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Potential Artifacts in Sample Preparation Methods Used for Imaging Amyloid Oligomers and Protofibrils due to Surface-Mediated Fibril Formation

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Abstract

Accurate imaging of nanometer-sized structures and morphologies is essential to characterizing amyloid species formed at various stages of amyloid aggregation. In this report, we examine the effect of different drying procedures on the final morphology of surface-mediated fibrils formed during the incubation period, which may be then mistaken as oligomers or protofibrils intentionally formed in solution for a particular study. AFM results show that some artifacts, such as globules, flake-like structures, and even micrometer-long fibrils, can be produced under various drying conditions. We also demonstrate that one can prevent drying artifacts by using an appropriate spin-coating procedure to dry amyloid samples. This procedure can bypass the wetting/dewetting transition of the liquid layer during the drying process, and preserve the structure of interest on the substrate without generating drying artifacts.

Graphical Abstract

Potential artifacts in the AFM study of surface-mediated fibrils due to sample preparation protocols.

Keywords

amyloid fibrils; amyloid beta peptide; surface effect; surface-mediated fibrillization; drying artifacts

Introduction

The formation of amyloid fibrils is one of the hallmarks of neurodegenerative diseases (*e.g.* Alzheimer's disease and Parkinson's disease) and other conditions such as type II diabetes.¹ In most *in-vitro* experiments, the fibril formation in bulk is through nucleation and growth mechanisms, and requires the sample concentration to be higher than the critical concentration for nucleation.^{2,3} Structural evolution of these fibrils can be monitored by spectroscopic techniques (*e.g.* thioflavin T, or ThT, fluorescence assay, CD spectroscopy, IR spectroscopy, and NMR spectroscopy)^{4,5}, and then visualized by single molecule techniques, such as transmittance electron microscopy (TEM) and atomic force microscopy (AFM).⁶⁻⁹ For both popular tools, samples are typically dried at various aggregation stages for further characterization of the amyloid species prepared in solution. Among different types of aggregates, structured oligomers have been recognized as potentially more toxic species than other structures.¹⁰⁻¹² However, characterizing these small structures is still challenging, particularly due to challenges associated with proper sample preparation.

One of the primary challenges in sample preparation stems from surface effects catalyzing amyloid fibril formation and generating oligomeric and/or fibrillar structures during drying. Recent studies indicate that the liquid-substrate interface can provide an alternative route to form amyloid fibrils that differs from bulk mechanisms, often allowing aggregation at concentrations well below the critical concentration for amyloid nucleation.¹³⁻¹⁷ Under such conditions, the mobility of surface-bound proteins or peptides as well as high surface concentration are important factors in determining the rate of aggregation.^{13,14} As demonstrated in our previous work, in the presence of a mica substrate, surface-mediated fibrils (mostly in mono-layer form with maximum height of ~ 0.5 nm) can form quickly (within 30 minutes).¹³ Since these surface-mediated fibrils are themselves mobile on the surface, specific attention must be paid during sample preparation to retain structures of interest and prevent potential artifacts due to aggregation during drying. Advanced fast-scanning AFM¹⁸ can bypass the drying procedure, but imaging such tiny features with high accuracy is very difficult at high imaging speeds and mobile species can potentially move on the surface during imaging.

For studies of the morphologies of amyloid species formed in the bulk (*e.g.* mature fibrils or toxic oligomers), during sample preparation one needs to incubate the sample solution on the substrate prior to the drying and subsequent imaging processes. The underlying assumption is that the amyloid structures formed in solution can physically deposit on the substrate in ratios similar to those found in bulk. However, if the monomers are not completely depleted, the surface-mediated fibrillization process can potentially compete with fibril deposition during the incubation period and generate new species that did not exist in the bulk solution. Consequently, some fine structures formed by the surface-mediated process can be misinterpreted as the products of bulk aggregation. Furthermore, inappropriate drying procedures can also introduce morphological changes during drying due to self-concentration and surface rearrangement of the deposited fibrils.^{19,20} Therefore, understanding the potential drying-artifacts in studies of surface-mediated fibril formation and for oligomer imaging becomes crucial in avoiding these situations.

In this study, various drying procedures are employed to prepare surface-mediated fibrils for AFM imaging. We demonstrate that spin-coating at slow rates can prevent drying artifacts for the surfaces that are already covered by some physically adsorbed protofibrils during incubation. In contrast, fast-spinning without incubation can help to trap larger species of fibrils, but may also produce aggregates due to the formation of small droplets that can be mistaken as oligomers. By establishing procedures for proper drying, one can prevent the formation of such artifacts and enhance the ability to accurately identify the existence of oligomers and protofibrils in bulk samples.

Materials and Methods

Sample Solutions

Synthetic A β _{12–28} peptide (purity > 95%, rPeptide) was directly used to prepare sample solutions without further purification. The A β _{12–28} peptide was first dissolved in 1 % NH₄OH_(aq) at a concentration of 1 mg/ml. The solution was sonicated for 2 minutes, and subsequently diluted with Milli-Q water to a concentration of 10 μ M. To remove large aggregates, the solution was filtered using a 0.22 μ m PTFE membrane syringe filter. Since A β _{12–28} does not have an absorbance band in the UV-VIS region, this commonly used method could not be employed to determine the exact concentration of the sample. The real sample concentration is expected to be lower than the reported value, as it is likely that some peptides were absorbed on the vial's walls. The peptide solutions were incubated at room temperature without agitation for durations of three hours (3H), two days (2D), or three days (3D) for further experiments. The aggregation of the mature fibril sample was performed by shaking a 100 μ M peptide solution (same preparation procedure as used for the low-concentrated peptide solution) at 37 °C and 800 rpm on an IKA MS3 digital orbital shaker in parafilm-sealed 1.7 mL Eppendorf tubes to ensure minimal solvent evaporation.

Cryogenic Transmission Electron Microscopy (CryoTEM)

An aliquot volume of 5 μ L sample solution (10 μ M, 3H or 3D peptide solution) was deposited on a lacy carbon-coated copper grid (Ted Pella, Inc.) in a humidity and temperature controlled environment vitrification system (Vitrobot, FEI, Inc.). The humidity was controlled at 100% and the temperature of the chamber was 22.0 °C. The sample was then blotted to remove excess solvent and create a thin film of the peptide on the grid surface using a 2 mm blotting force, a 1.0 s blot time, and a 10 s drain time to equilibrate any stresses in the sample caused by blotting. The resulting sample grid was vitrified by plunge freezing into liquid ethane (–183 °C). A Tecnai T-12 TEM operating at 120 kV was used to obtain the cryoTEM images.

Thioflavin T (ThT) Fluorescence Measurements

30 μ L peptide solutions (3H and 2D) were separately mixed with 100 μ L ThT solution (20 μ M in PBS buffer solution). The fluorescence spectra were recorded on a Tecan M1000 multimode plate reader, with excitation at 440 nm.

Spin Coated Samples

Three droplets of as-prepared incubated peptide solutions (3H or 2D) were placed on fresh-cleaved mica and were either spin-coated immediately, or incubated for 30 minutes before the spin-coating process. Spin-coating (WS-650 MZ-23, Laurell Technologies Corp.) was used to dry the samples under various spinning rates in the range of 1000 to 3000 RPM. The acceleration rate was 250 RPM/s for all spun-cast samples, except for the fast-spinning samples displayed in Figures 2B and 2F, where 400 RPM/s was used.

Kimwipe-prepared and Nitrogen-dried Samples

Three droplets of peptide solution (3H or 2D) were incubated on the mica substrate for 30 minutes. To remove the excess solution, the samples were blotted by Kimwipe or gently dried using a weak nitrogen stream after rinsing with 0.2 mL Milli-Q water. The Kimwipe-prepared samples dried rapidly without the need for nitrogen blowing. During both procedures, the substrate was tilted 30–45° to prevent the solution from flowing backwards. The total drying times for Kimwipe and nitrogen-dried procedures were estimated to be 5 and 8 minutes, respectively.

Atomic Force Microscopy (AFM)

The samples prepared using various drying procedures were imaged in tapping mode using a Keysight 5500 AFM instrument (Keysight Technologies) equipped with a closed-loop scanner. Rotated silicon probes with aluminum reflex coating (BudgetSensors, Tap-300G, resonance frequency ~300 kHz, tip radius <10 nm, force constant 40 N/m) were used to record topographic, amplitude, and phase images with 512×512 pixel resolution. For each sample, various areas on the sample were imaged and statistically relevant images were chosen to present in this manuscript (more examples shown in Figure S1 of SI). For the spinning samples, the images were taken from the central region of the substrate to avoid the potential effects at the edges of the sample, where the drying rate could be slower. An example of such an effect is reported in our previous report¹⁴. The AFM images were analyzed by Gwyddion package. A third-order polynomial was used to flatten the background for topographic images. The analyses of globular structures in spinning control section were performed by the grain analysis tool with structural mask in the Gwyddion software.

Experimental Results

Structural Characterization of Peptide Solutions

To study potential artifacts of preparing dry samples, we first examined amyloid structures formed in the as-prepared low-concentration peptide solutions using cryoTEM and ThT fluorescence measurements. CryoTEM can directly visualize structures present in the solution and ThT examines the β -sheet content, *i.e.* the existence of amyloid fibrils, in solution. In cryoTEM images (Figure 1A), only spherical dispersions with average diameter of 49.4 ± 7.9 nm and 27.2 ± 6.1 nm were observed in three hour (3H) and three day (3D) sample solutions, respectively. No fibrillar structures or larger aggregates were detected in either sample. We note that since no fibrillar structures were observed in the cryoTEM

imaging of the 3D solution, they should also be absent in the two day (2D) solution because the bulk aggregation mechanism is a time-dependent nucleation-growth process and should proceed further after 3 days. As shown in Figure 1B, the ThT fluorescence experiments display a weak increase of intensity peaked at 480 nm, roughly 2–3 times higher than the control solution (pure water mixed with ThT solution). When amyloid fibrils existed in the solution, the ThT fluorescence increased more than one hundred-fold for the ThT concentration used here. Therefore, the ThT fluorescence measurements also suggest no significant amounts of fibrils were formed in the solution. Both cryoTEM and ThT results are consistent with the fact that the peptide solutions were prepared with a concentration below the critical concentration for nucleation (see discussion in Supporting Information), and as such the peptides were not expected to aggregate into amyloid fibrils prior to drying.

Surface-Mediated Fibrils under Various Drying Processes

In the absence of surface-mediated fibrillization, samples prepared using these peptide solutions should only yield globular aggregates as suggested by the cryoTEM results. However, recent studies show that fibrils can quickly form in the presence of surfaces.^{13–16} To investigate the potential artifacts induced by surface-mediated fibrillization during the incubation and drying processes, all samples studied in this section were first incubated on the substrate for 30 minutes, before drying under various methods. We have previously demonstrated that this incubation period will result in the formation of short protofibrils that are only a monolayer thick.¹⁴ The samples were then subjected to various drying processes, including slow-spinning (1000 RPM), fast-spinning (3000 RPM), Kimwipe-drying, or nitrogen-blowing methods, and then imaged by AFM under ambient conditions. More details of these procedures are described in the *Materials and Methods* section. Statistically representative morphologies observed for each drying method are displayed in Figure 2, with some characteristic features analyzed in Figure 3. The control experiments of the non-incubated samples prepared using various drying processes are demonstrated in Figure 4A, 4C, and S7 of SI.

In the top row of Figure 2, images of slow-spinning samples are shown with isotropically distributed protofibrils on the mica substrate with feature heights of ~0.5 nm (a1 and b1 features in Figures 2A and 2B). This is expected as we have previously demonstrated that surface diffusion of adsorbed peptides can result in the formation of surface-mediated fibrils under these conditions.¹⁴ The 2D solution formed slightly longer fibrils compared to the 3H solution. When the spinning speed is increased to 3000 RPM, the main features observed on the surface are globular structures (c2 and d2 in Figure 2C and 2D). Some surface mediated fibrils (c1 and d1 structures) could also be observed on these samples. The fibrils in the background are the surface-mediated fibrils formed on the surface during the incubation period. At 3000 RPM, the solution droplet ruptures during spinning and the globular structures are attached to the surface as the small droplets on the surface dry to leave their content behind. The morphologies observed on the slow-spinning and fast-spinning samples are consistent with our previous work, and demonstrate that the 1000 RPM speed, which is below the rupture threshold of the solutions used here, can preserve the surface-mediated fibrils on the substrate and avoid the deposition of peptides and aggregates from the solution

during spinning.¹⁴ A more detailed comparison of the effect of spin speed on morphology will be addressed in the next section.

Kimwipe-drying or nitrogen-blowing processes are also widely used to prepare samples for TEM and AFM imaging.^{13,15,21–23} Figures 2E and 2F display images of the surface after a Kimwipe was used to blot the excess solution after the formation of surface-mediated fibrils such as those shown in Figures 2A and 2B. The Kimwipe-dried samples exhibited sparse, short protofibrils (Figure 2E for the 3H peptide solution) and long, well-oriented fibrils (Figure 2F for the 2D peptide solution), covering the entire substrate. Despite changes in the length of the fibrils observed in both samples, the heights of the protofibrils were near 0.5 nm, a signature of surface-mediated fibrils,¹⁴ as shown in Figure 3A (e1 and f1). It is important to note that the surface morphologies of the Kimwipe-prepared samples prepared by 2D peptide solution were not homogeneous. In some areas, the fibrils' length and density were like the slow-spinning samples (Figure S1B). This is an indication that the surface-mediated fibrils could rearrange during Kimwipe drying to form longer fibrils. Since the heights of the structures didn't change, it is unlikely that the fibrils were detached from the surface during the drying process. It is also unlikely that any of the content from the solution, such as aggregates, were attached to the surface during this procedure.

Nitrogen-dried samples showed an even more dramatic change in their surface morphologies, showing diverse morphologies such as fibrillar structures (h1), small (g2' and h2') and large globular structures (g2 and h2), and flake-like geometries (g3 and h3) as shown in Figures 2G and 2H. The heights of fibrillar and large globular structures were 0.5 nm and 2.0 nm, respectively (Figure 3A and 3B), and were similar to the ones observed in the slow- and fast-spinning samples. Two other types of structures, small globular structures and flake-like geometries, were scarcely observed in the samples prepared by other drying procedures. The heights of these structures are measured to be near 1.2 nm and 0.5 nm for the samples prepared by 3H and 2D peptide solution, respectively. As such, it is likely that some form of deposition from the solution occurred during nitrogen drying, likely due to the formation of small droplets on the surface. More AFM images of these samples in different areas are provided in Figure S1.

The Influence of Spinning Speed on Sample Preparation

As demonstrated in the previous section, different spinning speeds can influence the morphologies observed on the substrate. To further study the influence of spinning speed, the morphologies of the samples dried at various spin speeds were studied under two different conditions. One set of samples were spun-cast without any incubation on mica (Figure 4A–C); another set were incubated for 30 minutes before spinning (Figure 4D–F). The difference between these two sets of samples is the formation of surface-mediated fibrils on the second set before the start of the spin casting process.

In Figure 4A–C, in the absence of surface-mediated fibrils, only globular structures are observed in the samples that were not incubated. In contrast, with increased spinning speed, the incubated samples with surface-mediated fibrils show morphological changes from fibrils to globular structures (Figure 4D–F). Several features are notable in these samples. First, globular structures are observed in all spun-dried samples, except for the one imaged

in Figure 4D. There, surface-mediated fibrils are present on the surface when the spinning speed is below the rupture threshold of the solution, a speed between 1000 and 1500 RPM (based on the observed morphological change from surface-mediated fibrils to globular structures, Figure S3). This observation indicates that the globular structures are the residual peptides and aggregates that are left behind once small droplets of water are dried on the substrate. Second, the sizes of these structures vary slightly different among these samples. After tracking thousands of globular structures in each sample, the average size and volume of the globular structures were measured as plotted in Figure 5 and summarized in Table S1. The data presented in Figure 5 clearly indicate that the dimensions of the aggregates became smaller when the drying speed is increased. At higher spin speeds, the size of the water droplets is smaller, leaving smaller amounts of residual peptides behind, resulting in smaller-sized aggregates. Overall, these observations further confirm that inside the water droplets left on the surface, there are no fibrils that had previously formed in the solution. Furthermore, the size of the aggregates are defined by the droplet size and as such, these globular structures are not the well-defined oligomers that had formed in solution. Third, besides the globules, self-assembled structures are also observed in the samples with 30 min incubation. For example, the zoomed-in image in the inset of Figure 4F shows a self-assembled structure that is composed of multiple surface-mediated fibrils, aggregated together. This is consistent with observations in Kimwipe and nitrogen drying procedures where the surface-mediated fibrils can rearrange during the drying of larger water droplets. Again, it is worth emphasizing that none of these features belong to the state of the peptide aggregation in the solution.

Discussion

The results presented in Figures 2 and 3 show that the method of sample preparation can have a strong effect on the morphology observed on the surface, despite identical initial sample preparation conditions. Under the conditions chosen for this study, in the solution phase, the amyloid structures are spherical dispersions with an average size smaller than 50.0 nm, or individual peptides that cannot be observed in cryoTEM. This result is consistent with the fact that a 0.22 μm size exclusion filter was used to remove large, insoluble amyloid species during the preparation of the peptide solution. Furthermore, in agreement with previous reports, the peptide concentration is too low for nucleation and growth of fibrils in solution, and the preparation time is shorter than the typical lag times for nucleation-limited aggregation mechanism (several hours to days)³. We attribute the slight increase of ThT fluorescence to the formation of the spherical objects observed in cryoTEM as previous reports indicate²⁴, as the fluorescence is not strong enough to originate from long mature fibrils. No evidence of the existence of smaller stable oligomers with well-defined sizes is observed in these samples. As such, all the protofibrils and aggregates observed under various drying conditions, after 30 min incubation period, are formed through surface-mediated fibril formation on the mica substrate and drying induced artifacts. When the samples were dried by spin-coating without incubation on the surface, only globular structures were observed, as shown in Figures 4 A–C with an average size that depends on the spin speed as shown in Figure 5. The average sizes at all speeds are slightly smaller than those measured in bulk by cryoTEM, suggesting that most of these aggregates

are formed by the peptide content left over after drying of small droplets, and that most of the peptides are in aggregates smaller than those observed by cryoTEM (most probably just single peptides). Thus, no stable well-defined oligomeric species is observed under the preparation conditions of this study.

Once surface-mediated fibrils are formed on a substrate, there are some limitations in the current techniques to directly image them in aqueous environments. First, their dimensions are too small to be effectively imaged with the more limited resolution of wet AFM, *e.g.* ~ 0.5 nm in height and 10–20 nm in width (Figure 3A). Second, they may be too mobile on the surface to be imaged in fluid for most imaging tools. This mobility can be seen in the change in peptide morphology and length upon drying using different methods as shown in Figure 2. Therefore, the sample needs to be dried for the operation of high-resolution AFM measurements, and also for preventing the mobility or growth of those fibrils during the imaging process. Here, four distinct procedures were used to dry the samples. Based on the experimental results, only the slow-spinning condition can gently remove peptide solution and excess material while keeping the surface-mediated fibrils intact on the substrate with isotropic distribution. The other drying procedures also had some surface-mediated fibrils dispersed on the substrate; for example, the morphologies of a1–h1 species labeled in Figures 2. However, other types of structures, such as globules, μm -long fibrils, and flake-like structures, were also observed in those samples, all due to various drying artifacts resulting from the motion of the surface-mediated fibrils during drying, as well as deposition of material from the solution.

For the slow-spinning samples, the one prepared by 3H peptide solution (Figure 2A) has relatively shorter fibrils and less surface coverage compared with the one prepared by 2D peptide solution (Figure 2E). This is most probably due to a difference in concentration between the solutions used to prepare the samples that would change the rate of peptide deposition and thus fibril growth on the surface. Unfortunately, we are unable to precisely define the final solution concentration due to the lack of chromophores in this peptide sequence. Instead, an example of the concentration effect on the length of surface-mediated fibrils is shown in Figure S4 to explain the dependence of the fibril length and surface coverage on the concentration of the bulk solution. Based on these results, the concentration of 3H peptide solution is expected to be slightly lower than that of the 2D peptide solution, for which longer fibrils are observed, despite their absence in either the 3H or 3D solution cryoTEM images.

The surface morphologies observed for the other samples are not only influenced by the peptide concentration, but also by the drying method. Assuming that the slow-spinning procedure can preserve surface morphologies at the drying stage, the morphological differences between slow-spinning samples (top row of Figure 2) and the other samples reflect the impacts of the different drying procedures. Under the Kimwipe-drying procedure, as shown in Figures 2A and 2B, the fibril distribution becomes less isotropic on the surface and areas with either low coverage (Figure 2E) or highly crowded areas with well-oriented long fibrils (Figure 2F) can be observed on the surface. In particular, the fibrillar patterns displayed in Figure 2F suggest that the induced liquid flow during the blotting step allows the fibrils to move around on the surface, merge to form longer fibrils, and orient along a

particular direction, presumably along the direction of blotting. These data also indicate that the surface-mediated fibrils are loosely bound to the substrate and can move with the flow, consistent with our earlier observations¹⁴. There is, however, no evidence that the fibrils can detach from the surface during this procedure, or that additional material from the solution is deposited on the surface during blotting.

For nitrogen-dried samples, large morphological changes are observed. The major morphologies observed on the surface are flake-like and globular structures that are completely different than the slow-spinning samples. The flake-like structures had been reported as intermediate species in an early aggregation stage based on *in-situ* AFM measurements.²⁵ However, in our case, they are evidently formed due to motion of surface-mediated fibrils during drying, as our control experiments indicate that these structures did not exist in solution. As such the flake-like structures observed here are formed during the drying period and are artifacts of the method used to prepare them. Unlike the self-assembled surface-mediated fibrils in the inset of Figure 4F, the flake-like structures have no fibrillar sub-morphologies, instead, only several size-mismatched globules can be found inside them. These structures are similar in size to globular structures (g2' and h2') observed in Figure 3C. These globules are likely formed due to droplets produced by the nitrogen-drying procedure that were then blown along the surface and stretched before drying.

Based on the differences observed between the morphologies of the slow-spinning and fast-spinning samples, the spinning process is not always appropriate for retaining the surface-mediated fibrils. The fast spin speed can deposit globular artifacts on the surface and eliminate or displace the surface-mediated fibrils. The formation of surface-mediated fibrils potentially modifies the surface chemistry, resulting in different morphologies and sizes of the globular objects. This is likely because changes in surface chemistry result in formation of slightly larger droplets during fast-spinning, so that larger globular structures are formed. Therefore, we prepared two sets of samples with two different incubation periods (0 or 30 minutes) before performing experiments at different spinning speeds. Surface-mediated fibrils were only observed in samples with pre-incubation on mica (Figure 4D–F). This is consistent with the fact that the surface-mediated fibril formation mechanism is a kinetically-controlled process, which is on the time scale of the 30 minute incubation. The usage of 1000 RPM, a speed below the rupture threshold (1500 RPM), seems to be the best condition to keep these surface-mediated fibrils undisturbed on the surface. When the speed is above 1500 RPM, the globules appear and become the major species observed on the surface. A dimensional analysis (Figure 5) indicates the dimension of the globules decreases with increasing spinning speed. Finally, the distribution of globular structures, especially the network-like, polygonal geometries displayed in Figure 4E, are like the dewetting pattern. Such topographies have been widely observed in thin polymer or glass films, caused by the balance between interfacial tensions.^{26,27} The wetting or dewetting behavior during spin-coating may explain why some spinning speeds are not appropriate to dry the samples. The detail of this process merits further investigation and will be addressed in the future.

It is worth noting that if one desires to measure a species already formed in solution, as opposed to the surface-mediated fibrils, then the best strategy is to avoid any incubation time

on the surface, or ensure that no oligomers are present in the solution. One can also try multiple incubation times, to distinguish surface-mediated structures that grow with time from those that do not change and are formed in solution. It is also important to note that if spin-coating is used to dry such samples, the spin speed can affect the process, and some globular structures can be due to the aggregation of oligomers or single peptides as small droplets are dried. Such structures would change size with spin-speed, while well-defined oligomers formed in solution are expected to be invariant and insensitive to spin-speed. Using such control experiments are imperative for proper identification of important oligomeric species in experiments performed on dried samples. Besides the hydrophilic mica substrate presented in this study, other types of substrates – such as highly oriented pyrolytic graphite (HOPG), silicon, and cover glass – are also widely used to prepare dried samples for various studies. Drying can be different on various substrates due to the variations in substrate interactions, which may cause different types of artifacts than those presented in this study. In any case, these potential artifacts need to be carefully examined when preparing samples.

Conclusions

In summary, we have shown how the drying procedure can influence the morphologies of surface-mediated fibrils of A β _{12–28} peptides. Under the conditions used in this study, these self-assembled fibrils are loosely bound on the substrate, and can be either washed or rearranged and aligned to form μ m-long fibrils in the direction of flow. Other artifacts, including globules and flakes, can also be observed on samples prepared by two widely used procedures, Kimwipe and nitrogen drying. Spin-coating provides a path to preserve these structures on the surface. If spun below the rupture threshold of the solution, between 1000 RPM and 1500 RPM, surface-mediated fibrils stay intact during the drying stage. These results suggest the importance of the choice of the drying procedure and control experiments for preparing peptide samples that have weak interactions with the substrate, or include large concentrations of individual peptides. If the aim is to image amyloid species already formed in the solution, *e.g.* oligomers and mature fibrils, the best strategy is to avoid long incubation times on the surface prior to the drying stages in order to prevent self-assembled fibrils from forming. Furthermore, the structural variations under different drying methods can be compared to distinguish the species from the solution from those formed on the surface due to drying of droplets and other similar factors. Our measurements provide procedures and guideline to properly prepare samples both for studies of the formation of surface-mediated fibrils, as well as imaging of oligomers and fibrils formed in solution that need to be dried prior to imaging.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Chiti F, Dobson CM. Protein Misfolding, Functional Amyloid, and Human Disease. *Annu. Rev. Biochem.* 2006; 75(1):333–366. [PubMed: 16756495]
2. Hamley IW. The Amyloid Beta Peptide: A Chemist's Perspective. Role in Alzheimer's and Fibrillization. *Chem. Rev.* 2012; 112(10):5147–5192. [PubMed: 22813427]
3. Knowles TPJ, Vendruscolo M, Dobson CM. The Amyloid State and Its Association with Protein Misfolding Diseases. *Nat. Rev. Mol. Cell Biol.* 2014; 15(6):384–396. [PubMed: 24854788]
4. Nilsson MR. Techniques to Study Amyloid Fibril Formation in Vitro. *Methods.* 2004; 34(1):151–160. [PubMed: 15283924]
5. Yoo, SII, Yang, M., Brender, JR., Subramanian, V., Sun, K., Joo, NE., Jeong, SH., Ramamoorthy, A., Kotov, NA. Inhibition of Amyloid Peptide Fibrillation by Inorganic Nanoparticles: Functional Similarities with Proteins. *Angew. Chemie - Int. Ed.* 2011; 50(22):5110–5115.
6. Eva A, Vestergaard B, Langkilde AE, Vestergaard B. Methods for Structural Characterization of Prefibrillar Intermediates and Amyloid Fibrils. *FEBS Lett.* 2009; 583(16):2600–2609. [PubMed: 19481541]
7. Bhatia NK, Deep S. Diagnostic Tools for Structural Characterization and Elucidation of Fibrils and Their Precursors in Amyloid Fibril Formation Pathway. *J. Proteins Proteomics.* 2013 Dec.4:149–164.
8. Raigoza AF, Dugger JW, Webb LJ. Review: Recent Advances and Current Challenges in Scanning Probe Microscopy of Biomolecular Surfaces and Interfaces. *ACS Appl. Mater. Interfaces.* 2013; 5(19):9249–9261. [PubMed: 23848270]
9. Morton KC, Baker LA. Atomic Force Microscopy-Based Bioanalysis for the Study of Disease. *Anal. Methods.* 2014; 6(14):4932–4955.
10. Chen SW, Drakulic S, Deas E, Ouberaï M, Aprile FA, Arranz R, Ness S, Roodveldt C, Williams T, De-Genst EJ, et al. Structural Characterization of Toxic Oligomers That Are Kinetically Trapped during α -Synuclein Fibril Formation. *Proc. Natl. Acad. Sci.* 2015; 112(16):E1994–E2003. [PubMed: 25855634]
11. Ono K, Condron MM, Teplow DB. Structure-Neurotoxicity Relationships of Amyloid Beta-Protein Oligomers. *Proc. Natl. Acad. Sci. U. S. A.* 2009; 106(35):14745–14750. [PubMed: 19706468]
12. Nagel-Steger L, Owen MC, Strodel B. An Account of Amyloid Oligomers: Facts and Figures Obtained from Experiments and Simulations. *ChemBioChem.* 2016; 17(8):657–676. [PubMed: 26910367]
13. Shen L, Adachi T, VandenBout D, Zhu X-Y. A Mobile Precursor Determines Amyloid- β Peptide Fibril Formation at Interfaces. *J. Am. Chem. Soc.* 2012; 134(34):14172–14178. [PubMed: 22867029]
14. Lin Y, Petersson EJ, Fakhraai Z. Surface Effects Mediate Self-Assembly of Amyloid- β Peptides. *ACS Nano.* 2014; 8(10):10178–10186. [PubMed: 25229233]
15. Charbonneau C, Kleijn JM, Cohen Stuart Ma. Subtle Charge Balance Controls Surface-Nucleated Self-Assembly of Designed Biopolymers. *ACS Nano.* 2014; 8(3):2328–2335. [PubMed: 24571369]
16. Gao G, Zhang M, Lu P, Guo G, Wang D, Sun T. Chirality-Assisted Ring-Like Aggregation of $A\beta$ (1–40) at Liquid-Solid Interfaces: A Stereoselective Two-Step Assembly Process. *Angew. Chemie.* 2015; 127(7):2273–2278.
17. Shezad K, Zhang K, Hussain M, Dong H, He C, Gong X, Xie X, Zhu J, Shen L. Surface Roughness Modulates Diffusion and Fibrillation of Amyloid- β Peptide. *Langmuir.* 2016; 32(32):8238–8244. [PubMed: 27466062]

18. Watanabe-Nakayama T, Ono K, Itami M, Takahashi R, Teplow DB, Yamada M. High-Speed Atomic Force Microscopy Reveals Structural Dynamics of Amyloid β 1–42 Aggregates. *Proc. Natl. Acad. Sci.* 2016;201524807.
19. Deegan RD, Bakajin O, Dupont TF, Huber G, Nagel SR, Witten TA. Capillary Flow as the Cause of Ring Stains from Dried Liquid Drops. *Nature.* 1997; 389(6653):827–829.
20. Michen B, Geers C, Vanhecke D, Endes C, Rothen-Rutishauser B, Balog S, Petri-Fink A. Avoiding Drying-Artifacts in Transmission Electron Microscopy: Characterizing the Size and Colloidal State of Nanoparticles. *Sci. Rep.* 2015; 5:9793. [PubMed: 25965905]
21. Jiménez JL, Nettleton EJ, Bouchard M, Robinson CV, Dobson CM, Saibil HR. The Protofilament Structure of Insulin Amyloid Fibrils. *Proc. Natl. Acad. Sci. U. S. A.* 2002; 99(14):9196–9201. [PubMed: 12093917]
22. Paulite M, Fakhraai Z, Li IT, Gunari N, Tanur AE, Walker GC. Imaging Secondary Structure of Individual Amyloid Fibrils of a beta2-Microglobulin Fragment Using near-Field Infrared Spectroscopy. *J. Am. Chem. Soc.* 2011; 133(19):7376–7383. [PubMed: 21524071]
23. Yates EA, Owens SL, Lynch MF, Cucco EM, Umbaugh CS, Legleiter J. Specific Domains of Abeta Facilitate Aggregation on and Association with Lipid Bilayers. *J. Mol. Biol.* 2013; 425(11): 1915–1933. [PubMed: 23524134]
24. Younan ND, Viles JH. A Comparison of Three Fluorophores for the Detection of Amyloid Fibers and Prefibrillar Oligomeric Assemblies. ThT (Thioflavin T); ANS (1-Anilinonaphthalene-8-Sulfonic Acid); and bisANS (4,4'-Dianilino-1,1'-Binaphthyl-5,5'-Disulfonic Acid). *Biochemistry.* 2015; 54(28):4297–4306. [PubMed: 26087242]
25. Lin D, Luo Y, Wu S, Ma Q, Wei G, Yang X. Investigation of the Aggregation Process of Amyloid- β -(16–22) Peptides and the Dissolution of Intermediate Aggregates. *Langmuir.* 2014; 30(11): 3170–3175. [PubMed: 24588450]
26. Cai Y, Newby BZ. Dewetting of Polystyrene Thin Films on Poly(ethylene Glycol)-Modified Surfaces as a Simple Approach for Patterning Proteins. *Langmuir.* 2008; 24(10):5202–5208. [PubMed: 18407678]
27. Liu D, Wang T, Keddie JL. Protein Nanopatterning on Self-Organized Poly(styrene-B-Isoprene) Thin Film Templates. *Langmuir.* 2009; 25(8):4526–4534. [PubMed: 19275129]

Figure 1. Structural characterization of the peptide solutions. (A) Representative cryoTEM images of the 3H and 3D sample solutions. Red arrows indicate the globular aggregates. (B) Fluorescence signal of ThT solution mixed with different sample solutions, compared with ThT solution mixed with pure water. The concentrations of 3H and 2D peptide solutions were 10 μ M. The inset figure displays the ThT fluorescence response of the fibril sample.

Figure 2.

Representative AFM images of surface-mediated fibrils dried under slow-spinning (A and B), fast-spinning (C and D), Kimwipe (E and F), and nitrogen-blowing (G and H) procedures. the left column shows samples produced from the 3H solution and the right column shows samples prepared from the 2D peptide solution. All samples were incubated on mica substrate for 30 minutes before drying. The scale bars in each panel are 500 nm and 2 μ m for the left and the right images, respectively. The dimensions of morphologies highlighted using white rectangles are extracted and plotted in Figure 3.

Figure 3.

Cross-sectional line profiles along characteristic morphologies observed in Figure 2. The heights and widths of the (A) fibrillar structures, (B) large globular structures, (C) small globular structures, and (D) flake-like geometries are extracted from one of the features labeled in Figure 2 using white rectangles. The colors are consistent with labels in Figure 2.

Figure 4.

Spinning speed control for the spin-coating process. The 3H peptide solution was used to deposit on mica, and dried immediately (A–C) or dried after 30 minutes of incubation (D–F) at different spinning speeds. The spin speed was 1000 RPM for (A and D), 2000 RPM for (B and E), and 3000 RPM for (C and F), respectively. The inset figure in (F) is the zoomed-in image of self-assembled fibrils (2 $\mu\text{m} \times 2 \mu\text{m}$). The results for other spinning speeds, 1500 and 2500 RPM, are provided in Figure S3 of SI. The scale bar in each image is 2 μm .

Figure 5.

The average size (A) and volume (B) of the globular structures formed under different spinning speeds. R_{eq} is the average equivalent radius of the globular structures that is proportional to the size of globules. The error bars are the root mean square of the size variations and the lower bound of the error in volume is zero.