fibrils. The entire process can be inhibited and, at least in early stages, partly reversed by A β ligands.

Introduction

Deposition of amyloid β -peptide (A β) fibrils in brain parenchyma and vasculature is an invariable feature of Alzheimer's disease [1–3]. There is substantial evidence that the formation of these fibrillar deposits is a central event in the disease pathology. For instance, all known mutations that segregate with familial Alzheimer's disease lead to increased production of A β ^{1–42} and deposition of amyloid plaques early in life [4–9]. Transgenic mice expressing these mutated genes show neuropathological lesions similar to those found in Alzheimer's disease [10,11], and A β has been shown to become toxic to cells upon polymerization [12–14]. Consequently, the nature of the polymerization process and its potential inhibition are relevant topics to study, and small molecules capable of binding to A β are potential drugs for the prevention and treatment of the disease [13,15–19].

The polymerization process has been studied *in vitro* using different techniques, including turbidimetry [20–23], light scattering [23–28], analytical ultracentrifugation [20], circular dichroism spectroscopy (CD) [29–31],

electron microscopy (EM) [32], atomic force microscopy [33–35], polyacrylamide gel electrophoresis (PAGE) [29,36,37], size-exclusion chromatography (SEC) [25,38] and quantitative fluorimetry [32,39]. Only the spectroscopic techniques are able to measure the polymerization process directly in solution. So far, quasielastic light scattering has provided the most detailed description of this process [27,28]. In order to follow the polymerization of A β in solution we used fluorescence correlation spectroscopy (FCS). In FCS, the fluorescence of single dye-labeled molecules excited by a sharply focused laser beam is observed. From the intensity fluctuations, which are due to varying numbers of molecules in the volume element of observation, the average number of molecules can be obtained directly using an intensity correlation function. Components with different molecular weights and correspondingly different diffusion times can be analyzed using FCS without prior separation [40–45]. The high sensitivity of FCS, which allows analysis in the nanomolar range and below, opens up various new possibilities in the analysis of molecular interactions. In this paper, FCS was used to follow the polymer-

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ization of A β in solution, observing monomers/dimers and aggregates of different sizes simultaneously. FCS was also applied to investigate how the presence of A β ligands affects the polymerization process. The ligands used were peptides capable of binding to the Lys-Leu-Val-Phe-Phe (A β ^{16–20}) sequence, previously shown to be critical for A β A β binding and fibril formation [18,19]. Part of these results were reported previously in a thesis [46].

Results

FCS studies of A β polymerization using rhodamine-labeled A β as fluorescent probe

In the first set of experiments, the polymerization of A β ^{1–40} in Tris-buffered saline (TBS), pH 7.4, was studied with rhodamine-labeled A β as fluorescent probe. From the fluorescence intensity fluctuations of free rhodamine and of rhodamine-labeled A β ^{1–40}, the diffusion times (τ_D) were calculated using the autocorrelation function and found to be 40 μ s and 90 μ s, respectively. When compared with rhodamine (443 Da), which was used as a reference, the diffusion time of labeled A β corresponded to a molecular weight of about 5 kDa (theoretical value 4870 Da), assuming diffusion of spherical particles. There was no evidence to suggest that aggregates were present at the start of incubation. It has been shown that A β rapidly forms dimers in solution [28,38] and it is possible that the population of molecules observed in the freshly prepared solutions represents a mixture of monomers and dimers. Within the first 2 min after dissolution of A β ^{1–40} (80 μ M), no change in diffusion time was observed (Figure 1a–c), indicating that no aggregation occurred during this period. After 10 min plenty of large aggregates were detected and after 40 min the aggregation reached a maximum (Figures 1d–f and 2). After 40 min of incubation, the fluorescence intensity fluctuations exhibited several peaks with highly increased heights (Figure 1d). These peaks, with up to three times higher intensity than the baseline level, clearly represent Brownian motion of A β aggregates containing several rhodamine-labeled A β molecules. The diffusion time for these aggregates was found to be 3 s (Figure 1e). Similar diffusion times were obtained when the intensity autocorrelation functions (Figure 1b,e) were analyzed using the CONTIN algorithm [47,48] (Figure 1c,f). After 80 min of incubation the diffusion time of the aggregates had changed from 3 s to 0.3 s. No further changes in diffusion time and the amount of aggregates were detected between 2 h and 24 h (data not shown). From the diffusion times of around 0.3 s and 3 s, the hydrodynamic radii of equivalent spheres (Equation 5; see the Materials and methods section) were calculated to be 6 μ m and 60 μ m, respectively. For the 0.3 s species, the length of a rigid rod with a diameter of 100 nm (corresponding to the diameter of the fibril bundles detected using electron microscopy, EM, Figure 3c) was calculated to be 55 μ m (Equation 7; see the Materials and methods section).

Electron microscopy

Samples were prepared for EM after incubating solutions of A β for 1 min, 40 min or 1 day. No material was detected after 1 min of incubation, but after 40 min an amorphous network and short fibrils could be seen (Figure 3a,b). Mature fibrils, but no amorphous network or short fibrils, were shown to be present in the sample incubated for 1 day (Figure 3c). The fibrils were often arranged in bundles, about 100 nm wide and 5–20 μ m long, or in an irregular network. Without centrifugation preceding the EM analysis, only a few fibrils were detected, indicating that fibrils remained in solution and did not sediment to any major extent at 1 \times g. When 80 μ M A β ^{1–40} was incubated in the presence of 320 μ M LBMP1620 (a peptide capable of binding to A β ; see below), only a few occasional fibril bundles could be detected by EM, which instead revealed small amorphous aggregates (not shown).

The influence of concentration on A β polymerization

A β ^{1–40} was dissolved directly in 50 mM Tris-HCl and 150 mM NaCl (TBS) at concentrations ranging from 1 μ M to 300 μ M. The aggregation was strongly dependent on concentration and the critical concentration for rapid polymerization was found to be 50 μ M (Figure 4). Below this concentration only monomers/dimers were detected. Above this concentration the polymerization to large aggregates proceeded rapidly after a lag phase of a few minutes.

Quantification of peptides in supernatants using high performance liquid chromatography

Aliquots of 5 μ l were aspirated from the upper half of the FCS samples and 15 μ l formic acid was added (in order to dissolve aggregates) prior to injection into the reverse-phase column. The samples containing up to 100 μ M A β showed no significant decrease in A β concentration,

Figure 1

The polymerization of A β studied by FCS using rhodamine-labeled A β as fluorescent probe. A β (80 μ M) was incubated with rhodamine-labeled A β (10 nM). The fluorescence intensity fluctuations (due to rhodamine-labeled A β molecules diffusing through the volume element of observation) were measured after (a) 2 min and (d) 40 min. The corresponding intensity autocorrelation functions, $G(\tau)$, were calculated using Equation 1. The observed and calculated data points are completely overlapping. Their difference is shown as residuals underneath (Figure 1b,e). (b) The monophasic form of the curve indicates that the diffusion times for the different species present are similar. The diffusion time, τ_D , was found to be 90 μ s. (e) After 40 min of incubation, the calculated autocorrelation function has a biphasic appearance. Species with a very high diffusion time ($\tau_D = 3$ s), corresponding to very large aggregates, as well as the initial monomers/dimers ($\tau_D = 90$ μ s) can be observed. (c,f) The distribution of diffusion times, $P(\tau_D)$, was calculated from the autocorrelation function using the CONTIN algorithm (Equation 3). Note that only few, if any, intermediate-sized aggregates were present.

indicating that the decrease in large aggregates in those samples not was due to precipitation. Samples with over 100 μM A β and samples with ligand concentrations over 320 μM showed a decline in peptide concentration over time (data not shown).

The effect of A β ligands on polymerization

The experiments were repeated in the presence of peptides capable of binding to A β (and blocking its aggregation); LBMP1620, Lys-Leu-Val-Phe-Phe, Asn-Lys-Leu-Val-Phe-Phe-Ala, and leu-phe-leu-arg-arg (all D-amino

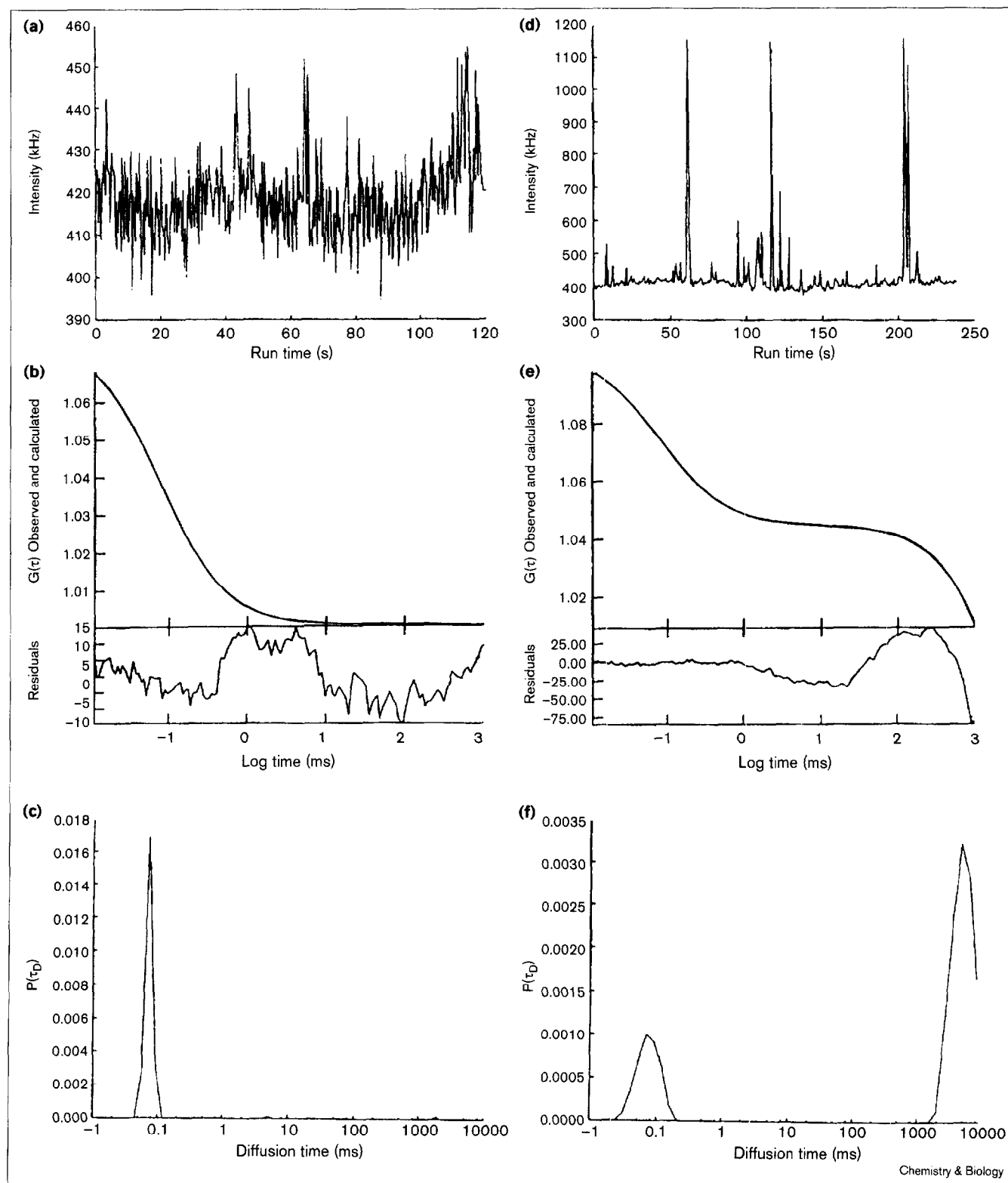
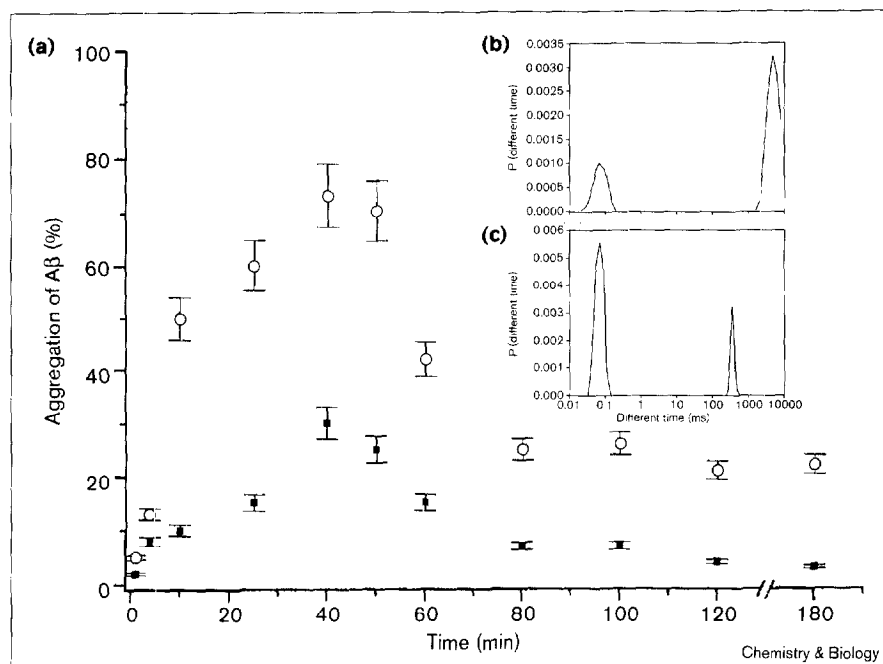


Figure 2



Time course of A β polymerization. A β (80 μ M) was incubated with rhodamine-labeled A β (10 nM) and the intensity fluctuations were measured at several time points ranging from 2 min to 24 h. The diffusion times and the percentage of A β aggregates were calculated by Equations 1 and 2. (a) The relative amount of aggregates was plotted as a function of incubation time (open circles). The effect of the A β -binding heptapeptide LBMP1620 on polymerization was studied by incubating 80 μ M A β and 10 nM rhodamine-labeled A β in the presence of 320 μ M LBMP1620 (filled squares). Distribution of diffusion times of A β aggregates after (b) 40 min and (c) 120 min incubation.

acids), and the nonbinding peptide Lys-Ala-Leu-Val-Phe-Phe-Ala. The heptapeptide LBMP1620 was found to inhibit aggregation most efficiently. A significantly lower amount of aggregates was formed in the presence of ligand. The effect of LBMP1620 on aggregation over time is shown in Figure 2. After 3 h of incubation the amount of aggregates was 20–25% in the absence of ligand (Figure 5b), and 3–4% in the presence of ligand (Figure 5d). Some of the aggregates, with diffusion times in the range of 1–20 ms, were not observed in the absence of ligand. The nonbinding peptide Lys-Ala-Leu-Val-Phe-Phe-Ala had no effect on the aggregation of A β (data not shown). When LBMP1620 was dissolved at a concentration of 320 μ M in a solution containing aggregated A β (80 μ M A β , aged for 1 h), a depolymerization process was initiated. The monomer/dimer concentration was ~95% after 2 h of additional incubation, with a concomitant disappearance of large aggregates (Figure 5c).

FCS studies of A β polymerization using labeled ligand as fluorescent probe

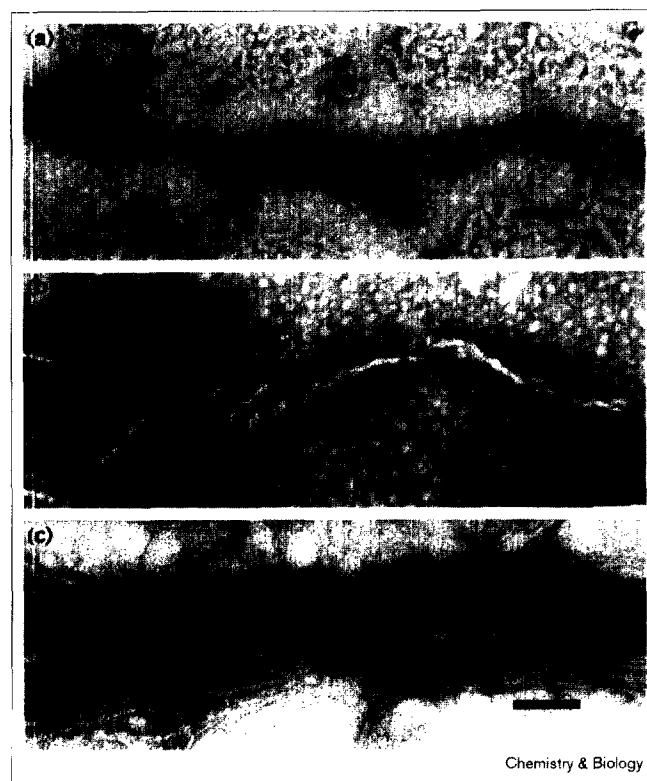
To investigate the binding characteristics of the A β ^{16–20} sequence, labeled ligand (rhodamine-Lys-Lys-Leu-Val-Phe-Phe) was used as fluorescent probe in one set of experiments. The diffusion time for labeled ligand dissolved in TBS, pH 7.4, was found to be 60 μ s, which corresponds to a molecular weight of 1.5 kDa (theoretical molecular weight = 1298 Da). As for A β , therefore, the ligand can exist as a mixture of monomers and dimers. When labeled ligand was incubated in the presence of 1 mM LBMP1620, no change in diffusion time was detected

(data not shown). When solutions containing A β (100 μ M) were incubated in the presence of labeled ligand, large aggregates were observed. The diffusion times of these aggregates were found to be the same as those detected with labeled A β (Figure 5a,e). When LBMP1620 was dissolved at 400 μ M in an aggregated solution of 100 μ M A β (aged for 1 h), the diffusion time changed from 3 s to 0.1 ms, and no aggregates ($\tau_D = 3$ s) were observed after incubation with the ligand (Figure 5f). When labeled A β ^{1–40} was used, aggregates could still be observed after the addition of an excess of LBMP 1620 (Figure 5c). This difference (compare Figure 5c with 5f) indicates that the labeled ligand is displaced by LBMP 1620.

Circular dichroism spectroscopy

In order to further characterize the starting material and to follow possible changes in A β conformation during the formation of the large aggregates detected with FCS, CD analysis was performed. CD spectra taken at 20 and 80 μ M A β , which corresponds to concentrations below and above the cooperative transition as observed by FCS (Figure 4), were similar, both showed typical disordered conformation (random coil; Figure 6a). Also, under conditions that show the time-dependent aggregation process by FCS (Figure 2), no changes occurred in the CD spectrum during incubation between 2 min and 24 h (Figure 6b, inset). Even in this case, the CD spectrum is of random-coil type. CD spectra are recorded in the same buffer as used in FCS experiments (TBS). For comparison, spectra are also recorded in phosphate buffer, which allows the CD spectra to be analysed in a low wavelength range (Figure 6b).

Figure 3

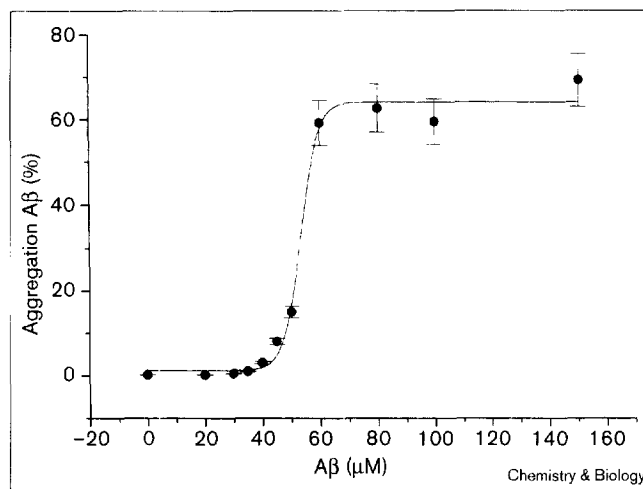


Electron microscopic examination of samples obtained after incubation. A β (80 μ M) was dissolved and incubated in TBS, pH 7.4, for 40 min or 1 day and then centrifuged at 20,000 \times g for 20 min. The resulting pellet was resuspended in water by brief sonication and 8 μ l of this suspension was placed on a grid. Excess fluid was withdrawn after 30 s, the grid was negatively stained with 3% uranyl acetate in water, and examined in a JEOL EM 100CX. After 40 min of incubation, (a) a diffuse network of aggregated A β was observed, possibly representing a precursor of (b) the diffuse fibrils detected on the same grid. (c) After one day of incubation, only mature fibrils of A β , often packed in thick bundles, were detected. It is suggested that the diffuse fibrils in (b) are precursors of these mature fibrils. Scale bars, 100 nm.

Discussion

Previous studies have provided limited information on the early (1–120 min) phase of A β polymerization, largely due to problems in measuring monomers, oligomers and large aggregates simultaneously. We choose to study A β polymerization using FCS, as this technique is not limited in this way. In addition, measurements are made directly in solution without any further sample preparation, minimizing the risk for artefacts. Using FCS, we found that the polymerization of A β rapidly proceeds from monomers/dimers into large aggregates without significant amounts of free oligomer at any stage of the process. The absence of oligomers suggests that such intermediates are short-lived species in the initial phase of the aggregation process. It is possible that oligomers are formed within the large aggregates, however. These oligomers would not be detected

Figure 4



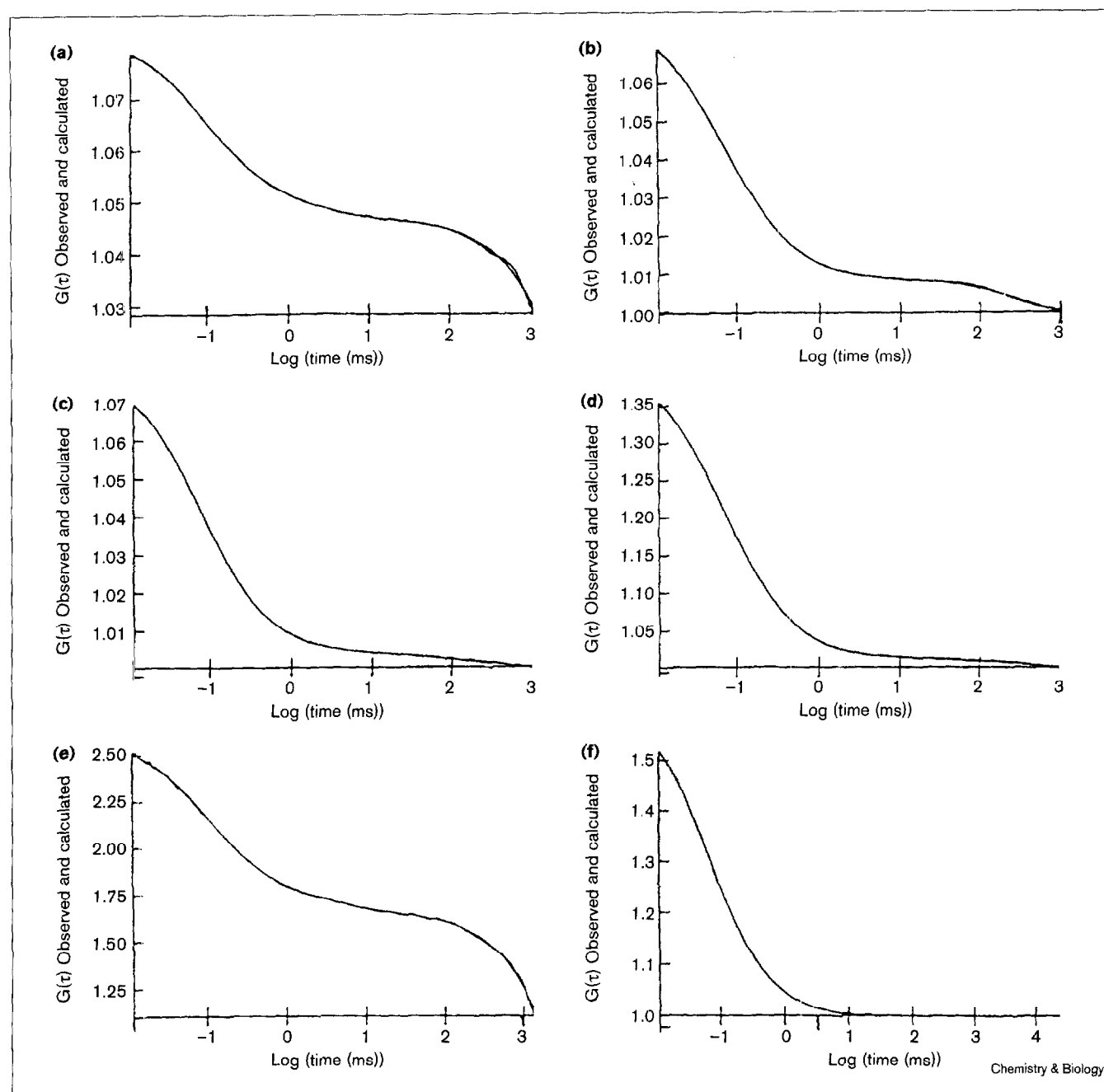
A β polymerization is strongly concentration dependent. Different concentrations of A β (20, 30, 35, 40, 45, 50, 60, 80, 100 and 150 μ M) were incubated with 10 nM rhodamine-labeled A β . After a 40 min incubation, the percentage of A β aggregates was calculated as described in Figure 2.

with FCS because they would be 'trapped' in the aggregates. Very low concentrations of free oligomer (not bound to the large aggregates) could have escaped detection. To investigate their possible existence, cross-correlation spectroscopy could be used [49].

The aggregation of A β reached a maximum after 40 min, followed by a decrease towards a steady-state equilibrium after 2 h (Figure 2). From the diffusion time of the aggregates, the hydrodynamic radius was calculated to be \sim 60 μ m. EM examination of samples incubated for 40 min revealed a network of amorphous structures and diffuse short fibrils. It is possible, therefore, that the large aggregates observed with FCS correspond to the amorphous networks seen in Figure 3a. The three-dimensional structure of such aggregates in solution is unclear, and, therefore, calculations that assumed shapes other than a sphere were not performed.

After incubation for 1 day, the diffusion time of the aggregates had decreased and corresponded to a hydrodynamic radius of \sim 6 μ m. Only mature fibrils, often arranged as networks or in bundles with a diameter of about 100 nm, were observed with EM (Figure 3c). The diffusion time obtained by FCS was used to calculate the length of a rigid rod with a diameter of 100 nm. This length was found to be around 55 μ m, somewhat larger than the lengths (about 5–20 μ m) of the fibril bundles as estimated from EM. The possibility that the arrangement of the fibrils is altered during preparation for EM cannot be excluded, however. When samples incubated for 1 day were fixed with glutaraldehyde,

Figure 5



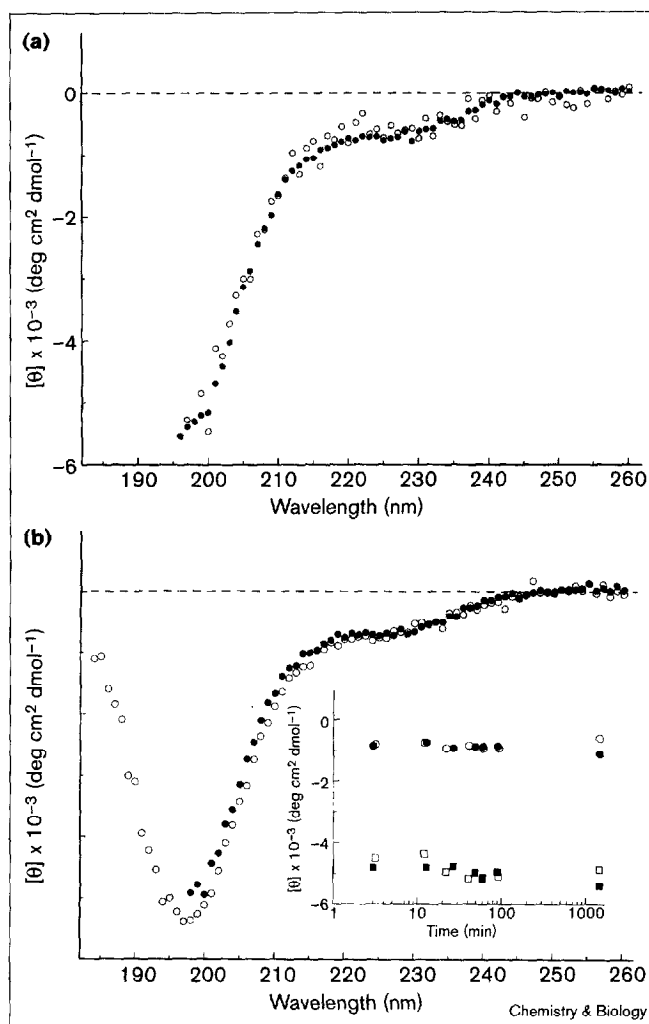
Dissolution of A β aggregates and displacement of rhodamine-labeled ligand with an excess of LBMP1620. Autocorrelation functions, $G(t)$ after incubation of A β (80 μM) and rhodamine-labeled A β (10 nM) for (a) 1 h (b) plus 2 h in the absence of or (c) in the presence of LBMP 1620 (320 μM). The proportion of large aggregates was found to be 20–25% and 5–6% after the additional 2 h of incubation without and with LBMP 1620, respectively. (d) If A β (80 μM) was dissolved in a solution containing LBMP 1620 (320 μM), only low amounts of aggregates (3–4%) were observed after 3 h of incubation.

(e) The polymerization process can be followed with rhodamine-labeled ligand. Here, 100 μM A β was incubated for 1 h with 10 nM rhodamine-labeled ligand as a fluorescent probe. The autocorrelation function was apparently identical with that obtained using rhodamine-labeled A β , compare (a) with (e). (f) Rhodamine-labeled ligand could be completely displaced by incubating the solution in (e) with an excess of LBMP1620 (400 μM) for 2 h. The observed and calculated data points are completely overlapping.

embedded in plastic, sectioned and stained, spherical and ellipsoid fibril aggregates with diameters in the range of

1–5 μm were observed by EM (data not shown). The size of these aggregates is consistent with the diameter calculated

Figure 6



(a) CD spectra of $A\beta^{1-40}$ at 20 (open circles) and 80 μM (filled circles) 90 min after dissolution in TBS, pH 7.4. (b) Comparison of the spectra of $A\beta^{1-40}$, at 80 μM in TBS, pH 7.4 (filled circles) and 10 mM PO_4 , 100 mM NaF, pH 7.4 (open circles) 90 min after dissolution. Time dependence of the CD signal in TBS, pH 7.4 (open symbols) and 10 mM PO_4 , 100 mM NaF, pH 7.4 (filled symbols) at 217 (circles) and 200 nm (squares) is shown (inset).

from the diffusion times obtained with FCS. It is possible therefore that the fibrils are present in solution as more or less spherical aggregates.

Taken together, the data suggest that the formation of the large aggregates, possibly corresponding to the networks in Figure 3a, is one of the first important steps in $A\beta$ fibril formation. The high local concentration of $A\beta$ in these aggregates increases the probability of $A\beta$ - $A\beta$ interactions, followed by energetically favourable conformational changes and fibril growth. Eventually, these networks either dissolve into monomers/dimers (Figure 2b,c) or proceed into diffuse short fibrils (Figure 3b) and further into mature

fibrils arranged in bundles (Figure 3c) or spherical aggregates. Findings similar to ours have recently been reported from electron spin resonance studies of the prion protein H1 peptide [50], in which it was also noticed that the fibrils were preceded by the formation of large amorphous aggregates, without any detectable amount of oligomers. The transient formation of very large aggregates could, therefore, be a general principle of amyloid assembly.

The early-stage aggregates observed using FCS have apparently escaped detection in previous light scattering and turbidimetric studies. Large aggregates of $A\beta$ (with apparent molecular weights of 10^6 Da and 10^{10} Da) that might correspond to those observed using FCS and EM have previously been detected using analytical ultracentrifugation, however [20]. Analogous experiments on transthyretin also provide evidence for the existence of large soluble aggregates that do not scatter light [51]. Light-scattering methods are dependent on the refractive index of the species to be studied, so aggregates with a refractive index close to that of the aqueous buffer are not observed. FCS is not limited in this way. CD spectroscopy provides information about protein secondary structure and the time scale of the analysis is compatible with that of the early phase of $A\beta$ polymerization. We could not detect any changes in secondary structure with CD spectroscopy within the first 24 h after dissolution of $A\beta$ at 80 μM . The formation of the early large aggregates observed by FCS and EM was not revealed by CD analysis. Under the experimental conditions in which large aggregates of $A\beta$ peptide were found by FCS (Figures 1,2), as well as the cooperative transition from monomers/dimers to large aggregates (Figure 4), no changes were observed in the CD spectra. The CD spectra were of random-coil type. We have found conditions at high peptide concentrations (150–300 μM) in which a transition to a typical β type CD spectrum similar to that reported by Seelig's group [31] was observed, and which might be connected to the formation of fibrillar structures. Given the behaviour of the CD spectra it is clear that the early larger aggregation phase is not connected to a change in the secondary structure of the $A\beta$ peptide. The early larger aggregation phase can be envisaged as a transient stage preceding the fibril formation that might be linked to a concomitant β transition [31]. The finding that the Lys-Leu-Val-Phe-Phe peptide is able to dissolve the early $A\beta$ aggregates more easily than the fibrillar forms could be connected to this behaviour.

The aggregation of $A\beta$ was highly concentration dependent. No aggregates or oligomers were detected at concentrations below 50 μM $A\beta$ and a sharp increase in aggregation occurred between 50–60 μM (Figure 4), which is in the range of the critical concentration (10–100 μM) reported by others [27,52–54]. The sharp phase transition observed (Figure 4) and the fact that practically no intermediate components in the aggregation process could be detected

by FCS indicate a highly cooperative interaction, as predicted by the seeding theory [55]. This theory postulates the formation of a short-lived multimeric aggregate, called a seed or a nucleus, to be the rate-limiting step. When a seed has formed, usually depicted as an aggregate of about eight monomers, the polymerization proceeds rapidly into fibrils. Our data indicates that the large diffuse networks are formed first; formation of oligomeric seeds could be facilitated within these networks. Using quasielastic light scattering, other investigators suggest that A β can form micelles in solutions at low pH. The micelles, formed at a critical concentration of $\sim 100 \mu\text{M}$, are either dissolved or transformed into seeds for fibril formation [27,53]. Although the aggregates we observe are much larger than micelles, they could represent clusters of micelles, each micelle being a possible seed for a fibril.

Incubation of A β in the presence of ligands, capable of binding to the Lys-Leu-Val-Phe-Phe motif, has been shown to inhibit fibril formation [18,19]. One of these ligands, LBMP 1620, was labeled and shown to bind both to monomeric/dimeric A β and aggregates of A β . Addition of an excess of LBMP1620 was shown to compete with labeled ligand, indicating a saturable and specific binding to A β . LBMP 1620 was found to decrease the amount, and to change the size distribution of the A β aggregates. The effect was most pronounced when the ligand was present from the start of the incubation. Interestingly, there was also a significant decrease in the amount of aggregates when the ligand was added after 1 h of incubation (Figure 5), indicating that the early large aggregates can be partly dissociated. The small populations of aggregates with diffusion times between 1 and 10 ms correspond to hydrodynamic radii of 3–35 nm. This is in the range of the size of protofibrils observed using atomic force microscopy [34,35], aggregates reported using quasielastic light scattering [27,28], and the diffuse fibrils reported here. The aggregates formed in the presence of ligand were found to be small and amorphous when examined using EM (data not shown), however, indicating that the species observed with FCS have structures less ordered than those of protofibrils or diffuse fibrils. Apparently, LBMP1620 acts in an early stage of the polymerization process by inhibiting the formation of the precursor of mature A β fibrils.

Significance

Accumulation of fibrillar deposits of A β in the brain is a crucial step in the pathogenesis of Alzheimer's disease. A detailed understanding of the molecular interactions of A β will facilitate the development of fibrillation inhibitors with potential to delay or prevent progression of the disease. Fluorescence correlation spectroscopy (FCS) provides fast and specific analysis of A β polymerization, with high sensitivity over a wide size distribution range. The polymerization proceeds from monomers/dimers to

two populations of large aggregates, and fibrils are formed after a decline in the total amount of large aggregates, indicating a precursor-product relationship. Interfering with the formation of these large aggregates is therefore a potential strategy for inhibiting A β fibril formation. The effects of A β ligands can be studied with a fluorescently labeled ligand; FCS could therefore be used as a highly sensitive and specific competition assay to identify potential inhibitors of fibril formation. The appearance of A β aggregates in cerebrospinal fluid of Alzheimer's patients using FCS was reported recently and is suggested as a diagnostic tool [56].

Materials and methods

Materials

Rhodamine (tetramethylrhodamine-5-(and-6)-isothiocyanate) was purchased from Molecular Probes Europe BV, Leiden, The Netherlands. Synthetic A β ^{1–40} was obtained from David Teplow, the Biopolymer Laboratory at Harvard University. All other peptides (rhodamine-Lys-A β ^{1–40} (labeled only at the amino terminus in order to minimize the effects of the label), rhodamine-Lys-Lys-Leu-Val-Phe-Phe (labeled ligand), Lys-Lys-Leu-Val-Phe-Phe-Ala (LBMP1620), Lys-Leu-Val-Phe-Phe, Asn-Lys-Leu-Val-Phe-Phe-Ala, Lys-Ala-Leu-Val-Phe-Phe-Ala and the all D-amino acid peptide leu-phe-leu-arg-arg) were obtained from Research Genetics, Huntsville, AL, USA. Peptides were dissolved in 70% formic acid and purified on a polystyrene-divinylbenzene column (PLRP-S 150 \times 25 mm, Polymer Laboratories, Church Stretton, UK) using a water-acetonitrile gradient with 0.1% trifluoroacetic acid. The peak fraction, peptide concentration $\sim 30 \mu\text{M}$ in $\sim 30\%$ acetonitrile, was collected and immediately frozen. The purified peptides were lyophilized in the presence of dry ice (to avoid thawing and thus minimizing the risk of aggregation) and their identity and purity were checked using electrospray mass spectrometry. The mass spectrometer was a Quattro triple quadrupole (Micromass, Altrincham, UK) fitted with an electrospray interface and spectra were recorded in the positive ion mode in the mass range 700–1700 m/z. Purity was also checked using HPLC using a C18 column (150 \times 4.6 mm, Valco, USA). Purity was in all cases greater than 95%.

Fluorescence correlation spectroscopy instrumentation

FCS was performed with confocal illumination of a volume element of 0.23 femtolitres (fl) in an instrument as described previously [43]. As focusing optics a Zeiss Neofluar 63 \times NA 1.2 was used in an epi-illumination setup. For separating excitation from emission radiation a dichroic filter (Omega 540 DRL PO²) and a bandpass filter (Omega 565 DR 50) were used. Tetramethylrhodamine-labeled A β was excited with the 514.5 nm line of an Argon laser. The intensity fluctuations were detected by an avalanche photo-diode (EG & G SPCM 200) and were correlated with a digital correlator (ALV 5000, ALV, Langen, Germany). The ConfoCor instrument of Zeiss-Evotec (Jena, Germany), built according to the principles described previously [43], was used for the analysis as well. In this instrument a Neofluar 40 \times NA 1.2 is used. The volume element in the ConfoCor was 0.19 fl.

FCS data evaluation

The intensity autocorrelation function $G(\tau)$ was analyzed according to a model describing the diffusion of a defined number of molecular species (two or three). For molecules diffusing in a finite three-dimensional Gaussian element [43] $G(\tau)$ is given by:

$$G(\tau) = 1 + \frac{1}{N} \sum_{i=1}^{2,3} \frac{x_i}{1 + \frac{\tau}{\tau_{D_i}}} \sqrt{\frac{1}{1 + \left(\frac{\omega}{z}\right)^2 \frac{\tau}{\tau_{D_i}}}} \quad (1)$$

where N is the number of fluorescent molecules, x_i is the fraction of species i , with diffusion time τ_{Di} , ω (0.5 μm) the radius and z (2 μm) the length of the volume element. The fraction of aggregation (F) was determined by:

$$F = \frac{x_2 + x_3}{x_1 + x_2 + x_3} \quad (2)$$

A more detailed analysis will have to take into account that x_i is also dependent on the number of labeled A β molecules incorporated, as well as on the quantum yield.

In order to assess the validity of the presence of discrete aggregates we analyzed $G(\tau)$ representing the distribution of diffusion times according to a model of multiple components:

$$G(\tau) = 1 + \frac{1}{N} \sum_{i=1}^m \frac{P_i}{1 + \frac{\tau}{\tau_{Di}}} \sqrt{\frac{1}{1 + \left(\frac{\omega}{z}\right)^2 \frac{\tau}{\tau_{Di}}}} \quad (3)$$

where P_i represents the distribution of diffusion time τ_{Di} .

The diffusion time is related to the cross-section of the illuminated volume element and the diffusion constant D by:

$$\tau_D = \omega^2/4D \quad (4)$$

With the assumption that the studied species are spherical, the hydrodynamic radii, R_h , can be obtained from the Stoke–Einstein equation:

$$D = kT/(6\pi\eta R_h) \quad (5)$$

where η is the solvent viscosity, k is Boltzmann's constant and T is the absolute temperature. For the calculations we used $T = 293$ K and $\eta = 1$ cP (which corresponds to water).

For rod-like objects the translational diffusion constant D and rodlength L at given length/diameter, $p = L/D$, is obtained from [57]:

$$\frac{3\pi\eta LD}{kT} = \ln p + \gamma \quad (6)$$

Where $\gamma = 0.38$ is an end-effect correction at $p = \infty$ [57]. Substitution of Equations 4 and 6 yields

$$\tau_D = \frac{3\pi\eta\omega^2 L}{4kT(\ln p + \gamma)} \quad (7)$$

Thus, for a known diameter, for example, from EM of the fibril bundles, the corresponding length can be calculated from the diffusion time.

For parametrization and fitting of the autocorrelation function $G(\tau)$ non-linear least squares minimization according to the Marquardt algorithm [58] was used. For evaluation of the distribution $P(\tau_{Di})$ the CONTIN algorithm [47,48] was applied.

FCS experiments

Free rhodamine was used to calibrate the instrument and as a reference to estimate the molecular weights of the dissolved peptides. The lyophilized peptides were dissolved directly in a buffer containing 50 mM Tris-HCl and 150 mM NaCl (TBS), pH 7.4, to concentrations ranging from 1 μM to 300 μM . The effect of A β ligands on polymerization was investigated by first dissolving the ligand in TBS and then adding the solution to lyophilized A β . The effect of ligands on preformed aggregates was studied by incubating a solution of 80 μM A β for 1 h and then dissolving the ligand in this solution. The ratio of A β : ligand ranged from 1:1 to 1:10. The labeled probe, rhodamine-Lys-A β or

rhodamine-Lys-Lys-Leu-Val-Phe-Phe, was in all experiments added to the buffer before the unlabeled peptide was dissolved. The final concentration of labeled probe was 10 nM. Droplets (15 μl) of the samples were placed in a 8-well Nunc chamber (Nalge Nunc International, IL USA) covered with a lid, and analyzed for 30 s up to 10 min in the FCS instrument, at several time points of incubation, ranging from minutes to weeks. Both siliconized and non-siliconized sample chambers were used, without any detectable differences in results. All experiments were performed at 20°C.

Electron microscopy

The samples analyzed by FCS were centrifuged at 20,000 $\times g$ for 20 min, the supernatants were aspirated and the pellets were resuspended in 100 μl water by a brief sonication. In one set of experiments, the supernatants were aspirated without prior centrifugation. Aliquots (8 μl) of the resuspended material were placed on grids covered by a carbon-stabilized formvar film. After 30 s, excess fluid was withdrawn and the grids were negatively stained with 3% uranyl acetate in water. Finally, the specimens were examined and photographed in a JEOL EM 100CX instrument at 60 kV.

Quantification of supernatants using HPLC

Five μl of the samples were aspirated from the upper half of the solution. Prior to injection onto the HPLC column (Polymer Laboratories PLRP-S, 250 \times 4.6 mm, 5 μm , 300 Å), 15 μl formic acid was added in order to dissolve aggregates of A β . The UV detector was set to 254 nm and the calculations were based on peak heights.

CD spectroscopy

Lyophilized A β 1–40 was dissolved in either: TBS, pH 7.4, 10 mM PO₄, 100 mM NaF, pH 7.4, or 5 mM MOPS, pH 7.4. CD spectra were recorded at 25.0°C in 0.1 cm cuvettes using an Aviv 62 DS spectropolarimeter (Aviv Assoc. Inc, Lakewood, NJ) between 260 and 180 nm with a spectral resolution of 1 nm and a spectral bandwidth of 1.5 nm. Data at time points < 20 min were recorded using an averaging time of 1 s/point (scan time < 160 s/spectrum), between 20 and 40 min, 8 s/point and at times > 40 min, 24 s/point. Raw CD data were converted to mean residue ellipticity, θ , in units of deg cm² dmol^{−1}.

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