Real-time and Single Fibril Observation of the Formation of Amyloid β Spherulitic Structures

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In Alzheimer disease, amyloid β, a 39–43-residue peptide produced by cleavage from a large amyloid precursor protein, undergoes conformational change to form amyloid fibrils and deposits as senile amyloid plaques in the extracellular cerebral cortices of the brain. However, the mechanism of how the intrinsically linear amyloid fibrils form spherical senile plaques is unknown. With total internal reflection fluorescence microscopy combined with the use of thioflavin T, an amyloid-specific fluorescence dye, we succeeded in observing the formation of the senile plaque-like spherulitic structures with diameters of around 15 μm on the chemically modified quartz surface. Real-time observation at a single fibrillar level revealed that, in the absence of tight contact with the surface, the cooperative and radial growth of amyloid fibrils from the core leads to a huge spherulitic structure. The results suggest the underlying physicochemical mechanism of senile plaque formation, essential for obtaining insight into prevention of Alzheimer disease.

In Alzheimer disease, amyloid β (Aβ)3 peptide forms amyloid fibrils that deposit in the extracellular space of the brain as senile amyloid plaques, pathological hallmarks of Alzheimer disease, and also in the walls of cerebral blood vessels (1–5). The formation of Aβ amyloid fibrils is considered to be a nucleation-dependent process in which Aβ peptides slowly associate to form a nucleus, which then grows via an extension reaction involving the sequential incorporation of Aβ peptides, producing rigid and straight morphology consisting of several layers of cross-β sheets (6, 7). This process is influenced by several factors, i.e. peptide concentration, pH, ionic strength, and interactions with other components (8, 9). The interactions with lipid membranes in particular have received attention because the membrane surface might be responsible for both neurotoxicity and senile plaque formation (10–12).

For several proteins including Aβ, amyloid fibrils prepared on the solid substrates such as mica or quartz produce radial assemblies (13–15). Considering that in several neurodegenerative diseases, radial and spherical aggregates of amyloid fibrils are found in tissue deposits (5, 16), surface interaction may play dominant roles in the formation of amyloid fibrils and their deposition in vivo (7, 17). Moreover, amyloid deposits are found in a specific tissue region, suggesting that a specific surface chemistry is involved in the fibril formation and deposition processes in general. However, the behavior of amyloid fibrils on solid surfaces is still far from clear. To obtain further insight into the mechanism of senile plaque formation and the effects of surface, direct observations are needed.

We previously developed a unique approach to monitoring fibril growth in real time at the single fibril level (18, 19), in which TIRFM was combined with the use of thioflavin T, an amyloid-specific fluorescence dye (20). With this approach, we focused on the effects of the physicochemical properties of surface on the growth of amyloid fibrils of Aβ.

EXPERIMENTAL PROCEDURES

Chemical Modification of Surfaces—Octadecyltriethoxysilane (OTS) was purchased from Shin-Etsu Chemical (Tokyo, Japan). Aminopropyltriethoxysilane (APTS) was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). Polyvinylsulfonate (PSS; molecular weight (Mw) 70,000), polyacrylic acid (PAA; Mw 30,000), and polyvinylsulfonate (PVS; Mw unknown) were purchased from Sigma. Polyethyleneimine (PEI; Mw 60,000) was obtained from Nacalai tesque (Kyoto, Japan). 10-(Carboxymethyl)decylmethylchlorosilane (CMDDS: Mw 292.92) was purchased from Gelest (Morrisville, PA).

The surface of quartz slides was modified either by adsorbing polyelectrolytes or by grafting a silane monolayer with a functional terminal group (21). In both cases, either positively or negatively charged surfaces were generated by the chemical species directly exposed to the solution (the uppermost polyelectrolyte layer or the terminal group of silane). Quartz slides were first cleaned with 0.5% (v/v) Hellmanex (Hellma, Müllheim, Germany)/water and then treated with a solution of...
NH₄OH (28% w/v)/H₂O₂ (30% w/v)/H₂O (0.05:1:5, v/v/v) for 15 min at 65 °C, rinsed extensively with distilled water, and finally dried in a vacuum oven at 110 °C (21). Hydrophobic substrates were prepared by the silanization of OTS (21, 22). For the silanization, quartz slides were incubated in an OTS solution (0.04% w/v in tetrahydrofuran/cyclohexane (1:20, v/v)) and subsequently annealed at 110 °C for 30 min. Positively charged surfaces were prepared by the silanization of APTS (23) or adsorption of PEI by incubating quartz in aqueous solutions of APTS (1% w/v) or PEI (0.1% w/v) (21, 24). Negatively charged surfaces were prepared either by layer-by-layer deposition of polyelectrolytes or by using a self-assembled monolayer of silanes (21, 25). The former type was created by the adsorption of PSS, PAA, or PVS onto positively charged surfaces of PEI or APTS. The other type of negatively charged surface was prepared by the silanization of the substrate with CMDDS followed by hydrolysis of the ester terminal group, forming a monolayer of 10-(carboxy)decyldimethylchlorosilane.

Water contact angles were determined with a contact angle meter (Kyowa Interface Science, Saitama, Japan) at room temperature. Data were averages of the measurements of at least three spots.

**Direct Observation of Amyloid Fibrils**—The TIRFM system used to observe individual amyloid fibrils was developed based on an inverted microscope (IX70, Olympus, Tokyo, Japan) as described (18, 19, 26). The ThT molecule was excited at 442 nm by a helium-cadmium laser (IK5552R-F, Kimmon, Tokyo, Japan). The fluorescence image was filtered with a bandpass filter (D490/30, Omega Optical, Brattleboro, VT) and visualized using a digital steel camera (DP70, Olympus).

Aβ-(1–40) peptides were purchased from the Peptide Institute (Osaka, Japan). The purity was >95% according to the elution pattern of high performance liquid chromatography. Aβ-(1–40) was dissolved in a 0.02% ammonia solution to 500 μM at 4 °C. Aβ-(1–40) amyloid fibrils were prepared by the fibril extension method as described previously (27). Seeds were prepared by the fragmentation of amyloid fibrils for 5 min with a TAITEC VP-30S sonicator (Saitama, Japan) equipped with a microtip. The seeds were added at a final concentration of 5 μg/ml to 50 mM monomeric Aβ-(1–40) in 50 mM sodium phosphate buffer at pH 7.5 and 100 mM NaCl. After 3 h at 37 °C in the test tube, 100 μM ThT was added at a final concentration of 5 μM. An aliquot (14 μl) of sample solution was deposited on each microscopic slide, and an image of the fibrils was obtained with TIRFM. The formation of Aβ-(1–40) fibrils on various surfaces was observed with negative charges (panels A–F), whereas the formation of fibrils was suppressed on hydrophobic (panel G) or positively charged (panels H and I) surfaces.

![Figure 1](image_url)

**TABLE 1**

| Surface materials | Surface charge | Water contact angle° | Fibril growth pattern |
|-------------------|----------------|----------------------|----------------------|
| Quartz            | Negative       | 0                    | Linear and radial    |
| CDDS*             | Negative       | 10                   | Linear and radial    |
| PEI/PVS           | Negative       | 0                    | Spherical            |
| APTS/PVS          | Negative       | 6                    | Spherical            |
| PEI/PAA           | Negative       | 24                   | Spherical            |
| PEI/PSS           | Negative       | 58                   | Spherical            |
| OTS               | Non-charged    | 105                  | No growth            |
| APTS              | Positive       | 62                   | No growth            |
| PEI               | Positive       | 35                   | No growth            |

*° The water contact angle is the maximal angle between the surface of the substrate and that of the water droplet: Repulsion between the substrate and water makes the angle larger, thus giving a measure of the hydrophobicity of the substrate.

*10-(carboxy)decyldimethylchlorosilane.
Single Fibril Observation of Amyloid β Spherulitic Structure

For the observation of Aβ-(1–40) spherulitic structures, the microscopic slide and incubated at 37 °C for 3 h.

For the real-time observation of Aβ-(1–40) amyloid fibril growth, the seed fibrils were added at a final concentration of 0.5 μg/ml to 50 μM monomeric Aβ-(1–40) in polymerization buffer (50 mM sodium phosphate buffer at pH 7.5 and 100 mM NaCl). The ThT solution was then added at a final concentration of 5 μM, sample mixtures were deposited on the PEI/PVS surface, and fibrils were observed every 2 min under TIRFM at 37 °C.

Laser Scanning Confocal Microscopy and Three-dimensional Reconstruction of Spherulitic Structures—For the confocal image observation, Aβ-(1–40) amyloid fibrils were prepared under the same conditions as used for the TIRFM observation. Confocal images were taken with a laser scanning confocal microscope (Fluoview FV1000, Olympus, Tokyo, Japan). ThT was excited by 458 nm light of a multi-argon laser. For through-focus imaging, all optical sections were collected with 0.25 μm z axis steps and were clarified through Kalman filtration. The three-dimensional reconstruction of stacked images was performed with Amira 4.0 (Mercury Computer Systems, Chelmsford, MA). Quartz crystal microbalance measurements are provided in Supplemental Fig. 3.

Transmission Electron Microscopy—The presence of amyloid fibrils in the spherulitic structures was examined by electron microscopy. After confirming the formation of spherulitic structures under TIRFM, the coverslip was carefully removed from the quartz substrate. An aliquot (5 μl) of distilled water was deposited on the substrate, and then carbon-coated copper grids (400 mesh) were put on the substrate for 3 min so as to transfer the fibrils. Then, the grids were stained with a 2% (w/v) uranyl acetate solution. Electron micrographs were acquired using a transmission microscope (100CX, JEOL, Tokyo, Japan) at 80 kV with magnification ×29,000.

RESULTS

Effects of Various Surfaces on the Formation of Aβ Fibrils—We observed the seed-dependent formation of Aβ-(1–40) fibrils on the surface of various chemically modified substrates that were created either by alternative adsorption of polyelectrolytes or with self-assembled monolayers of silanes. The results are compiled in Fig. 1 and Table 1. In the presence of the Aβ-(1–40) seed fibrils, enhanced fibril formation was observed on negatively charged surfaces, including quartz and PEI/PVS. On quartz, intense growth led to remarkably long fibrils as reported previously (Fig. 1A) (19). We often observed radial growth patterns suggesting the presence of clustered seeds. Real-time observation showed that once growth started, unidirectional growth continued until the depletion of monomers, producing long fibrils of uniform width (Supplemental Fig. 1). Such long fibrils were not observed in solution, indicating that moderate interaction with the surface prevents the lateral association of growing fibrils, thus enabling the persistent growth.
Formation of Aβ Spherulitic Structures—Fibril growth was especially prominent on the surfaces covered with PEI/PVS, highly negatively charged and hydrophilic polyelectrolytes (Fig. 1D). We initially presumed that the growth of fibrils on the PEI/PVS initiated from large clustered seeds attached to the surface. However, the real-time observation revealed striking images of fibril growth, producing huge spherical assemblies with a densely packed radial pattern (Fig. 2A and Supplementary Movie 1). Importantly, no branching of the growing ends was observed as on quartz (see also Figs. 3 and 4, below).

We also examined the spherical assemblies by a laser scanning confocal microscopy; a three-dimensional reconstruction image (Fig. 2B) and all sectional images (Supplemental Movie 2) indicated that they are built up with ThT-positive amyloid monomers (Fig. 4). Incidentally, the rates of growth were similar (∼0.3 μm/min) for both types of fibrils (19). This result supports morphological similarity of individual fibrils. Thus, once the fibril growth started, the rate of fibril growth might be less affected by the physicochemical properties of the surface, although the extents of seed clustering and adsorption significantly depend on the surface.

Considering that TIRFM illumination has a depth of penetration of ∼150 nm and the depth of focus on the objective lens is about 100 nm, the large clusters of seeds formed at first in solution and were not in contact with the substrate. The hazy areas observed at the initial stages, as indicated in Fig. 2A by arrows, may represent the clustered seeds or aggregated intermediates formed in solution. Since the thickness of the water layer estimated from the fine focus stroke between the quartz slide and the coverslip is about 10 μm, the spherical assemblies observed here are in fact flattened spheres. However, three-dimensional reconstruction image based on confocal microscopy observation suggests that the core region is indeed spherical (Fig. 2B). The surface used for TIRFM observation was located on the upper side of the cell, so the clustered fibrils on the surface are not deposited by gravitational force.

As for other substrates, extensive fibril formation was generally observed on the surfaces with negative charges, regardless of whether they were modified by a polyelectrolyte or silane (Fig. 1, A–F). In contrast, fibril growth was largely suppressed on positively charged or hydrophobic surfaces (Fig. 1, G–J). The image obtained with hydrophobic surface suggested that the
The binding efficiency of seed fibrils are less than that of other surfaces. Thus, the efficiency of seed adsorption may be an important factor determining the fibril growth (see below). To examine the effects of seeding, we studied the spontaneous fibril formation with variously modified surfaces. Although a similar massive growth of fibrils was observed even for spontaneous fibril formation on the negatively charged surfaces, spontaneous fibril formation was also suppressed on the positively charged surfaces (Supplemental Fig. 2). We then confirmed that the observed morphological differences were induced during the growth of fibrils on the surface; loading of the fibrils preformed in a test tube to various substrates did not induce the morphological changes (Fig. 5).

**Interaction of Aβ with Various Surfaces**—The Aβ-(1–40) sequence is divided into the polar region (1D-28K), with both positive (5R, 13H, 14H, 16K, 28K) and negative (1D, 3E, 7D, 11E, 22E, 23D) charges, and the hydrophobic transmembrane regions (29G-40V) (4). The net charge of Aβ-(1–40) at pH 7 is slightly negative. To address the interaction of Aβ with various surfaces, we used quartz crystal microbalance with dissipation monitoring, by which the mass and viscoelastic properties of bound ligands can be estimated (29). The results indicated that although Aβ monomers and fibrils bind to a variety of surfaces at pH 7, the binding of seed fibrils to the hydrophobic surface is less than that of others (Supplemental Fig. 3), consistent with the TIRFM observation (Fig. 1G). Importantly, the bound fibrils are more flexible on the negatively charged surface, probably because of the charge repulsion.

**DISCUSSION**

**Formation of Senile Plaque-like Aβ Spherulitic Structures**—Most importantly, the assemblies of Aβ-(1–40) fibrils observed in the present study resemble the amyloid core of senile plaques observed in the cerebral cortices of patients suffering from Alzheimer disease (5). Similar spherical amyloid deposits are observed in a mouse model of Alzheimer disease (16), in patients with Creutzfeldt-Jakob disease (17), and in several other neurodegenerative diseases (7). Furthermore, spherulites were observed in vitro in many systems including natural and synthetic polymers (see Ref. 30 for detail), for example in insulin (30, 31), pathogenic immunoglobulin chains (32), β-lactoglobulin (33), and synthetic peptides (34), indicating that they are a common architectural feature of fibers. We consider that the senile plaque-like spherical objects observed here correspond
to spherulites, a higher order spherical assembly of amyloid fibrils ranging in diameter from 10 to 150 μm. In a polarizing light microscope, spherulites exhibit a typical “Maltese-cross” extinction pattern (30). In the present study, we did not examine the presence of Maltese-cross since we could not isolate the spherical objects to perform the measurements under cross-polarizers. Thus, additional studies are necessary to conclude that the observed amyloid assemblies are indeed spherulites.

However, the similarity of the amyloid core of senile plaques and the spherulitic assemblies observed in the present study suggest that senile plaques in patients also develop through the cooperative growth and association of amyloid fibrils as visualized here, together with interactions between Aβ monomers/fibrils and various biological molecules. During concurrent fibril growth from clumped seeds, moderate repulsion between the fibrils may keep the growing ends separated and thus active. At the same time, moderate attraction between growing fibrils is presumably important to maintain the spherical shape. Thus, the formation of senile plaques is likely to be governed both by the physicochemical properties of Aβ amyloid fibrils per se and by the molecular environment in situ.

Effects of Surface on the Fibril Formation—Our observations with TIRFM are consistent with various reports studying the effects of surface on fibril formation. In situ atomic force microscopy of the amyloidogenic immunoglobulin light chain variable domain SMA showed that negatively charged SMA formed fibrils on the negatively charged mica surface, whereas fibrils did not form on the positively charged or hydrophobic surfaces (15). It has been reported that the formation of Aβ fibrils was facilitated by the negatively charged acidic phospholipids rather than neutral phospholipids (11). These reports also proposed that the strong electrostatic attractions tightly trap the seed fibrils on the surface so that the subsequent growth is inhibited. The inhibitory role of hydrophobic surface might be explained in a similar manner. Moreover, the slightly low efficiency of binding of amyloid precursors in the forms of monomers or seed fibrils to the hydrophobic surfaces might contribute to the suppressed fibril formation. Taken together, the effects of surface on the formation of Aβ-(1–40) fibrils can be classified into three types (Fig. 6).

First, tight electrostatic attraction between negatively charged Aβ-(1–40) and a positively charged surface is unfavorable for efficient fibril growth (Fig. 6A). Tight hydrophobic interaction is also unfavorable. Tight interactions may mask the growing ends. Flexibility of the growing ends might also be required for fibril growth. Moreover, adsorption to the surface decreases the concentration of active monomers, thus inhibiting the growth.

Second, even when the surface charge is moderately negative as in the case of quartz, Aβ-(1–40) with a net negative charge can bind to the surface through local electrostatic attraction and hydrophobic interaction. However, since the interaction is weaker, Aβ-(1–40) seeds expose their growing edges, ensuring efficient fibril growth (Fig. 6B). Weak binding of Aβ-(1–40) fibrils to quartz was evidenced by the transient disappearance of fibrils from evanescent fields monitored by TIRFM (19).

Third and most interestingly, PEI/PVS with a strongly negative and hydrophilic surface produced a huge spherical object, a spherulitic structure (Fig. 6C). The real-time observation showed that the main body of fibril growth indeed takes place
some distance from the surface (beyond the depth of penetration of the evanescent field) (Fig. 2). Thus, although overall observations point to a clear influence of solid surfaces on the formation of spherulitic structures, it remains unknown whether direct interactions with the surface are essential. One possibility is that increasing the local concentration of seed fibrils on the PEI/PVS surface, as revealed by the quartz crystal microbalance measurements, leads to the clustering of seeds. Then, the clustered seeds may detach from the surface, promoting the formation of spherulitic structures in solution. A similar effect has been suggested in the spontaneous formation of spherical aggregates of Aβ termed “β-amyl balls” (35). Once fibril growth started from the clustered seeds, the combination of intense growth and the delicate balance of repulsions and attractions enables the massive growth of fibrils in a radial pattern, producing a huge and harmonic object. Finally, we consider that the above models on the basis of the results with Aβ-(1–40) will be applicable to Aβ-(1–42), which is suggested to be the main component of senile amyloid plaques.

Conclusion—The surface properties have crucial roles in both promoting and suppressing the fibril growth and interactions. By controlling the surface properties, we reproduced the senile-plaque-like spherulitic assemblies of Aβ-(1–40). The real-time images at the single fibrillar level revealed that a balance of attractive and repulsive interactions coupled with intense growth without branching produces a huge spherical object. On the other hand, on a quartz surface, intense growth without branching produces a huge spherical object. On the other hand, on a quartz surface, intense growth without branching produces a huge spherical object. Finally, we consider that the above models on the basis of the results with Aβ-(1–40) will be applicable to Aβ-(1–42), which is suggested to be the main component of senile amyloid plaques.

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