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Two Distinct Amyloid β -Protein ($A\beta$) Assembly Pathways Leading to Oligomers and Fibrils Identified by Combined Fluorescence Correlation Spectroscopy, Morphology, and Toxicity Analyses^{*S}

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Nonfibrillar assemblies of amyloid β -protein ($A\beta$) are considered to play primary roles in Alzheimer disease (AD). Elucidating the assembly pathways of these specific aggregates is essential for understanding disease pathogenesis and developing knowledge-based therapies. However, these assemblies cannot be monitored *in vivo*, and there has been no reliable *in vitro* monitoring method at low protein concentration. We have developed a highly sensitive *in vitro* monitoring method using fluorescence correlation spectroscopy (FCS) combined with transmission electron microscopy (TEM) and toxicity assays. Using $A\beta$ labeled at the N terminus or Lys¹⁶, we uncovered two distinct assembly pathways. One leads to highly toxic 10–15-nm spherical $A\beta$ assemblies, termed amylospheroids (ASPDs). The other leads to fibrils. The first step in ASPD formation is trimerization. ASPDs of ~330 kDa in mass form from these trimers after 5 h of slow rotation. Up to at least 24 h, ASPDs remain the dominant structures in assembly reactions. Neurotoxicity studies reveal that the most toxic ASPDs are ~128 kDa (~32-mers). In contrast, fibrillogenesis begins with dimer formation and then proceeds to formation of 15–40-nm spherical intermediates, from which fibrils originate after 15 h. Unlike ASPD formation, the Lys¹⁶-labeled peptide disturbed fibril formation because the $A\beta$ ^{16–20} region is critical for this final step. These differences in the assembly pathways clearly indicated that

ASPDs are not fibril precursors. The method we have developed should facilitate identifying $A\beta$ assembly steps at which inhibition may be beneficial.

Conversion of disease-specific amyloid proteins from the native forms into fibrillar assemblies is a common feature of a wide range of human pathologies, including neurodegenerative diseases such as AD,³ Parkinson disease, prion diseases, and the polyglutamine diseases (1–5). On the other hand, smaller, non-fibrillar assemblies, which might be early precursors in fibrillogenesis, have recently been considered to be more proximate mediators of neurotoxicity (1–5). However, it is unclear whether these originate from a linear process or a series of parallel processes involving different intermediates. Thus, ordering the assembly pathway(s) is essential for understanding disease pathogenesis and developing rational therapeutics, as inhibiting inappropriate step(s) could increase the level of the toxic assemblies (2, 5).

In AD, various forms of toxic $A\beta$ assemblies, ranging in mass from dimers to multimers of ~1 MDa, have been reported (6–14). These assemblies may play the key role in AD pathogenesis by causing synaptic impairment (12, 13, 15–17). Indeed, $A\beta$ dimers that induce synaptic impairment and not neuronal loss have been reported to be isolated from AD brains (18). In contrast, the molecular natures of the $A\beta$ assemblies that directly cause neuronal loss in human AD remain to be elucidated. Because neuronal loss causes cognitive deterioration in AD patients (19), we sought to isolate such $A\beta$ assemblies *in vivo*. As a first step, we prepared highly toxic 10–15-nm spherical $A\beta$ assemblies termed ASPDs using an *in vitro* assembly

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^SThe on-line version of this article (available at <http://www.jbc.org/>) contains supplemental "Experimental Procedures," equations, Table S1, Figs. S1–S8, and additional references.

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³ The abbreviations used are: AD, Alzheimer disease; $A\beta$, amyloid β -protein; FCS, fluorescence correlation spectroscopy; TEM, transmission electron microscopy; ASPD, amylospheroid; ADDLs, $A\beta$ -diffusile ligands; TMR, tetramethylrhodamine; ha, hamster monoclonal; PBS, Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+} ; IP, immunoprecipitation.

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system (10). ASPDs are not fibril intermediates because they are not incorporated into mature fibrils and continue to exist after fibril formation ceases (5, 10). They also differ from protofibrils and A β -derived diffusible ligands (ADDLs) in morphology and size (10) (see reviews in Refs. 5 and 20)). Recently, we have produced ASPD-specific antibodies and used them to selectively immunoprecipitate highly neurotoxic ASPDs from human AD brains (21), strengthening the hypothesis that ASPDs are effectors of neurodegeneration *in situ* in humans. We also found that ASPD concentration correlated with the pathological severity of AD (21). These findings suggest that native ASPDs might be a candidate for A β assemblies that directly cause neuronal loss in human AD brains. The immunoreactivity profile of ASPD-specific antibodies in comparison to that of anti-A β antibodies or of an anti-oligomer A11 antibody suggests that ASPDs are structurally distinct from dimers, ADDLs, and A11-reactive entities (21). Taken together, data extant suggest that distinct types of A β assemblies, with distinct neurotoxic activities, exist in the AD brain. Therefore, elucidation of the assembly state-neurotoxicity relationships of these assemblies is important for understanding AD pathogenesis.

Here, we use combined FCS, TEM, and toxicity analyses to address these issues and to provide a highly sensitive and reliable method for *in situ* monitoring of the assembly process. At present, the assembly process cannot be monitored directly *in vivo* and much higher sensitivity is required for reliably monitoring *in vitro* formation of nonfibrillar A β assemblies, which occurs at low protein concentration (5, 20, 22–25). Our approach should therefore be useful in studies of other amyloid proteins.

FCS involves autocorrelation of fluctuations of fluorescence intensity, which gives the average number and average diffusion time (*i.e.* molecular size) of fluorescent molecules as they diffuse through an illuminated volume (26). We employed FCS because FCS provides the highest sensitivity for detecting small assemblies in dilute solutions among available analytical techniques (small angle x-ray diffraction, ultracentrifugation, laser light scattering, etc.). In addition, FCS does not require physical separation of metastable assemblies, and its noninvasive character allows monitoring without perturbation of monomer-nonfibrillar assembly-fibril equilibria (27–29). Although FCS has been employed to detect A β aggregates in cerebrospinal fluid of AD patients (30) and the formation of large (>10⁴ kDa) fibrillar assemblies from A β ^{1–40} *in vitro* (29, 31), little is known about the formation of neurotoxic nonfibrillar A β assemblies. Here, by using combined FCS, TEM, and toxicity analyses, we uncovered two distinct assembly pathways, one leading to ASPDs and the other to fibrils. We also determined the mass (128 ± 44 kDa) and height (7.2 ± 2.6 nm) of the most toxic ASPDs. The observed differences in the assembly pathways clearly indicated that ASPDs are not fibril precursors.

EXPERIMENTAL PROCEDURES

Materials—ASPD-specific antibodies, a rabbit polyclonal rpASD1 ($K_d \approx 5$ pM) and a hamster monoclonal haASD1 ($K_d \approx 0.5$ pM), have been produced in our laboratory and recognize epitopes distinct from those present on dimers, A11 antibody-reactive 12-mers, or fibrils (21). The characteristics of these

ASPD tertiary structure-dependent antibodies are summarized in [supplemental Table S1](#).

Sample Preparations—Fluorescent probes, A β ^{1–40} site-specifically labeled with tetramethylrhodamine (TMR) either at the N terminus (NTR) or Lys16 (K16TR), were synthesized ([supplemental “Experimental Procedures”](#)). ASPDs were prepared *in vitro* from 50 μ M solutions of A β ^{1–42} (with or without 0.1 μ M NTR or K16TR; a probe ratio of 1/500) in F12 buffer without riboflavin, L-glutamine, and phenol red by slowly rotating the solutions at 4 °C for 16.5 h (10). Their quality was confirmed by dot blotting, TEM, and toxicity assays. Spherical assemblies 10–15 nm in diameter, with rare fibril-like structures, were usually produced as major components (10). Fibrils were prepared from 100 μ M solutions of A β ^{1–40} (with or without 0.1 μ M NTR or K16TR; a probe ratio of 1/1000) in 0.5 \times Dulbecco’s phosphate-buffered saline without Ca²⁺ and Mg²⁺ (PBS) at pH 3.5 by slowly rotating the solutions at 4 °C for 2 days. Fibrils without ASPDs were detected by TEM. A β concentration of each preparation was determined by quantitative amino acid analysis (Waters AccQ-Tag system) (10).

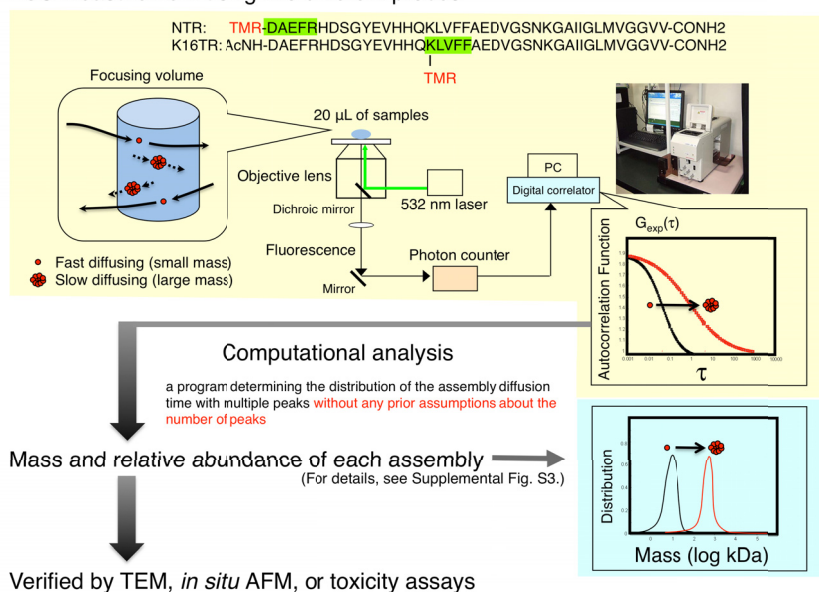
FCS—FCS was performed with a confocal volume element of 0.3 femtoliters using a preproduction prototype apparatus (Hamamatsu Photonics K.K.). The fluorescence intensity fluctuations were detected using a photomultiplier tube, and the autocorrelation function was calculated using a digital correlator (Fig. 1A; see also [supplemental “Experimental Procedures”](#)). Each sample (20 μ l) was measured at room temperature for 3 s \times 10 times using free rhodamine 6G (479 Da in mass, 2.8×10^{-10} m² s⁻¹ in the diffusion coefficient (D)) or Alexa Fluor 532 C5 maleimide (813 Da, 2.8×10^{-10} m² s⁻¹) as reference dyes.

FCS Data Evaluation—At the onset of the assembly process, A β solutions mainly contain rapidly diffusing A β assemblies ranging in mass from monomers to trimers. Some other assemblies may also be present, but their concentrations are unpredictable. Therefore, the constrained regularization program CONTIN (32, 33), combined with least-squares fitting, was utilized to determine the distribution of the diffusion times so that the mass of the rapidly diffusing A β assemblies fitted the dimer/trimer range (8–15 kDa), without any prior assumption about the number of the assembly types in solutions. From the determined distributions, the relative abundance and diffusion time of each assembly were calculated. By comparing the diffusion time of each assembly with that of reference dyes with known mass and diffusion coefficient, the mass (for spherical assemblies such as ASPDs) or the diffusion coefficient (D) (for non-spherical assemblies such as fibrils) was determined ([supplemental Fig. S3](#)). The details are described in the [supplemental “Experimental Procedures.”](#)

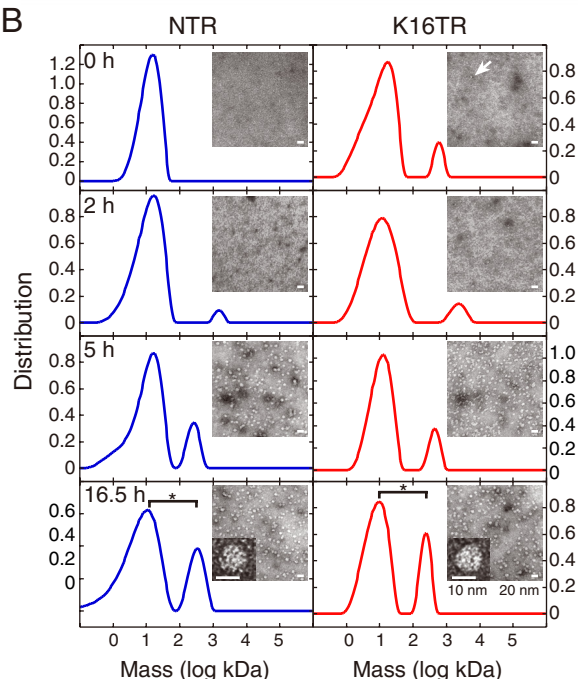
In FCS, the contribution of each component to the correlation curve is related to both its relative abundance and brightness (34). If all components have equal brightness, the relative abundance of each assembly can be obtained directly from the distribution of assembly diffusion time. This is true for ASPDs and early fibril intermediates, but not for fibrils containing more than one fluorophore. In the latter case, a rough estimate of the relative abundance of the fibrils was obtained from the distribution of the diffusion time (for details, see *“In Situ Monitoring of Fibril Formation”* under *“Results”*). To confirm the estimates thus obtained,

A

FCS measurement using two different probes



B



C

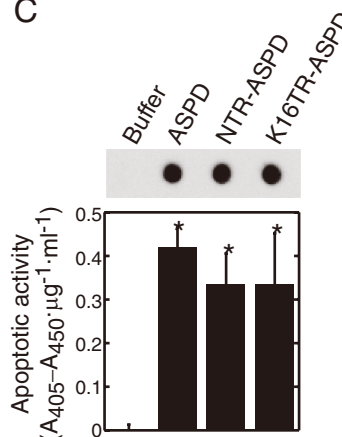


FIGURE 1. Time course of ASPD formation. *A*, schematic representation of FCS-based method. *B*, time course of ASPD formation. At the indicated time, aliquots were examined by TEM (*inset*) and FCS ($n = 10$). The distribution of assembly mass is shown (*, $p < 0.005$ by Scheffé post hoc test compared with the fast-diffusing assembly). Each distribution is normalized so that the total area becomes 1 (as described under "Experimental Procedures"). An *arrow* in TEM data indicates not-yet-dissolved amorphous aggregates. *C*, characterization of ASPDs formed with or without probes in dot blotting (1 pmol/dot) using 0.04 $\mu\text{g}/\text{ml}$ ASPD-specific rpASD1 antibody or specific apoptotic activity against rat primary septal neuronal cultures, determined by monitoring cytoplasmic histone-associated DNA fragments, and normalized by the amount of A β present (mean \pm S.D.; Games-Howell post hoc test. *, $p < 0.005$, $n = 6$; see supplemental "Experimental Procedures").

mature fibrils were separated in the retentate fractions of 0.1- μm filters (confirmed using TEM), and their amount was directly obtained from the fluorescence count using a fluorometer (Twin-*kle* LB970; Berthold Technologies GmbH).

Other Methods—Immunoprecipitation (IP), TEM, fluid-phase imaging of ASPDs by atomic force microscopy, toxicity assays, and statistics are described in the supplemental "Experimental Procedures."

RESULTS

Fluorescent Probes for FCS Measurements and Program for FCS Analysis—To monitor ASPD formation by FCS, we chemically synthesized fluorescent probes by labeling TMR site-specifically either at the N terminus (termed NTR) or at Lys¹⁶ (termed K16TR) of A β ^{1–40} (Fig. 1*A*). We consider TMR labeling at these sites to minimally affect ASPD formation because

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excess A β^{1-5} (DAEFR) or A β^{16-20} (KLVFF) had no effect on ASPD formation (supplemental Fig. S1), suggesting that amino acid residues around A β^{1-5} or A β^{16-20} are not involved in ASPD formation.

FCS was performed using a preproduction prototype apparatus (Hamamatsu Photonics K.K.) at 100 μ W optimum laser output power (supplemental Fig. S2). From FCS data, the relative abundance (%) and diffusion time of each A β assembly were obtained using CONTIN (32, 33) combined with least-squares fitting (Fig. 1A; see supplemental “Experimental Procedures”). By comparing the diffusion time of each assembly with those of reference dyes (rhodamine 6G and Alexa Fluor 532) with known mass and diffusion coefficient, either the assembly mass (for spherical assemblies such as ASPDs) or diffusion coefficient (D) (for nonspherical assemblies such as fibrils) was determined (supplemental Fig. S3, A and B).

First, we examined the masses of the probes by FCS. The average mass values of the probes, freshly dissolved at 0.1 μ M in 0.5 \times PBS, were calculated to be 7.9 kDa (NTR) and 8.6 kDa (K16TR), respectively ($n = 5$), assuming they behave as spherical structures. Thus, these probes (4.8 kDa nominal mass) behave as dimers, like unlabeled A β^{1-40} (35), and should be available for incorporation into ASPDs during assembly. The result validates the accuracy of our FCS-based analytical method.

In Situ Monitoring of ASPD Formation—Next, we examined whether ASPDs were formed in the presence of the probes. Consistent with our study using unlabeled A β^{1-42} (10), TEM revealed the formation of 10–15-nm ASPDs in the presence of NTR or K16TR at various ratios (1/500–1/10 of total A β ; 16.5 h in Fig. 1B, inset). FCS detected ASPD-sized structures in the range of 100–1000 kDa (16.5 h in Fig. 1B) (10) even at a 1/500 probe ratio. NTR- or K16TR-labeled ASPDs were indistinguishable from unlabeled ASPDs in immunoreactivity to anti-ASPD antibodies and in neurotoxicity to rat primary neuronal cultures (Fig. 1C). Accordingly, we set the probe ratio at 1/500 for subsequent experiments. Because the mass of ASPDs does not exceed 669 kDa (*i.e.* \sim 148-mer) (21), at 1/500 probe ratio, assemblies will contain a maximum of one fluorophore per assembly, so they will have equal brightness. This means that we can obtain the relative abundance of each assembly directly from the distribution of assembly diffusion time determined by CONTIN (see “Experimental Procedures”).

We then followed time-dependent changes in A β assembly state simultaneously using TEM and FCS (Fig. 1A). As shown previously (10), during up to 2 h of slow rotation, TEM detected few structures except for occasional amorphous structure (Fig. 1B, arrow at 0 h in inset). During this initial phase of ASPD formation, up to 2 h, A β trimers (12.7 ± 1.8 kDa, 92%, $n = 8$) predominated (Fig. 1B). This means the first step in ASPD formation involves trimerization. Besides trimers, FCS also detected small amounts of large assemblies with varying mass and relative abundance (for time 0, $10^2 \sim 10^7$ kDa in mass, $8.4 \pm 11\%$; for 2 h, $10^2 \sim 10^4$ kDa, $11.3 \pm 7.2\%$; $n = 8$) (supplemental Fig. S4). This is consistent with the observation of low numbers of cloud-like uranyl acetate staining structures (dotted line in supplemental Fig. S4, inset), distinct from the staining of buffer-derived salts, up to 2 h in TEM. We speculate that these large species are metastable and therefore may be destroyed during

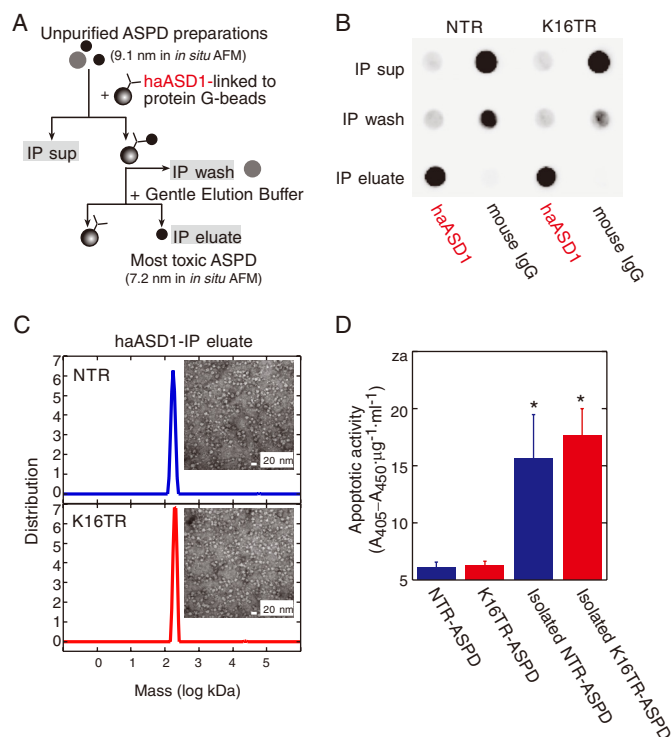


FIGURE 2. Isolation of the most toxic ASPD fraction. A, method for IP. B, dot blotting (using 0.04 μ g/ml rpASD1) of IP supernatant (*sup*), wash, and eluate fractions. C, the isolated ASPDs in haASD1-IP eluates examined by TEM (inset) and FCS ($n = 10$). The distribution is normalized as in Fig. 1B. ASPDs were undetectable in mouse IgG-IP eluates using TEM or FCS (data not shown). D, specific apoptotic activities of the isolated ASPDs and unpurified ASPDs were examined as in Fig. 1C (mean \pm S.D.; Games-Howell post hoc test; *, $p < 0.001$, $n = 6$).

sample preparation for TEM. Although it is unclear whether these species are intermediates of ASPDs, it is noteworthy that neither the cloud-like structures nor the large species with such variety in mass and relative abundance were absent at the onset of fibril formation (see “*In Situ Monitoring of Fibril Formation*”; see Fig. 3). After that, ASPDs appeared at 5 h of slow rotation. At this time, 5–20-nm spherical structures, mainly 10–15-nm ASPD-sized spheres, were observed (Fig. 1B, inset). ASPDs remained as the dominant structures from 5 h until at least 24 h (Fig. 1B, inset). In accordance with TEM observations, FCS detected an ASPD-sized assembly of $10^2 \sim 10^3$ kDa at 5 h, and its amount was increased at 16.5 h (Fig. 1B and supplemental Fig. S4). Its mass was 330 ± 58 kDa ($24 \pm 7.7\%$, $n = 5$), in good agreement with that of ASPDs (\sim 158–669 kDa) previously estimated from glycerol-gradient sedimentation assays (5, 10). Thus, we conclude we could monitor ASPD formation continuously and quantitatively using FCS. The two probes gave essentially identical results (Fig. 1B).

Mass and Height of Isolated ASPDs—We recently produced ASPD-specific antibodies (supplemental Table S1) (21) and used them to selectively immunoprecipitate ASPDs from AD brain extracts without affecting ASPD structure or neurotoxicity (21). With this method (Fig. 2A), we immunoprecipitated the most toxic fraction of ASPDs from unpurified ASPD preparations and determined the mass using our FCS method. Large amounts of 10–15-nm ASPDs were detected only in IP eluates of a monoclonal ASPD-specific haASD1 antibody, whereas ASPDs were undetectable in IP eluates of normal mouse IgG

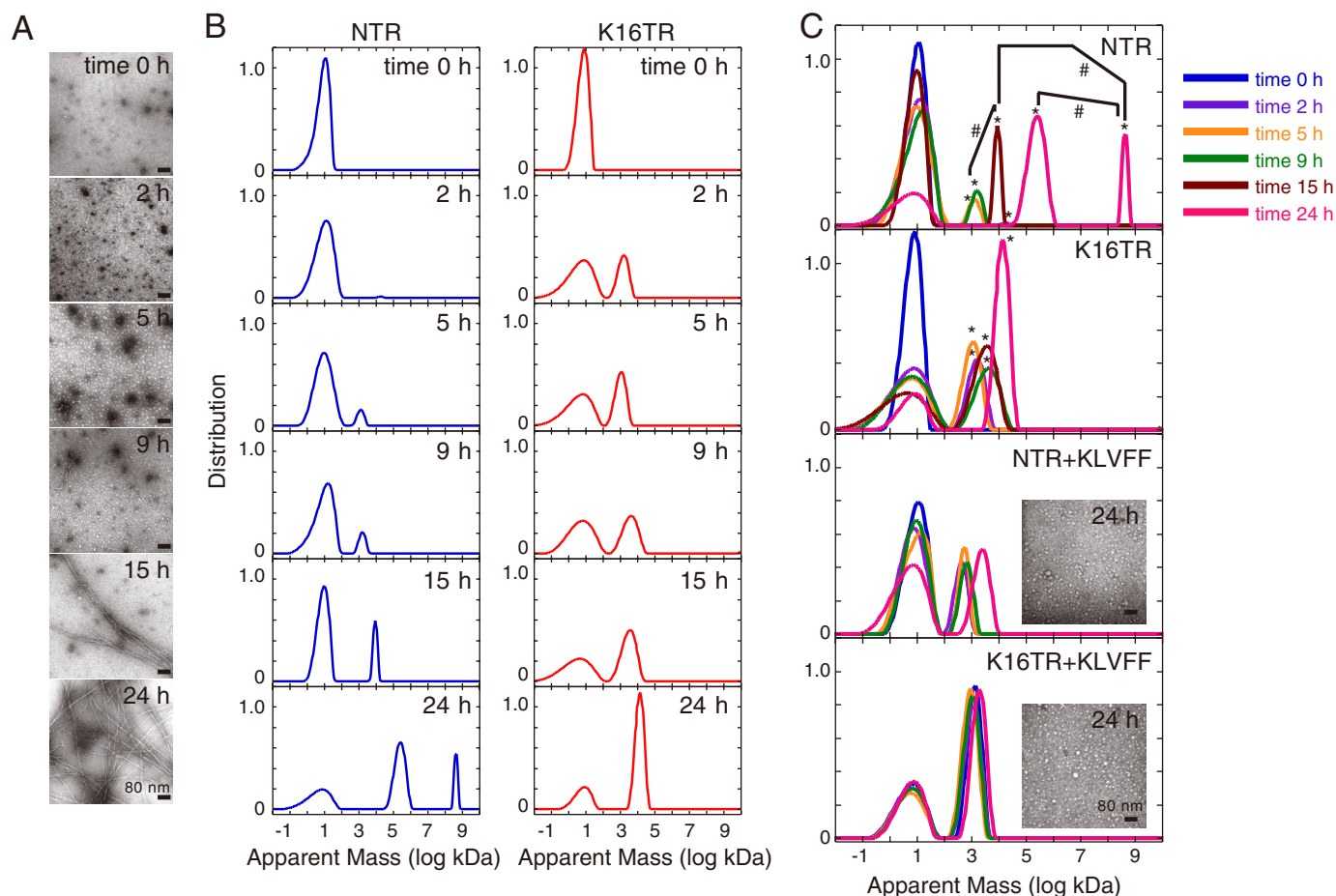


FIGURE 3. Time course of fibril formation. *A* and *B*, fibrils were formed from 100 μM solutions of A β^{1-40} with 0.1 μM NTR or K16TR at pH 3.5. At the indicated time, aliquots were examined by TEM (*A*) and FCS ($n = 5$) (*B*). As fibrillogenesis proceeded essentially in the same way with or without probes, representative TEM images of fibrils with NTR are shown. The normalized distribution of apparent assembly mass (see [supplemental Fig. S7](#) for diffusion coefficient) is shown, as in Fig. 1*B*. *C*, at the onset of fibril formation, a 10-fold molar excess of A β^{16-20} (KLVFF) was added to the A β^{1-40} solutions, and analyses using TEM (*inset*) and FCS were performed as above (*, $p < 0.005$ by Scheffé post hoc test compared with the fast-diffusing assembly corresponding to dimers; #, $p < 0.005$ by Scheffé post hoc test). The probability distribution is normalized as in Fig. 1*B*.

(Fig. 2, *B* and *C*, *inset*). In accordance with this, 97% of the fluorescent species in haASD1-IP eluates detected by FCS was ASPD-sized assemblies of 128 ± 44 kDa ($n = 6$) (Fig. 2*C*). The IP process mostly eliminated A β dimers (9.4 ± 4.0 kDa) (Fig. 2*C*), which had been a major component before IP (76%, $n = 5$, 16.5 h in Fig. 1*B*). The neurotoxicity of the purified ASPDs to rat primary neuronal cultures was >10 times greater than that of the unpurified ASPD preparations (Fig. 2*D* and [supplemental Fig. S5](#)). These data collectively indicated that the most toxic ASPDs were concentrated by the IP procedures. Their mass estimated from FCS analysis was in good agreement with our previous sedimentation results, which indicated that the highest toxicity exists in the fraction at the migration position of 158-kDa aldolase (10, 21). The most toxic ASPDs (128 ± 44 kDa; ~ 32 -mers) were smaller in mass than the unpurified ASPDs (330 ± 58 kDa; compare Fig. 2*C* with 16.5 h in Fig. 1*B*). Consistent with these data, *in situ* atomic force microscopy imaging in physiological solutions (10) revealed that the average height of the most toxic ASPDs (7.2 ± 2.6 nm, 94%, [supplemental Fig. S6](#)) was smaller than that of the unpurified ASPDs (9.1 ± 2.0 nm (10)). We thus have succeeded in determining the mass and height of the most toxic ASPDs for the first time.

In Situ Monitoring of Fibril Formation—We next monitored fibril formation ([supplemental Fig. S3B](#)). We used acidic solutions because fibrils are well formed from 100 μM A β^{1-40} at pH 3.5 (36), whereas ASPDs are hardly formed (24 h in Fig. 3*A*). Because fibrils contain higher numbers of A β monomers than ASPDs (10), we set the probe ratio for fibrils at 1/1000. As with ASPDs, if all components have equal brightness, the relative abundance of each assembly can be obtained from the distribution of assembly diffusion times determined by CONTIN. This is true for early assemblies before 15 h ($< 5,000$ kDa) that will contain a maximum of one fluorophore per assembly at 1/1000 probe ratio. However, this might not be the case for late stage assemblies (after 15 h), which include very large assemblies (probably mature fibrils) with more than one fluorophore per assembly (Fig. 3); here, as the contribution of a component to the FCS correlation curve scales with the brightness squared, the fraction of brighter component will be overestimated (34). The problem is that the number of fluorophores per fibril (*i.e.* fibril brightness) cannot be determined, because fibrils exist over a wide mass range. Furthermore, fibril formation may alter the fluorophore quantum yield (37). Therefore, a rough estimate of fibril amount was obtained from the distribution of

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assembly diffusion times and was confirmed by separating the mature fibrils in retentate fractions on 0.1- μ m filters and by directly measuring their fluorescence.

As with ASPDs, time-dependent changes in A β assembly state were monitored simultaneously using TEM and FCS (Fig. 3). At the onset of slow rotation, amorphous structures incorporating small globular structures (<3 nm) were occasionally detected in trace amounts with TEM (Fig. 3A). At 2 h, as reported previously (14), 15–40-nm spherical intermediates (larger than 10–15-nm ASPDs) appeared and increased until 5 h (Fig. 3A). Then, with the formation of short fibril-like structures, these spherical intermediates decreased at 9 h and were no longer detectable at 15 h (Fig. 3A). Mature fibrils appeared at 15 h, and their amount was markedly increased at 24 h. Subsequently, these fibrils grew into meshwork-like or bundled fibrils (Fig. 3A). These morphological changes generally occurred in this sequence, irrespective of the presence or absence of probes, although the size and morphology of mature fibrils varied somewhat among preparations.

TEM observations confirmed that the presence of either probe at 1/1000 did not affect overall fibrillogenesis (Fig. 3A). However, the two probes gave different FCS results at 24 h (Fig. 3B). To illustrate the time-dependent size changes, we show the diffusion coefficient (supplemental Fig. S7) and apparent mass (Fig. 3) of each structure both for spherical structures (5 h in Fig. 3A) and nonspherical structures (15 and 24 h in Fig. 3A). Unlike ASPD formation, at the onset of fibril formation, A β dimers (9.2 ± 1.7 kDa, 85% at time 0, $1.4 \pm 0.2 \times 10^{-10}$ m²s⁻¹, $n = 6$) were the dominant species (Fig. 3B), which decreased to 41% at 24 h. Consistent with the TEM results, large aggregates of $\sim 10^3$ kDa were occasionally detected by FCS at time 0. At 2 h, A β assemblies of ~ 2910 kDa in average mass appeared and remained detectable up to 9 h (29%, $2.2 \pm 0.7 \times 10^{-11}$ m²s⁻¹, $n = 18$; see NTR and K16TR in upper two panels in Fig. 3C). Their average masses likely correspond to the 15–40-nm spherical intermediates (5 h in Fig. 3A). At 15 h, consistent with their disappearance in TEM (Fig. 3A), they were hardly detectable with FCS, and other A β assemblies of 5.4×10^3 kDa in apparent mass, a slightly larger than the 15–40-nm intermediates, appeared (roughly 38%, $1.6 \pm 0.4 \times 10^{-11}$ m²s⁻¹, $n = 6$; Fig. 3B). Although the two probes, NTR and K16TR, behaved similarly up to 15 h, they gave different results at 24 h. NTR detected two kinds of A β assemblies, a very large A β assembly of 1.2×10^5 kDa in apparent mass (roughly 45%, $0.7 \pm 0.4 \times 10^{-11}$ m²s⁻¹, $n = 3$) and another much larger A β assembly of 3.9×10^9 kDa in apparent mass (roughly 7%, $1.9 \pm 0.9 \times 10^{-13}$ m²s⁻¹, $n = 3$) (Fig. 3B). However, K16TR only detected the former very large A β assembly of 1.4×10^4 kDa in apparent mass (roughly 64%, $1.3 \pm 0.7 \times 10^{-11}$ m²s⁻¹, $n = 3$), but failed to detect the latter huge A β assembly (Fig. 3B). As described above, to confirm the estimates at 15 and 24 h, the fibril amount was directly obtained by filtration using 0.1- μ m filters, followed by counting of fluorescence intensity. The fibril amounts in the 0.10- μ m retentates were $29 \pm 7\%$ (NTR) and $36 \pm 3\%$ (K16TR) at 15 h, and $74 \pm 7\%$ (NTR) and $61 \pm 6\%$ (K16TR) at 24 h ($n = 5$; supplemental Fig. S8). These data seem consistent with the FCS results (38% at 15 h ($n = 6$) and 52% for NTR and 64% for

K16TR at 24 h ($n = 3$)) (Fig. 3B), suggesting that the fibril amount can be estimated using FCS.

As for the difference between NTR and K16TR at 24 h, we speculate that TMR introduced at Lys¹⁶ disturbed incorporation of K16TR into mature fibrils, because the 16–20 region of A β is known to be critical for A β self-association and subsequent fibril formation (38). To examine this hypothesis, we added excess A β^{16-20} (KLVFF) at the onset of A β assembly. In TEM and FCS analyses (Fig. 3C), 15–40-nm spherical intermediates that had disappeared at 15 h without A β^{16-20} (Fig. 3, A and B) remained as the dominant structures in the presence of excess A β^{16-20} , up to 24 h (Fig. 3C, inset). NTR and K16TR both detected these spherical intermediates even at 24 h (2280 kDa, for $2.2 \pm 0.2 \times 10^{-11}$ m²s⁻¹ for NTR, 3370 kDa, $1.9 \pm 0.3 \times 10^{-11}$ m²s⁻¹ for K16TR, $n = 3$, Fig. 3C and supplemental Fig. S7). This result indicated that A β^{16-20} did not prevent assembly of A β dimers into spherical intermediates but blocked conversion of the latter into fibrils.

DISCUSSION

A large body of evidence supports the hypothesis that nonfibrillar A β assemblies play causative roles in AD (3, 5, 20, 22–25). Accordingly, A β assemblies other than fibrils have recently been proposed as therapeutic targets (39). However, A β monomers develop into various nonfibrillar A β assemblies differing in size and toxicity, which might represent distinct structural variants (5, 20, 24, 40). It is not clear whether and how these different types of assemblies are related to each other or indeed how they contribute to AD pathogenesis.

To elucidate the neurotoxic molecular entities responsible for AD pathogenesis, several approaches have been employed to reveal the formation of nonfibrillar A β assemblies *in vitro*. For example, studies using HPLC multiangle laser light scattering analysis (41) determined the size of certain forms of nonfibrillar A β assemblies. Studies using a UV-cross-linking method (42) or limited proteolysis with mass analysis (43) elucidated the roles of various amino acid residues in the formation of nonfibrillar A β assemblies, and solid-state NMR analysis revealed conformational changes in the early stage of fibril formation (14). Interestingly, in the case of cross-linked A β oligomers, higher order oligomers have stronger neurotoxicity (44). These studies and others together suggest that assembly may not be a linear process but may be the result of a series of multiple processes involving intermediates from side paths (5). However, these approaches could not follow the continuous changes of assembly state in solution and the assembly state–neurotoxicity relationship largely remained to be uncovered.

Here, we have developed a highly sensitive *in vitro* monitoring method using combined FCS, TEM, and toxicity analyses. Applying this method to A β labeled with TMR at the N terminus or Lys¹⁶, we uncovered two distinct assembly pathways, one leading to highly toxic 10–15-nm spherical A β assemblies termed ASPDs (10), which we showed to exist *in vivo* (21), and the other to fibrils. The first step in ASPD formation is trimerization. ASPDs of ~ 330 kDa in mass originate from these trimers after 5 h of slow rotation (Fig. 1B). At least until 24 h, ASPDs remained the dominant structures in these reactions. Previously, we reported that ASPDs are formed from 50 μ M

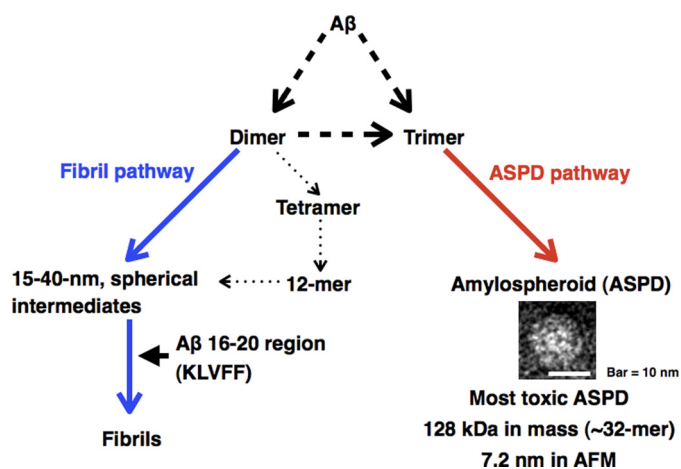


FIGURE 4. **Two distinct A β assembly pathways, one leading to ASPDs (red) and the other to fibrils (blue).** The first step in ASPDs formation is trimerization (Fig. 1), whereas the pathway to fibrils begins with dimers, which further assemble into 15–40-nm spherical intermediates, eventually leading to fibrils (Fig. 3B). The A β ^{16–20} region is critical for intermediate conversion into fibrils (Fig. 3C), but not for ASPD formation (supplemental Fig. S1). Although the assembly pathway to 12-mers is unknown, tetramers might serve as a precursor of 12-mers (45); if this is so, the assembly pathway to 12-mers (dotted line) differs at the initial step from that of ASPDs.

A β ^{1–42} within 14 h during slow rotation (10). With the present method, we could thus more easily and accurately monitor ASPD formation, as well as detect ASPDs themselves. Indeed, we found that the most toxic ASPDs are ~128 kDa in mass (~32-mers) (Fig. 2 and supplemental Fig. S5) and are 7.2 nm in height (supplemental Fig. S6).

Our previous data have strongly suggested that ASPDs have a distinct tertiary structure from other assemblies (21). Therefore, we were interested in whether or not ASPDs share building blocks with these assemblies. With respect to dimers (18), we found that they clearly are not building blocks *per se* because the initial step in ASPD formation is trimerization (Fig. 1B). We could not detect 12-mers during ASPD formation (supplemental Fig. S4). This suggests that 12-mers are not precursors of ASPDs. Although the assembly pathway to 12-mers is unknown, ion mobility-mass spectrometry studies have shown that tetramers might serve as a precursor of 12-mers (45), indicating that the assembly pathway to 12-mers may differ at the initial step from that of ASPDs. These results collectively demonstrated that the assembly pathway to ASPDs is distinct from those leading to dimers and to 12-mers. We propose a scheme of the assembly pathways based on these findings (Fig. 4).

In contrast to ASPD formation, the first step leading to fibrils consists in the assembly of A β monomers into dimers, which further assemble into 15–40-nm spherical intermediates, eventually leading to fibrils (blue arrows in Fig. 4). Although the A β ^{16–20} region is not involved in ASPD formation (supplemental Fig. S1), we found that this region is critical for conversion of the spherical intermediates to fibrils because probe labeling at Lys¹⁶ was found to disturb fibrillogenesis (Fig. 3C). Excess amounts of the A β ^{16–20} (KLVFF) peptide in fibril formation reactions inhibited the process, supporting this conclusion (Fig. 3C). As for 12-mers, we could not detect them as a single peak during fibril formation (Fig. 3B), which suggests that even though 12-mer formation appears to begin with dimer forma-

tion (45), the 12-mers do not directly serve as a primary fibril precursor (Fig. 4). This view is consistent with other studies showing that 12-mers are semistable structures that do not readily form fibrils (12), and it would likely take a slow transformation process for them to rearrange into fibrils (45). Of relevance to the role of dimers in fibrillogenesis are data recently published by the Walsh and co-workers (46) that show that dimers themselves are inert with respect to neurotoxicity but that their further assembly produces higher order toxic prefibrillar assemblies. Consistently with this, dimers in AD brains have been reported to be contained in amyloid cores as insoluble reservoirs that do not readily dissociate (18).

Previously, we have shown that both A β ^{1–40} and A β ^{1–42} form ASPDs of ~158–669 kDa in sedimentation assays, and among them, the highest toxicity exists in the fraction at the migration position of 158 kDa (aldolase) (10, 21). Epitope analysis of ASPD-specific antibodies has also suggested that A β ^{1–40} and A β ^{1–42} ASPDs share the same tertiary structures because essentially the same results are obtained in inhibition studies of ASPD-specific antibodies through the binding to either A β ^{1–40} or A β ^{1–42} ASPDs of pentapeptides derived from A β (21). However, there exists a critical difference between A β ^{1–40} and A β ^{1–42} in the rate and speed of ASPD formation. A β ^{1–42} ASPDs are formed more rapidly, after 5 h of incubation, and in greater quantities (24%). They induce neurodegeneration at lower concentration (~0.35 nM) and exhibit ~100-fold higher toxicity than A β ^{1–40} ASPDs (10). In contrast, A β ^{1–40} forms ASPDs after 3 days of incubation, but in lesser amounts (5% on average) than does A β ^{1–42} (10). These observations are consistent with the general consensus that A β ^{1–42} is more toxic than A β ^{1–40}. Interestingly, we found that the first step to A β ^{1–40} ASPDs also consists in the assembly of A β ^{1–40} monomers into trimers (12.7 ± 0.4 kDa, 93%, *n* = 4; data not shown). Although A β ^{1–40} seemingly behaves as trimers in FCS, we speculate that, probably because of the absence of the stabilizing influence of the hydrophobic carboxyl terminus, A β ^{1–40} trimers are more unstable than A β ^{1–42} trimers and therefore less likely to form ASPDs. This view is supported by other studies using different analytical methods, including SDS-PAGE, ion mobility-mass spectrometry, and size exclusion spectroscopy (45, 47, 48), all of which have indicated the absence of trimers/tetramers in A β ^{1–40}. Certain differences in the initial folded structures and properties between A β ^{1–40} and A β ^{1–42} might be reflected in the differences in their ability to form higher order assemblies (45, 47, 48). Although the conformation adopted by A β within the nonfibrillar A β assemblies is not known, a β -hairpin has recently been reported to be a building block of toxic A β assemblies; this was established by engineering a double-cysteine mutant in which the β -hairpin is stabilized by an intramolecular disulfide bond (48). Notably, the double-cysteine mutant A β ^{1–42} has been reported to form SDS-stable dimers/trimers, particularly trimers, which were absent in the mutant A β ^{1–40} (48). As a result, the mutant A β ^{1–42} forms highly neurotoxic, A11-negative, β -sheet-enriched assemblies of ~100 kDa (apparent mass on size exclusion chromatography) more readily than the mutant A β ^{1–40} does (48). Such conformational difference in A β ^{1–40} and A β ^{1–42} monomers might be reflected in the differences of ASPD formation. It is also possible that con-

Two Distinct A β Assembly Pathways to ASPD and Fibrils

formational differences in the initial monomer state might cause differences in dimer or trimer formation, leading to the distinct assembly pathways observed here. Further analysis, *e.g.* with NMR, is needed to elucidate the molecular mechanism of the differences.

To conclude, the observed differences in the assembly pathways clearly indicated that ASPDs are not fibril precursors, in accordance with the fact that ASPDs continued to exist after mature fibril formation without being incorporated into fibrils (5, 10). Our data also support the idea that the assembly pathway to ASPDs is different from the pathways leading to fibrils and to oligomers such as dimers and 12-mers (5, 10). As has also been suggested by others (45, 47, 48), our data further support the view that distinct assembly pathways lead to formation of assemblies with distinct tertiary structures (Fig. 4). We have thus discerned the different pathways by employing the combined FCS method, which should facilitate identifying the assembly steps where inhibition might be beneficial. This method should also be useful to find biological molecules or chemical factors that inhibit formation of these nonfibrillar A β assemblies and offers the potential for developing therapeutic agents based on this mechanistic understanding.

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**Protein Structure and Folding:
Two Distinct Amyloid β -Protein ($A\beta$)
Assembly Pathways Leading to Oligomers
and Fibrils Identified by Combined
Fluorescence Correlation Spectroscopy,
Morphology, and Toxicity Analyses**



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