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Atomic Force Microscopy of β-Amyloid

Static and Dynamic Studies of Nanostructure and Its Formation

Justin Legleiter and Tomasz Kowalewski

1. Introduction

Ordered aggregation of the β-amyloid (Aβ) peptide in the brain as plaques consisting of fibrils is an important characteristic of Alzheimer's disease (AD), a late onset neurodegenerative disease ***(1)***. Aβ derives from the endoproteolysis of the amyloid precursor protein (APP), which is a transmembrane protein containing 677–770 amino acids ***(2–9)***. The two most common forms of Aβ are the 40 and 42 residues long fragments respectively referred to as Aβ(40) and Aβ(42) (sequence shown in **Fig. 1**; **ref. *10***). The insoluble aggregated form of Aβ, which deposits in the extra cellular space in the brain and on the walls of cerebral blood vessels ***(6)***, exhibits an enhanced β-sheet conformation as opposed to the partially α-helical soluble form found in body fluids ***(11,12)***. Despite the lack of the definitive establishment of the causative role of Aβ in AD, evidence points to its aggregation and deposition in the pathogenesis of AD.

The formation of the ordered, β-sheet rich fibrils is believed to proceed via a slow nucleation-dependent mechanism that is followed by rapid "chain-growth" into protofibrils that eventually elongate and possibly coalesce to form mature amyloid fibrils (**Fig. 2; refs. *7,13–17***). The elongation of the protofibrils and fibrils appears to be of the first order ***(7,13,16,17)***. The slow step is the formation of Aβ oligomers that nucleate the process, but it is unclear what causes the formation of these small oligomers. It appears that a critical local concentration needs to be achieved. Such conditions can occur as the result of inefficient clearance of Aβ from the brain. Intra- and extracellular surfaces located inside the brain could also play a pivotal role by increasing local concentrations of Aβ to facilitate the formation of a stable nucleus. Understanding how the process of fibrillogenesis is nucleated and how it is facilitated could

From: *Methods in Molecular Biology, vol. 242: Atomic Force Microscopy: Biomedical Methods and Applications*
Edited by: P. C. Braga and D. Ricci © Humana Press Inc., Totowa, NJ

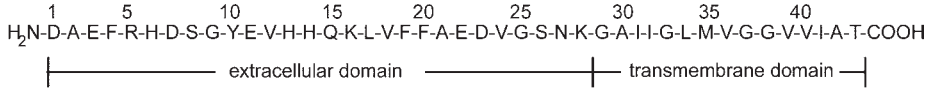

Fig. 1. The sequence of Aβ peptide ***(10)***.

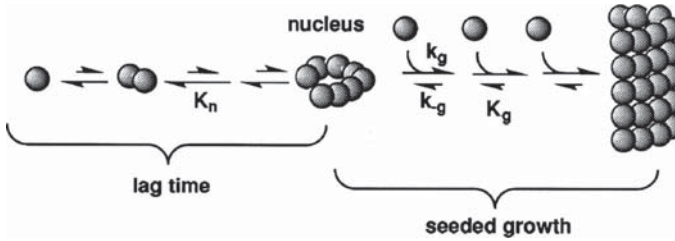

Fig. 2. A simple nucleation dependent mechanism for the growth of Aβ fibrils. A series of unfavorable protein-protein association equilibria with rate constant K_n lead to the formation of a stable nucleus. Once the nucleus is formed, growth into a fibril is achieved by a series of favorable equilibria with rate constant K_g. This shift from the unfavorable to favorable equilibria results in a critical concentration phenomenon. Once a stable nucleus is formed, fibril growth is first order ***(17)***. With permission, from the *Annual Review of Biochemistry*, Volume 66 © 1997 by Annual Reviews www.annualreviews.org.

offer valuable insights into possible targets along the disease pathway for novel treatments for AD.

Atomic force microscopy (AFM) can be used to image and study Aβ with resolution comparable to that achievable with transmission election microscopy (TEM). However, it does not require the extensive sample preparations, such as staining, that precludes the use of TEM in kinetic studies and could possibly alter the morphology of the Aβ fibrils. AFM can also obtain more complete 3D information than can be derived from the 2D cross sectional profiles obtained in TEM studies. *In situ* tapping-mode AFM ***(18,19)*** under liquids offers the ability to study Aβ fibrilization under physiological conditions in a time-dependent manner, which allows the monitoring of changes in conformation and aggregation of Aβ ***(20)***. AFM can also be used to gain insights into the interaction of Aβ with other materials that are of potential importance in AD that could either inhibit or promote Aβ self-assembly into fibrils. This type of information would be useful in evaluating specific drugs designed to inhibit the process and in the determination of where along the pathway they interact with Aβ. These types of studies could also lead to understanding of how other relevant factors (such as lipoproteins, lipid bilayers) affect fibril formation.

2. Materials

1. AFM capable of performing *in situ* operations. There are several systems commercially available from different vendors (Digital Instruments-Veeco, JEOL, Molecular Imaging, Omicron, Pacific Scanning, Quesant Instrument Corporation, Accurion Scientific Instruments, Asylum Research).
2. Standard contact mode fluid cell or fluid cell with piezoelectric accuator.
3. Low-spring-constant cantilever probes, for example, 100-μm wide-legged silicon nitride cantilevers with nominal spring constant of 0.58 N/m for *in situ* tapping mode atomic force microscopy (TMAFM) (commercially available from several vendors: Digital Instruments, Olympus, Bioforce).
4. Tapping mode. Tapping mode-etched silicon probes for *ex situ* studies. (Commercially available from several vendors: Digital Instruments, Olympus, Bioforce.)
5. Dimethylsulfoxide (DMSO).
6. Trifluoroacetic acid (TFA).
7. Phosphate-buffered saline (PBS) buffer of approx pH 7.4.
8. Mica.
9. Highly ordered pyrolitic graphite.
10. Aβ(40, 42), or other fragments that are commonly available from several vendors.
11. Nitrogen.
12. Ultra pure water.

3. Methods

The methods section discusses experimental topics (*see* **Note1**) such as preparation, handling, and incubation of Aβ samples (*see* **Note 2**), *ex situ* AFM studies of Aβ (*see* **Note 3**), *in situ* AFM studies of Aβ (*see* **Note 4**), common methods of analyzing data obtained form AFM studies of Aβ (*see* **Note 5**), and incorporating other factors into AFM studies of Aβ (*see* **Note 6**).

3.1. Preparation and Handling of Aβ Samples

3.1.1. Storage

1. Several different solvents can be used to prepare stock solutions of Aβ. Eventually, these stock solutions should be dissolved in a physiological buffer, such as phosphate-buffered saline (PBS) or Tris-HCl. Solvents that have been used to dissolve Aβ include dimethylsulfoxide (DMSO) ***(21,22)***, TFA ***(14)***, acetic acid ***(23)***, chloroform ***(24)***, physiological buffer ***(25,26)***, and deionized water ***(3,27)***. DMSO appears to be the most commonly used solvent, and the following procedure will involve the use of DMSO.
2. Aβ is easily dissolved in DMSO, and it can be used to make stock solutions that can be stored at –20°C for extended periods of time. Care should be taken to obtain accurate knowledge of the concentration of these stock solutions (usually 2–10 m*M* but this can vary). The stock solutions can also be filtered to remove any fibril seeds that may be present ***(21)***, but a larger initial concentration is

needed for this so that the final concentration remains in the approximate range indicated above.
3. Also, an independent method needs to be used to analyze the concentration of the stock solution after filtration since the removal of seeds will reduce the amount of Aβ in solution. This can be accomplished by quantitative amino acid analysis ***(21)***.
4. It is also useful to store the Aβ stock solution in smaller aliquots that will be used for individual experiments. The size of these aliquots depends on the concentration of the stock solution and the desired concentration for experiment once the stock solution is dissolved in physiological buffer as will be discussed in **Subheading 3.1.2., step 1**. This prevents waste and possible complications that may arise because of several cycles of thawing and refreezing stock solutions. Since these stock solutions are going to be dissolved into physiological buffer, it is important to keep the stock solutions concentrated enough so that upon dilution in the buffer the DMSO is diluted to less than 0.1% of the total volume. This limits the effect that the DMSO may have on the observed behavior of the Aβ in the study.

3.1.2. Incubation

1. In order to initiate the fibril formation, aliquots of the stock Aβ solution in DMSO need to be dissolved in physiological buffer. The new solution should be gently vortexed for approx 60 s to ensure thorough mixing. In order to ensure the solubility of the Aβ into physiological buffer, the buffer can initially be heated to approx 37°C prior to the addition of the DMSO Aβ stock solution.
2. Once the Aβ stock solution is dissolved in the buffer, the temperature should be held at 37°C for approximately another 30 min. These prepared incubation samples can range in concentration from 5–500 μ*M*. Lower concentrations may inhibit the formation of fibrils or may inhibit the ability to observe fibrils due to concentration depletion associated with aggregation of Aβ along the surfaces of the container.
3. Incubation of these samples can last from a few minutes to days and can be carried out at room temperature. These incubating samples should not be perturbed except to obtain aliquots for imaging to prevent the possibility of disrupting the process of self-assembly into fibrils.
4. Variation of the incubation process can easily be achieved by adding different elements to the Aβ solution, such as fibril seeds ***(28)*** or known amyloid inhibitors or promoters ***(25)***. Also, different pH, temperature ***(21)***, concentration, and other conditions can easily be varied.

3.2. Ex Situ *Studies of Aβ*

1. *Ex situ* AFM experiments have provided many insights into the fibrillization of Aβ ***(21,23,28,29)*** (*see* **Notes** and **Fig. 3**) and are especially useful as a complement to other techniques used to study the aggregation and self-assembly of Aβ. The major limitation of this technique is sample preparation, which ultimately

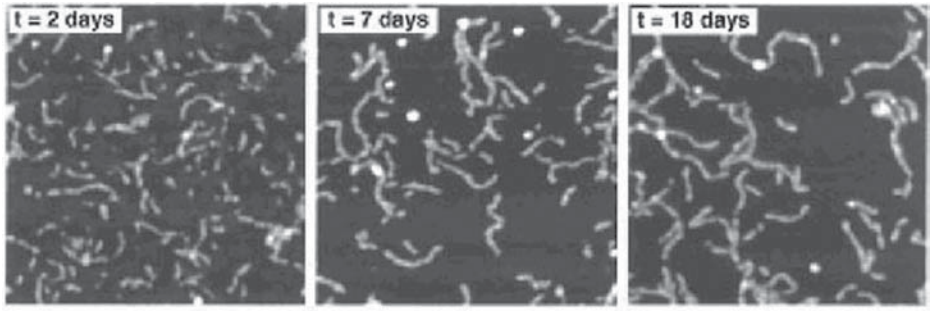

Fig. 3. *Ex situ* AFM images of an Aβ sample deposited on mica at different time intervals (2, 7, and 18 d). Each image is 500 nm by 500 nm. The development of longer protofibrils can be seen as the sample was allowed to incubate for longer times ***(28)***. Reprinted with permission from **ref. *21***. Copyright 1999 American Chemical Society.

carries the sample through a range of nonphysiological conditions, potentially leading to the perturbation of the original structure.

2. Moreover, *ex situ* AFM studies do not allow for the study of the development of the same Aβ structure over time that is possible, as will be discussed in **Subheading 3.3.**, with *in situ* AFM studies. However, by preparing several different aliquots from the same incubation at different time intervals, the development of protofibrils to mature fibrils can still be observed and studied.
3. *Ex situ* AFM is especially useful for observing changes in Aβ fibrillogenesis over extended periods of time (days).

3.2.1. Deposition

1. Deposition of Aβ onto a substrate for imaging is an important aspect of *ex situ* studies. Care must be taken to make the deposition process as noninvasive as possible as well as to reproducibly deposit the sample onto the surface. To ensure this, strict protocol should be used to deposit the sample onto the substrate.
2. To optimize the deposition process, concentrations can be adjusted to increase and decrease the amount of deposited peptide found on the surface.
3. The following is a brief procedure for depositing Aβ samples onto mica.
   a. Aliquots of 2–5 μL of incubated Aβ solution should be placed on freshly cleaved mica. Marking the backside of the mica with a small dot for sample placement is useful for locating the deposited Aβ later during imaging.
   b. The droplet is then left on the substrate for approx 30 s to 2 min, depending on the concentration of solution and the desired coverage. Once optimal conditions are found, the time the sample is allowed to incubate on the substrate should be held constant between depositions.
   c. After incubating the aliquot on the mica, the sample should be washed with 50–200 μL of ultra pure water to remove excess salts and unbound peptide. It is useful to tilt the substrate and deposit the wash above the sample on the

mica. Then, the wash can gently flow past the deposited peptide. This reduces the risk of damaging the deposited Aβ structures when applying the wash.

d. Allow the samples to dry under a gentle stream of nitrogen to prevent contamination and speed the drying process.
e. Once the mica is dry, the sample can be mounted onto a puck and imaged. Samples should be imaged as soon as possible to prevent any contamination or degradation over time.

3.2.2. Chemically Immobilized Aβ Deposition

1. *Ex situ* studies have also been carried out on thiol-based immobilization of Aβ on flat gold surfaces ***(23)***. Vapor deposition on mica can be used to prepare the gold substrates. After rinsing the substrates with ethanol, the gold substrate can be immersed in a 1 m*M* solution of 11-mercaptoundecanoic acid or a mixed solution of 11-mercaptoundecanoic acid and 3-mercaptopropionic acid (1 : 10).
2. The gold substrate should be soaked overnight and then placed in an aqueous solution of 1-ethyl-3-(3dimethlylaminopropyl)-carbodiimide (75 m*M*) and N-hydroxysuccinimide (15 m*M*) for 5 min.
3. After this, the substrates can be soaked in the diluted amyloid solutions for up to an hour. The substrates should be rinsed with deionized water after removal from the amyloid solution and allowed to dry. The substrates should be stored under argon.

3.3. In Situ *Studies of Aβ*

1. *In situ* TMAFM has been successfully applied to the study of Aβ aggregation and fibrillization ***(22,26)***. This technique offers the unique opportunity to image the fibrillization process in a dynamic way, and it can be used to study the interactions of Aβ with other important factors implicated in AD ***(14,24,25)***.
2. It can also be used in conjunction with other techniques used to study Aβ. These techniques include circular dichroism ***(14,25,30)***, fluorescence ***(30)***, and absorbance ***(30)***. It should be noted that the concentration of incubating Aβ solutions may need to be decreased. If the concentration is too large, the surface will be crowded, and the measurement of dimensions for individual particles will become difficult. However, the larger concentration of the incubating samples is also important to prevent the depletion of the sample from aggregation on the walls of the container and also to facilitate the fibrilization process. The concentration used for imaging needs to be systematically optimized.

3.3.1. Choice of Substrate

1. The choice of substrate to be used in the experiment is extremely important. Because of differing hydrophobicity and hydrophilicity of different surfaces, different effects on Aβ can be observed. The surface interactions play a significant role in aggregation and deposition, and thus in the self-assembly of Aβ into fibrillar species.

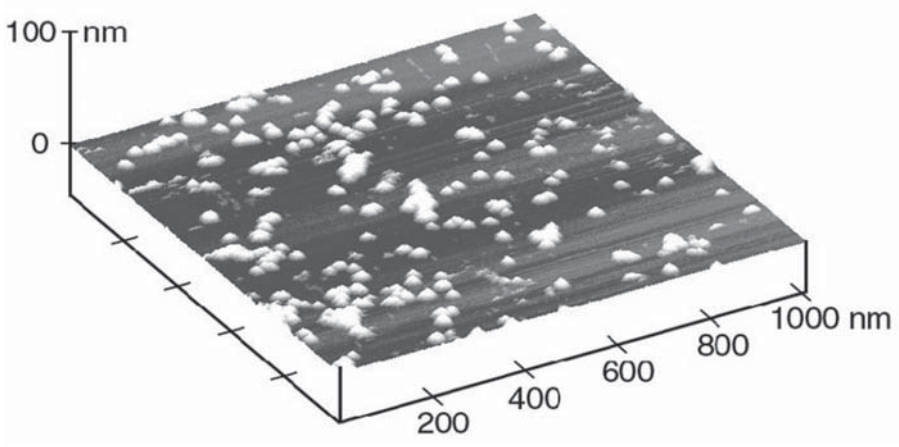

Fig. 4. 3D rendering of *in situ* AFM image of Aβ(42) on the hydrophilic surface of mica. This image was taken in PBS buffer with a peptide concentration of 500 μ*M*. The surface is covered with globular and protofibrillar aggregates of Aβ. The hydrophilic mica can be viewed as a model of the exterior of phospholipid bilayers that constitute cell membranes ***(22).***

2. Two commonly used substrates are mica and highly ordered pyrolitic graphite (HOPG) which can easily be cleaved to provide atomically flat surfaces.
3. Contrast between interaction of hydrophilic mica **(Fig. 4)** and hydrophobic graphite **(Fig. 5)** can also offer insights into the specific interactions that lead to Aβ self-assembly. The surface of mica is negatively charged in solution. Due to this negative charge, mica can be thought of as a surface that models the exterior of anionic phospholipid membranes. The interiors of phospholipid bilayers and lipoprotein particles can be modeled by the hydrophobic surface of graphite.

3.3.2. Imaging of Aliquots

Similar to the procedure briefly described in *ex situ* AFM experiments in **Subheading 3.2.**, aliquots of the same incubating sample can be imaged after different times to monitor the self assembly of Aβ into fibrils.

3.3.3. Time Lapse Imaging

1. *In situ* AFM can be used to study dynamic biological processes, including fibrilization, by time lapse imaging ***(20)***, which allows observation of the initial aggregation of Aβ into protofibrils and the elongation of these protofibrils into mature fibrils **(Fig. 6)**. In this technique, a freshly prepared sample is imaged in the same area of the surface at different time intervals, which can be hours in duration.

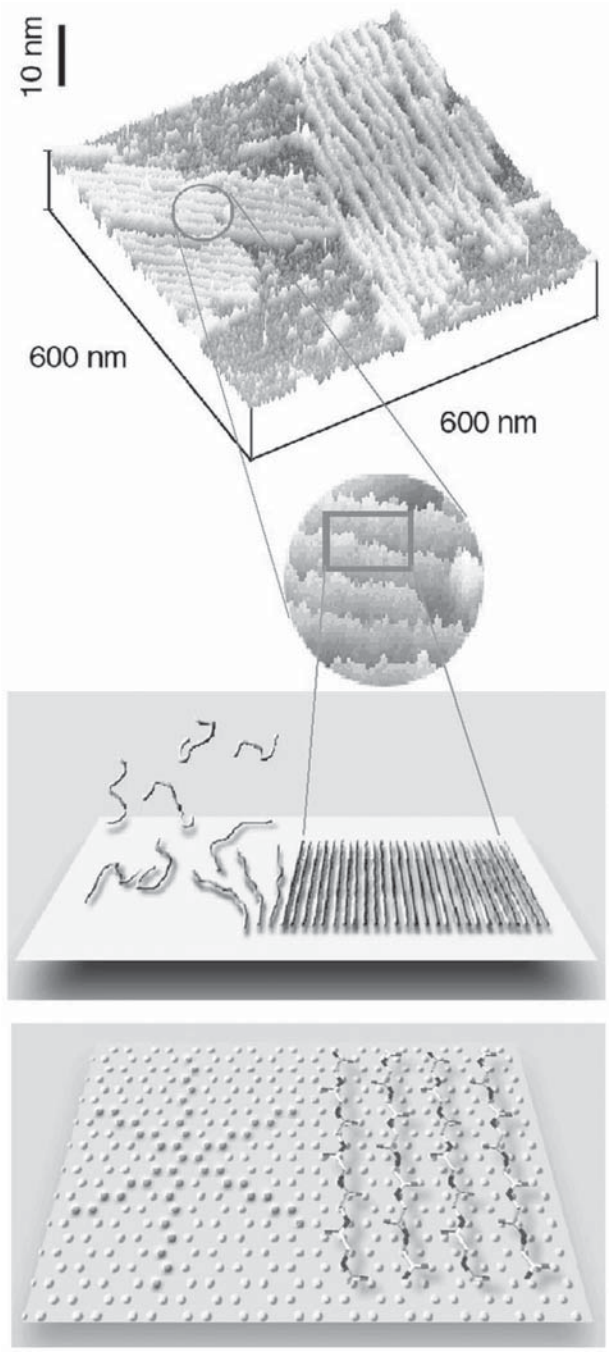

Fig. 5. 3D rendering of *in situ* AFM image of Aβ(42) on graphite. The sample was imaged in PBS buffer. The ribbon-like assemblies of Aβ preferentially orient along

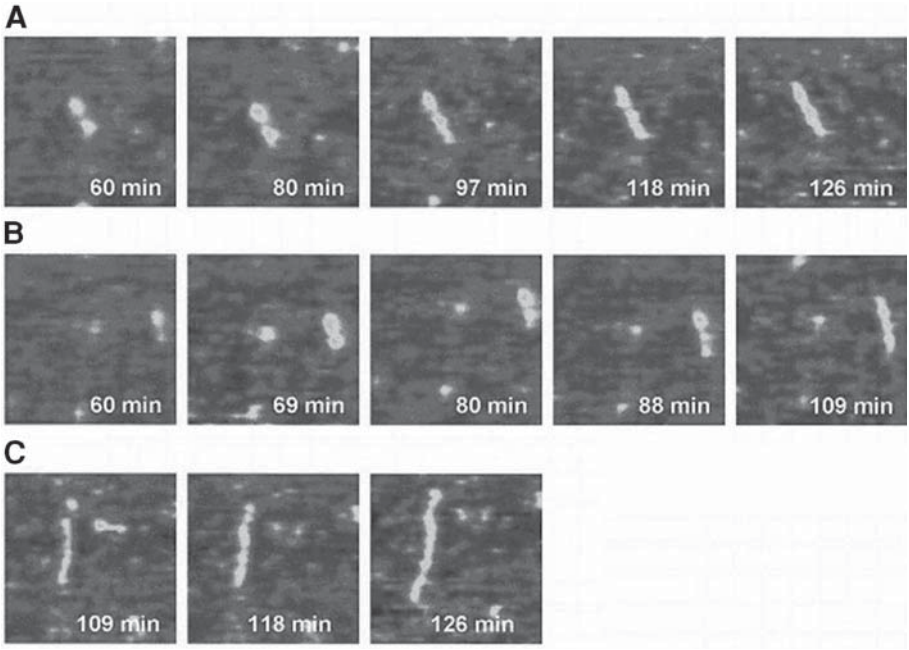

Fig. 6. *In situ* tapping mode AFM makes it possible to track the early steps of Aβ fibrillization. The above 1-μm by 1-μm images track the Aβ aggregates as they form protofibrils and elongate. **(A** and **B)** Images tracking the formation of a protofibril from two Aβ aggregates and the elongation of the protofibril by further addition of Aβ aggregates. **(C)** An Aβ protofibril is shown to elongate in two directions by the further addition of Aβ aggregates. From **ref. *26***. Copyright 2000, with permission from Elsevier.

2. It is important to maintain a good seal between the fluid cell and surface to prevent the evaporation of the solution. By monitoring the same area at different time intervals, it becomes possible to identify and track the development of individual fibrils, and to measure the rate of their elongation and detect morphological changes. Such direct observations provide insights into the mechanisms by which Aβ fibrils nucleate and grow. These insights may be then used in the determination of where along the pathway a specific compound may interfere with the fibril growth.

Fig. 5. *(continued)* crystallographic directions of graphite, presumably maximizing hydrophobic interaction with the surface. Hydrophobic graphite can be viewed as a model of the interior of phospholipid bilayers and the core of lipoprotein particles. The average lateral spacing of the aggregates is 18.8 ± 1.8 nm. The schematic illustrates the orientation of peptide chains in the aggregates based on their dimensions (bottom). The height of the aggregates above the graphite surface ranged from 1.0–1.2 nm. The dimensions of Aβ aggregates on graphite strongly suggest that Aβ adopts a β-sheet form with peptide chains perpendicular to the long axis of the ribbon ***(22)***.

3.4. Quantitative Analysis of AFM Images

1. Quantitative analysis of AFM images can easily be carried out with the aid of computer programs designed to measure heights, diameters, volumes and numbers of particles.
2. In such analysis, it is important to take into account the finite size and shape of the tip used in the experiment, since it may significantly contribute to the observed dimensions of imaged objects.
3. In time lapse *in situ* AFM, the tip contribution may remain relatively constant, provided that the tip is not damaged and that peptide molecules are not aggregating on the tip changing its size and shape. Hence when comparing results from different experiments, variability between the tips should be considered.
4. Common methods for tip characterization include the use of tip characterizers ***(31)*** and blind tip reconstruction ***(32)***.
5. A simple quantitative measurement involves monitoring the number of objects per unit area as a function of time **(Fig. 7; *21*,*22*,*26*)**. Different types of objects (i.e., oligomers, protofibrils, or mature fibrils) can be differentiated by a characteristic physical parameter like height or diameter **(Figs. 7–8; *22*,*26*)**.
6. Analysis of the population of these different forms of Aβ has shown that smaller aggregates tend to disappear as large protofibrils and mature fibrils are formed, indicating that smaller aggregates of Aβ are coalescing to form the larger structures ***(21*,*22*,*26)***.
7. Comparisons of these populations with the change in an average physical parameter (like diameter) also show if these particles are aggregating and coalescing into larger assemblies. A change in the average height or effective diameter of adsorbed material can also indicate different regimes of growth if plotted as a function of time **(Fig. 8; *22*)**.
8. Elongation rates can also be measured by an average measurement of fibril lengths as a function of time from aliquots of the same incubation or by the change in length of individual protofibrils as observed in time lapse AFM **(Fig 9; *21*,*26*)**.

3.5. Incorporating Other Factors into AFM Studies of Aβ

1. Once optimal conditions for imaging Aβ are determined, other variables can be added to gain insight into more specific interactions controlling fibrillization. For instance, the effects of glycerol and trimethylamine N-oxide, which act as chemical chaperones in the amyloid pathway, on the fibrillization of Aβ have been studied using *in situ* AFM ***(25)***.
2. AFM has also been used to study soluble Aβ oligomers by altering solution conditions ***(30)***. These oligomers may be important as intermediates between the monomeric form and the fibrillar form of Aβ and may be themselves physiologically active ***(30)***. The interaction of Aβ with planar bilayers of total brain lipid extract and DMPC deposited on a mica surface has also been reported ***(14)***.
3. The effects of Aβ on endothelial cells have been studied by imaging cells incubated with Aβ present ***(24)***. The Aβ was not directly observed in these images of endothelial cells. Rather, the vitality of the cell was monitored using AFM for cells that had been incubated with and without Aβ.

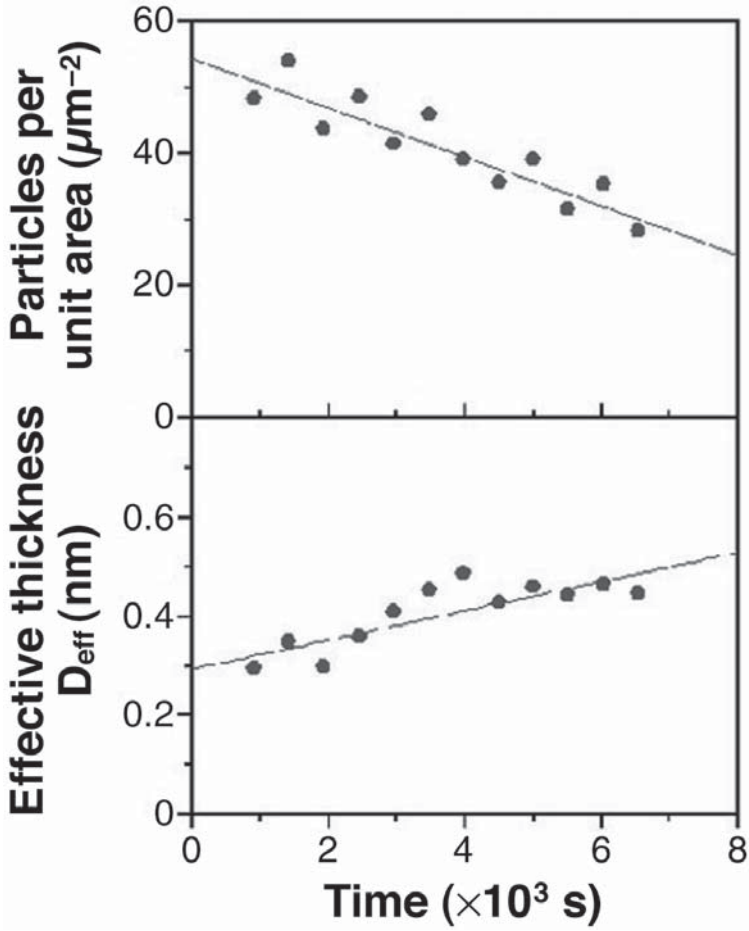

Fig. 7. Quantitative analysis of the aggregation of Aβ(42) on mica can be accomplished by studying the number of particles present in an area as a function of time. Comparison to some physical parameter (effective thickness in this instance) as a function of time can aid in determining quantitatively the aggregation of Aβ. In the above plots, it is shown that the number of individual aggregates of Aβ decreases as the effective thickness of the aggregates (corresponding to total volume) increases. This indicates that aggregates on the mica surface merge to form larger aggregates as a function of time *(22)*.

4. Notes

1. The choice of surface for AFM studies of Aβ is important in gaining insight into the role of surface interaction in the pathway of amyloid formation. Intra- and extracellular surfaces in the brain may play a role in the fibrilization of Aβ. Different surfaces can model different aspects of surfaces found in vivo. Mica is a

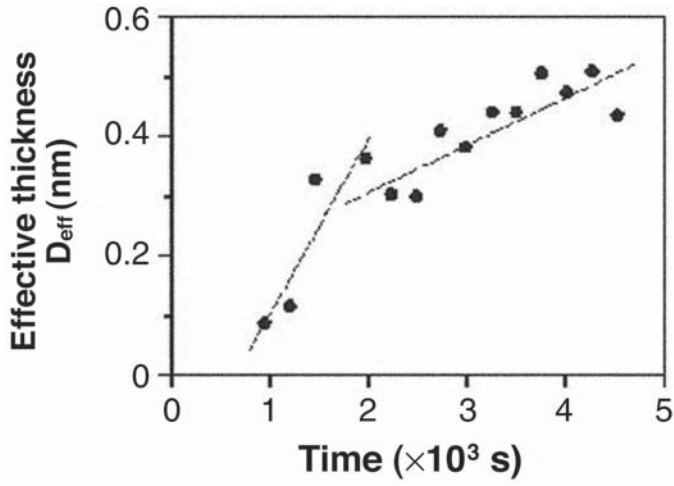

Fig. 8. Quantitative analysis of Aβ aggregation on graphite is accomplished by plotting effective diameter as a function of time for an *in situ* AFM experiment. The plot shows the appearance of an incubation time as well as two separate regimes of growth ***(22)***.

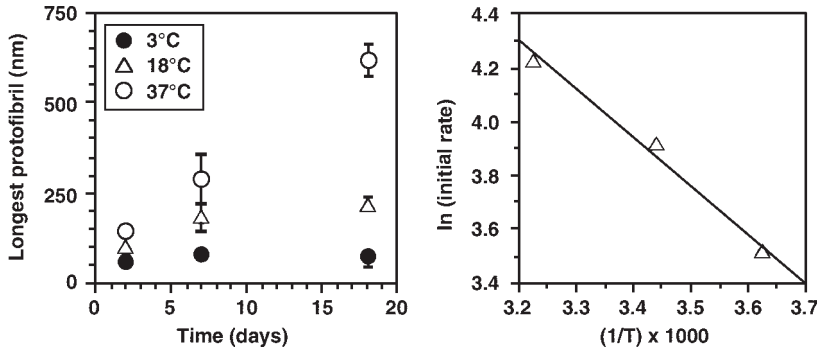

Fig. 9. The temperature dependence of protofibril growth is studied by tracking the longest protofibril as a function of time at various temperatures (left) using *ex situ* AFM. An arrhenius plot can be constructed from data of the longest protofibril. The plot on the right shows an Arrhenius plot constructed form data taken at two days for Aβ samples incubated at different temperatures. Reprinted with permission from **ref. *21***. Copyright 1999 American Chemical Society.

hydrophilic surface and can be used to model the outside of cell surfaces that are primarily composed of the hydrophilic head groups of phospholipids. Graphite, which is hydrophobic, can be used to model the interior of the lipid bilayers that

make up cell membranes. Different characteristics of aggregation have already been observed *in situ* using AFM for these surfaces **(Figs. 4–5)**. An important step towards more physiologically relevant surfaces has been recently made through the use of supported lipid bilayers ***(14)***. Substrates can provide insights into the potentially disruptive interactions of Aβ with cell membranes ***(14)***.

2. Aβ(42) has been shown to form fibrils at a higher rate than Aβ(40) ***(7,33)***. The difference between fibril formation by these two fragments could provide insights into an overall mechanism of the process. The different rates exhibited by the two different Aβ fragments can be taken advantage of in experimental design. For instance, the faster initial rate of Aβ(42) to form fibrils decreases the amount of time needed to observe the transformation of Aβ populations containing mostly protofibrils to a population containing mature fibrils. Conversely, Aβ(40), with its slower initial elongation rate, may be valuable in studying the initial formation of protofibrils and other Aβ oligomers that may be important in the amyloid pathway.
3. *Ex situ* AFM studies support the formation of protofibrils as an intermediate to the formation of mature amyloid fibrils, as protofibrils (3 nm by 20–70 nm) were observed and eventually disappeared as mature fibrils were formed ***(29)***. Subsequent *ex situ* AFM experiments showed that the formation of amyloid fibrils could be seeded by preformed fibrils, indicating the importance of nucleation ***(28)***. Another *ex situ* AFM study has shown that the elongation process of fibrilization is first order ***(21)***, and a different *ex situ* AFM experiment revealed protofibrils that were composed of individual aggregates of Aβ ***(23)***.
4. In situ AFM studies provide the unique opportunity to study Aβ fibrilization under physiological conditions at different stages of fibrillogenesis. This technique is useful for imaging initial aggregates of Aβ, the formation of protofibrils, and the maturation of fibrils. It has been shown that under physiological conditions, in contact with hydrophilic surfaces, Aβ will form 5–6 nm tall particulate aggregates that eventually form protofibrils **(Fig. 4; *22*)**. On hydrophobic surfaces, Aβ forms elongated approx 1 nm tall and approx 19 nm wide ribbons **(Fig. 5; *22*)**. *In situ* AFM studies have also been used to show the growth of protofibrils into mature fibrils over time **(Fig. 6; *22*,*26*)**.
5. Due to viscous damping when imaging in fluids, the mechanical quality factor, Q, of the cantilever is significantly reduced, resulting in larger imaging forces (tapping forces). The forces associated with imaging could disturb fragile aggregates or even inhibit their formation. Furthermore, tapping forces could be especially important in the studies of the interaction of Aβ with other molecules, such as potential drugs, since they could perturb or disrupt the interaction. In order to limit the invasiveness of the probe used to image Aβ and its interactions with other molecules *in situ*, the force used needs to be minimized. It has been demonstrated recently that tapping forces can be significantly reduced through the use of active resonance Q control ***(34–37)***. The lower force makes it possible to study weak interactions that are otherwise disrupted by the large forces normally associated with *in situ* imaging. Active Q control instrumentation is now commer-

cially available from different vendors ***(38,39)***. Lowering the tapping force could also open the way to the use of sharper tips (such as carbon nanotubes), by reducing the pressure exerted by the sharper tips on the samples, and the possibility of damage to the tips themselves.

6. Another important consideration when using *in situ* tapping mode AFM is the mode of excitation of the cantilever. There are currently two commonly used methods, acoustic excitation ***(18,19)*** and magnetic excitation ***(40,41)***. Acoustic excitation uses the sound waves produced by vertical oscillation of the piezoelectric scanner ***(18,19)***. In magnetic excitation, a magnetically coated cantilever is oscillated by passing an alternating current through the solenoid located in the vicinity of the cantilever ***(40,41)***. The advantage of this mode is that it does not rely on oscillation of the liquid, which could be potentially disturbing to the sample. Acoustic excitation is more economical since it does not require expensive magnetically coated tips.

Acknowledgments

Financial support from NIH (P50-AG05681) and from Carnegie Mellon University (start-up grant to T.K.) is gratefully acknowledged.

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