

Environmental Control of Amyloid Polymorphism by Modulation of Hydrodynamic Stress

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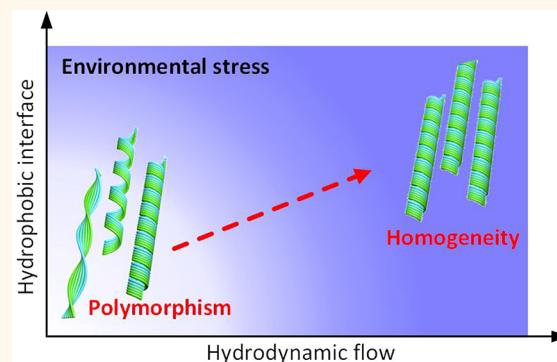
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ABSTRACT: The phenomenon of amyloid polymorphism is a key feature of protein aggregation. Unravelling this phenomenon is of great significance for understanding the underlying molecular mechanisms associated with neurodegenerative diseases and for the development of amyloid-based functional biomaterials. However, the understanding of the molecular origins and the physicochemical factors modulating amyloid polymorphs remains challenging. Herein, we demonstrate an association between amyloid polymorphism and environmental stress in solution, induced by an air/water interface in motion. Our results reveal that low-stress environments produce heterogeneous amyloid polymorphs, including twisted, helical, and rod-like fibrils, whereas high-stress conditions generate only homogeneous rod-like fibrils. Moreover, high environmental stress converts twisted fibrils into rod-like fibrils both in-pathway and after the completion of mature amyloid formation. These results enrich our understanding of the environmental origin of polymorphism of pathological amyloids and shed light on the potential of environmentally controlled fabrication of homogeneous amyloid biomaterials for biotechnological applications.

KEYWORDS: protein aggregation, amyloid polymorphism, environmental control, air–water interface, hydrodynamic flow



Pathological amyloid formation is a significant feature of many neurodegenerative disorders, such Parkinson's disease, that is related to the misfolding and aggregation of the α -synuclein protein.^{1–4} However, amyloids are not only related to disease, and biocompatible functional amyloid materials with exceptional mechanical properties have been gaining increasing interest in biomedical and biotechnological applications, such as biosensors and biomimetic materials.^{5–7}

Amyloid polymorphism refers to the diversity of amyloid fibrils' architecture and is a widespread phenomenon *in vitro* and *in vivo*.^{8–12} Understanding the underlying mechanisms and pathways of polymorphic amyloid formation is of great importance for both biological and materials science applications. On one hand, various polymorphs of pathology-related amyloid fibrils *in vivo*^{10,13} show distinct biological effects in living systems, *e.g.*, prion strain and cytotoxicity.^{12,14} On the other hand, the performances of functional amyloid materials strongly depend on homogeneity, mesoscale structure, and the deriving mechanical properties,^{9,11,15–17} and the control of amyloid polymorphism could contribute to the development of functional biomaterials.^{18–21}

Environmental conditions in the incubation solution are responsible for the modulation of the properties of many biological systems and amyloid formation.^{22–24} Various intrinsic and extrinsic environmental factors have shown significant influence on the process of fibrillar polymorphism²⁵ and their aggregation kinetics.^{26,27} It was previously suggested that the interplay among amyloid polymorphs can be controlled by intrinsic kinetics parameters²⁸ and induce amyloid polymorphs.^{8,9,29} Furthermore, extrinsic factors, such as hydrophobic air–water interfaces,^{30–32} have shown to significantly accelerate primary nucleation,^{30,33,34} which may impact the morphology of protein aggregates.^{25,30,32,35} Similarly, the presence of hydrodynamic flows strongly influences aggregation kinetics by promoting secondary

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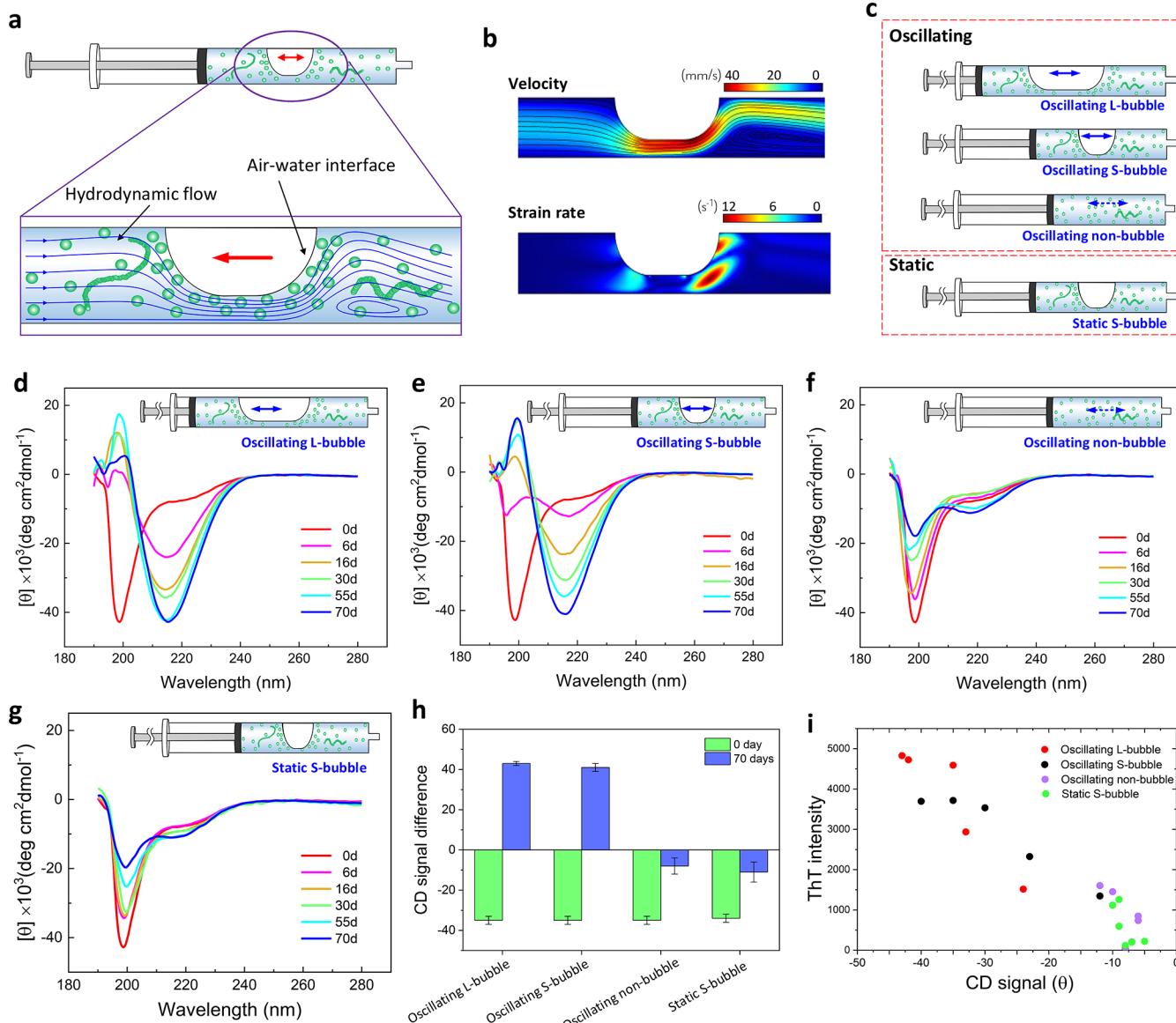


Figure 1. (a) Schematics of the oscillating bubble in the syringe of protein solution exposed to both hydrodynamic and interfacial effects. (b) CFD simulation of oscillating S-bubble condition showing the hydrodynamic flow field and strain rate distribution. (c) Schematics of experimental syringes in the oscillating and static conditions. (d–g) Evolution of CD spectra under the oscillating L-bubble (d), oscillating S-bubble (e), oscillating nonbubble (f), and static S-bubble (g) conditions. (h) Difference in CD signal between α -helix and β -sheet content after 0 and 70 days of incubation. Error bar represent standard error. (i) Correlation of the CD spectroscopy and ThT fluorescence to characterize the formation of amyloid fibrils.

nucleation^{33,34,36} and protein–protein interaction,^{26,36–38} suggesting a further influence on fibrillar polymorphism. Hydrodynamic and interfacial effects often show a synergistic impact in many physiological circumstances,^{33,39,40} such as bloodstream to cerebrovascular system.^{41,42} Thus, it is of fundamental importance to understand how these extrinsic environmental conditions affect the polymorphism of the aggregates. However, the influence of environmental factors on amyloid polymorphism is still not clear, and the precise modulation of their biophysical properties is still a fundamental challenge in bioscience.

In this work, we achieve the control of the polymorphism of α -synuclein (α -Syn) amyloid fibrils by modulation of the degree of hydrodynamic stress in solution. This approach is realized by regulating the size of an air–water interface introduced in the incubation tube in the form of an air bubble

and the strength of hydrodynamic flow induced by rotation of the tube in a microgravity random positioning machine. We then pursue a single aggregate structural characterization^{43–45} of polymorphism as a function of the variation of hydrodynamic stress in solution and demonstrate that the control of amyloid polymorphism can be achieved on-pathway or after the formation of mature amyloid fibrils. These results contribute to the understanding of the origin of pathological amyloid polymorphism, as well as shed light on controlling fibrillar polymorphism, in order to produce homogeneous amyloid-based functional biomaterials.

RESULTS AND DISCUSSION

Control of Environmental Hydrodynamic Stress during α -Synuclein Aggregation. The study of α -Syn amyloid formation was carried out by controlling the presence

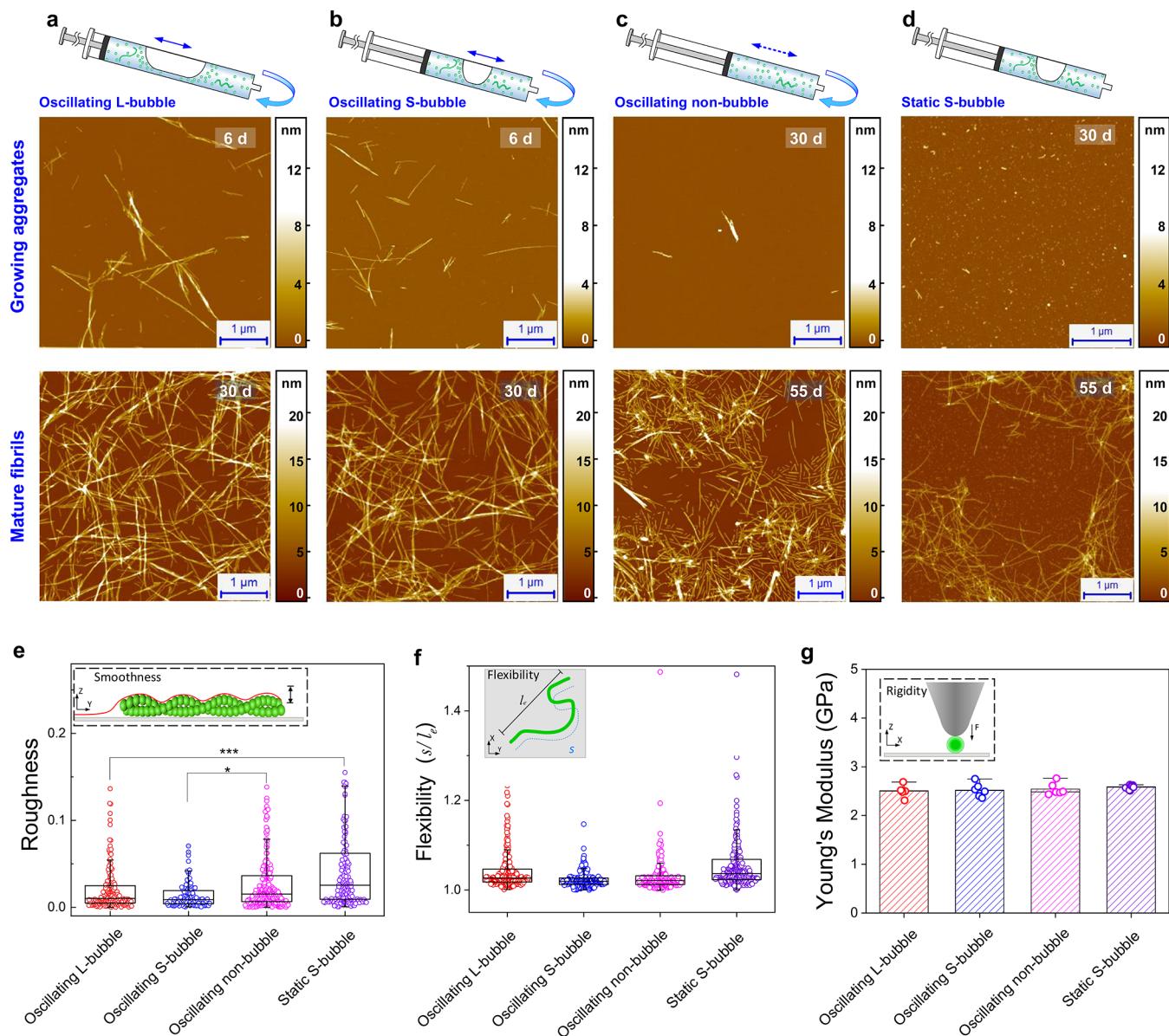


Figure 2. AFM images and statistical analysis on the representative features of amyloid fibrils. (a–d) AFM images of growing aggregates and mature fibrils from each studied condition at the concentration of 30 μM . **(e–g)** Statistical investigation on the morphological and mechanical properties of more than 150 mature fibrils in each condition, including the roughness (e), flexibility (f), and Young's modulus (g). Statistically significant differences are noted (*: $p < 0.05$, **: $p < 0.001$) that were determined by using two-way ANOVA with Tukey's posthoc correction. Error bar, SD.

and the motion of an air bubble introduced in the protein solution incubated in a syringe rotating in a random positioning machine (RPM) (Figure 1a,b).^{46–48} The bubble introduces an air–water interface, and the rotation at a low speed allows a gentle reciprocating motion of the bubble in the syringe, which further induces a hydrodynamic flow in solution (see Methods and Figure S1). The protein was incubated at the concentration of 30 μM .

To study the effect of the hydrodynamic flow and air–water interface systematically, three syringes of α -Syn solution were prepared with different bubble sizes: large bubble (oscillating L-bubble), small bubble (oscillating S-bubble), and no bubble (oscillating nonbubble), as shown in Figure 1c. The volume ratios of protein solution to bubble in the large and small bubble conditions were maintained 2:1 and 4:1, respectively, in the experiment (see Methods). Since both the hydrodynamic

flow and hydrophobic air–water interface could influence the kinetics of α -Syn fibril formation,^{34,35} we also studied as control the aggregation of α -Syn in another syringe with a small bubble and incubated in the static condition (static S-bubble) to discriminate the effect of the air–water interface alone.

In the case of the oscillating L/S-bubble conditions, the gentle bubble movement (Figure 1a) led to a combined environmental stress, originated from the air–water interface and the generated hydrodynamic flow. To quantify the strength of these hydrodynamic and surface effects, we performed a computational fluid dynamics (CFD) simulation (Figures 1b and S2). The simulation indicated the main hydrodynamic contribution, the strain rate (Methods), which refers to the elongation flow fields.³⁶ The simulation showed that the hydrodynamic flow is as large as ~45 mm/s and

generates high kinetics stress with a strain rate of $\sim 12\text{ s}^{-1}$, in both oscillating L-bubble and oscillating S-bubble conditions. No significant difference of flow field and strain rate values was observed between these two conditions (Figure S2). Therefore, the main difference between the oscillating L-bubble and oscillating S-bubble conditions was the different surface to volume ratio of the air–water interface. The volume of the air bubble in the former case was maintained at twice larger than that of the latter case in this experiment. We then refer to these two conditions as *high hydrodynamic stress environments*.

By contrast, the protein solution in the oscillating conditions in the absence of any air–water interface (oscillating nonbubble) was affected only by the residual hydrodynamic stress due to the agitation of dissolved microbubbles, which are always present in solution,^{49,50} and the insoluble protein aggregates forming as a function of time. Similarly, the protein solution in the static condition (static S-bubble) was only exposed to an air–water interface in the absence of any hydrodynamic effect. We therefore refer to these two conditions as *low hydrodynamic stress environments*.

Bulk Characterization of α -Syn Amyloid Fibril Formation. The structural evolution of α -Syn aggregates was investigated by circular dichroism (CD). This technique enables monitoring the secondary structure conformation of protein during amyloid formation. Prior to incubation, α -Syn showed a characteristic absorption at 199 nm (Figure 1d–g), indicating an intrinsic disordered random-coil structure that is characteristic of monomeric proteins. In all conditions, as a function of time, this peak gradually shifted to a minimum at 218 nm, demonstrating a conversion from random coil to β -sheet conformation and indicating the formation of cross β -sheet amyloid fibrils.

In the oscillating L-bubble condition (Figure 1d), already after 6 days, the absorption dip at 199 nm disappeared and the dip at 218 nm increased in the spectrum. The structural transition in oscillating S-bubble (Figure 1e) condition showed a slightly slower but similar trend. By contrast, the spectral variations in the oscillating nonbubble (Figure 1f) and static S-bubble (Figure 1g) conditions occurred at similar and much slower speed. During 70 days of incubation, a gradual decrease of the dip at 199 nm and a slight rise of the dip at 218 nm were simultaneously observed on the spectra. Furthermore, the smaller amplitude of the peak at 218 nm in these two conditions, after 70 days of incubation, suggested the formation of a lower quantity of amyloid fibrils when compared to the oscillating L/S-bubble conditions at the same time point of aggregation.

The CD results showed a significant formation of β -sheet content in the high hydrodynamic stress environment of the oscillating L/S-bubble conditions (Figure 1h), whereas this conversion was less extensive under low environmental stress conditions, *i.e.*, under RPM oscillation of the solution or in the presence of a static air–water interface. These results were confirmed by a thioflavin T (ThT) fluorescence assay (Figure S3 and Supplementary Text 1). The ThT signals reached the final plateau of the aggregation reaction after 25 days, in the oscillating L/S-bubble conditions, while the plateau was reached after more than 50 days in the oscillating nonbubble and static S-bubble conditions. We then plotted the intensity of ThT fluorescence *versus* the CD intensity at 218 nm (Figure 1i) to confirm the tendency of protein aggregate formation in both conditions of high and low hydrodynamic environmental stress.³⁴

Single Aggregate Characterization of α -Syn Amyloid Formation.

The morphological differences of amyloid aggregates play a critical role in determining various biological activities, *e.g.*, cytotoxicity,^{12,14} and biomaterial performance such as mechanical properties.^{18–21} After the bulk characterization, we then employed high-resolution atomic force microscopy (AFM) to assess morphological features of amyloid fibrils at the single-aggregate level.

We first imaged the process of amyloid formation as a function of the incubation time (Figure 2a–d), from the initiating monomeric protein (Figure S4). Then, we characterized the morphological characteristics of the formed fibrils. We evaluated the length and average height of the fibrils, as well as their smoothness, flexibility, and stiffness (Figure 2e–g and Figures S5–7). The smoothness described the surface roughness of the fibrils^{21,51} (Figure S5). The flexibility, characterized by the ratio of contour length s to end-to-end length l_e (s/l_e , Figure S8), is related to the fibril stiffness and to its interstrand cross-sectional arrangement.^{44,52–54} A lower value of flexibility is related to an increase of the bending rigidity, which is correlated to the mechanical properties of the aggregates. To correlate the flexibility to the Young's modulus of mature fibrils, we thus performed AFM quantitative nanomechanical mapping of Young's modulus²⁰ (Figure 2g, Figure S9 and Supplementary Text 2).

Morphological and kinetics differences in the aggregation of the protein under the conditions investigated (high and low hydrodynamic environmental stress) became gradually evident as a function of the incubation time, as shown in Figure 2a–d. Prior to incubation, α -Syn protein was in the monomeric and oligomeric state (Figure S4). In the oscillating L-bubble condition (Figure 2a and red boxes in Figure 2e–g), protofibrils and fibrils formed only after 6 days, while abundant mature fibrils were obtained after 30 days of incubation. Similarly, the oscillating S-bubble conditions (Figure 2b and blue boxes in Figure 2e–g) promoted fibril formation, and abundant mature fibrils were detected after 30 days of incubation. Interestingly, the morphological features of mature fibrils yielded in the two high-stress conditions are comparable, with similar fibril average length and diameter (Figure S6). In both conditions, the fibrils showed relatively low flexibility and low roughness (Figure 2e,f).

In the oscillating nonbubble condition (Figure 2c and magenta boxes in Figure 2e–g), the trend of amyloid fibril formation was slower. Protomembranous and fibrillar aggregates were detected after 30 days, and short mature fibrils (average length $0.2 \pm 0.1\text{ }\mu\text{m}$) were formed after only 55 days of incubation. We could demonstrate that these are mature fibrils since they possess high roughness and rigidity (Figure 2e,f), with a relatively uniform average height of $5 \pm 0.7\text{ nm}$ (Figure S6).

By contrast, the slower static S-bubble condition (Figure 2d and purple boxes in Figure 2e–g) showed the abundant formation of only protomembranous aggregates (height of peaking $3.1 \pm 0.2\text{ nm}$) after 30 days,^{30,34} and only after 55 days we had the formation of an abundant population of mature amyloid fibrils with an average height of $5.2 \pm 0.4\text{ nm}$ (Figures S6 and S7).

Overall, the protein solutions under high environmental stress showed the abundant formation of amyloid fibrils after 6 days of incubation, while the fibrils under low environmental stress formed amyloid fibrils only after 50 days of incubation.

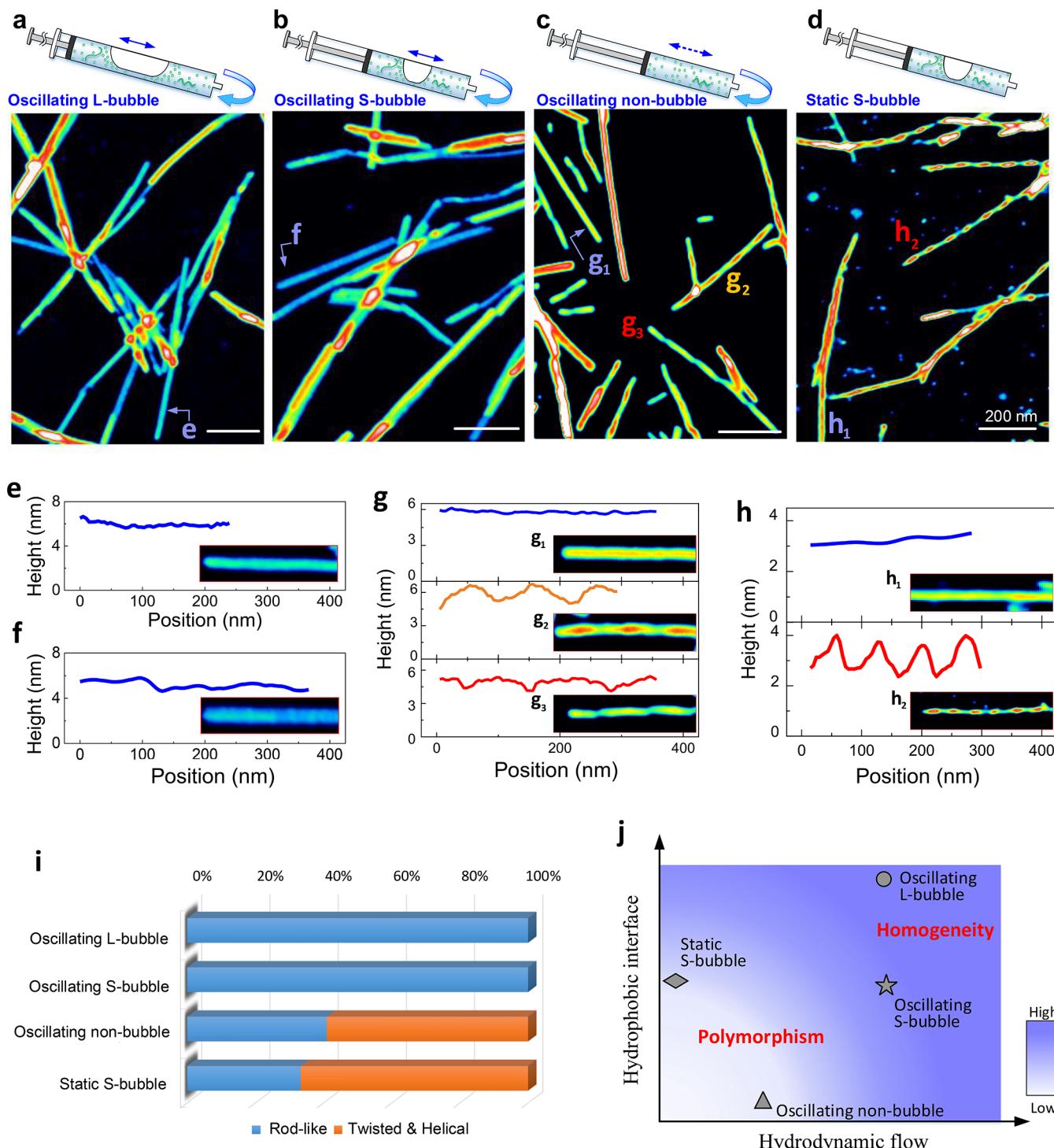


Figure 3. Fibril polymorphism and its correlation with environmental stress. **(a–d)** AFM images of fibrils formed. In the oscillating L-bubble (**a**) and oscillating S-bubble (**b**) conditions, we observed only rod-like fibrils (**e, f**). In the oscillating nonbubble (**c**) and static S-bubble (**d**) conditions, we observed a mixture of twisted, helical, and rod-like polymorphs (**g, h**). **(i)** Statistics of amyloid polymorphism in each condition from more than 100 mature fibrils. **(j)** Plot of environmental stress of each incubation condition originated from hydrodynamic flow and air–water interface to indicate the dependence between fibril polymorphism and environmental stress. The strength of the blue color indicates the strength of the environmental stress, where the deep-blue zone produces homogeneous fibrils and the light-blue zone generates polymorphic fibrils.

Single-Aggregate Characterization of α -Syn Amyloid Polymorphism. As a major advantage of AFM over other imaging methodologies, we performed nanoindentation measurements to prove that we were comparing only the morphology of mature amyloid fibrils. The measured Young's

modulus was \sim 2.5 GPa (Figures 2g and S9), as expected for mature amyloid fibrils and supported by the ThT assay (Figure S2). Being sure to compare the structural state of amyloid fibrils at the same final stage of amyloid formation, we performed the statistical investigation of their polymorphism

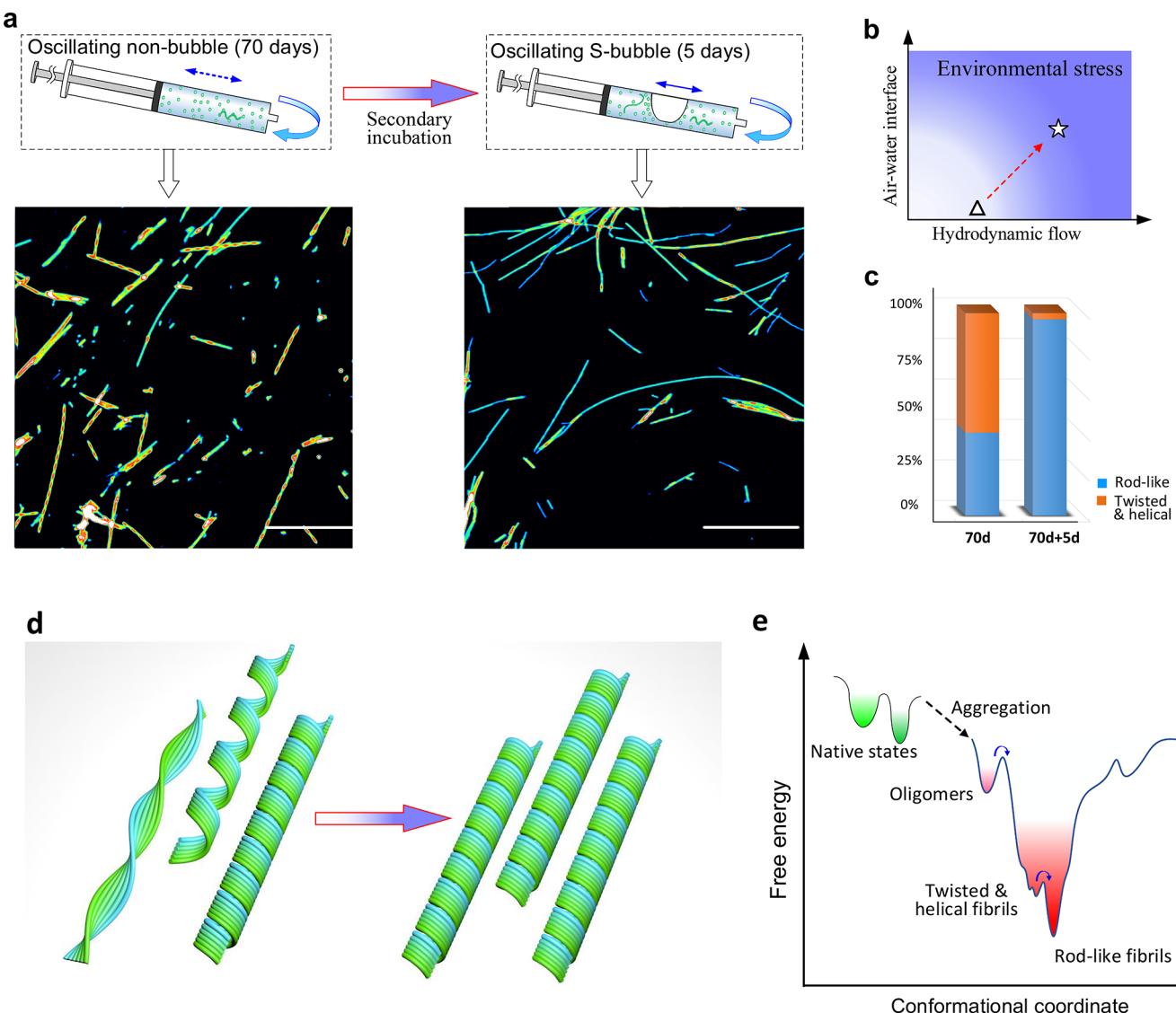


Figure 4. Order–order transition of amyloid polymorphs by mediating environmental stress. (a) Protein solution incubated after 70 days in the oscillating nonbubble condition yielded mature fibrils with heterogeneous morphologies (left). After the secondary incubation of 5 days in the oscillating S-bubble condition, these fibrils transformed into rod-like fibrils (right). (b, c) Variation of the environmental stress (b) and the statistics of fibril polymorphs (c) before and after the transition based on the observation of more than 150 amyloid fibrils. (d) Schematics of the transition from polymorphic fibrils to homogeneous rod-like fibrils. (e) Funnel-like energy landscape map of the amyloid polymorphs in this experiment.

(Figure 3a–d). The oscillating L-bubble and oscillating S-bubble conditions promoted the formation of homogeneous rod-like fibrils (Figures 3a,b and e,f), which indeed had very low surface roughness (Figure 2e). Multiple fibril polymorphs were found in the oscillating nonbubble and static S-bubble conditions, including rod-like,^{8,16,12,18} helical,^{8,9,16,18} and twisted^{8,15,16,18} fibrils (Figure 3c,d and g,h). Thus, the incubation conditions with high environmental stress generated homogeneous rod-like fibrils, whereas the conditions with weak stress could lead to polymorphic fibrils. This result is supported by the previous observations on the fibril formation influenced by hydrodynamic flow⁵⁵ or interfacial factor^{35,56} individually, as well as their synergistic effect.⁵⁷

The different degree of polymorphism in the different conditions is related to the environmental stress, which can thus be used to control the polymorphism of amyloid fibrils.^{11,17,28,58} In this designed experiment, the environmental

stress in the oscillating nonbubble and the static S-bubble condition were relatively weak, as shown on the environmental stress map (Figure 3j). This weak stress allows multiple fibrillization pathways and thus yields heterogeneous fibrils, including twisted, helical, and rod-like polymorphs, while the high environmental stress in the oscillating L-bubble and oscillating S-bubble conditions leads to the homogeneous formation of the most thermodynamically stable rod-like fibrils.

Controlling and Modulation of Amyloid Polymorphism. To further demonstrate the capability of environmental stress in controlling amyloid polymorphism, we considered the secondary incubation under high environmental stress of mature amyloid fibrils formed under low environmental stress. First, the protein solution was incubated for 70 days in the oscillating nonbubble condition (low environmental stress), which yielded mature twisted and rod-like amyloid fibrils, as confirmed also by the ThT fluorescence

assay (Figure S3). Then, this solution was further incubated for 5 days in the oscillating S-bubble condition (high environmental stress), as shown in Figure 4a. This approach resulted in changing environmental stress of the final fibrillar products of aggregation from low to high stress (Figure 4b). Prior to the secondary incubation, under low environmental stress, we detected the coexistence of multiple polymorphs including twisted and rod-like fibrils. However, after 5 days in the oscillating S-bubble condition, in high environmental stress, a clear transition was observed from heterogeneous polymorphs to homogeneous rod-like fibrils, as demonstrated by a single aggregate statistical investigation (Figure 4c). Thus, we demonstrated that the amyloid fibril polymorphism could be successfully modulated by varying environmental stress in solution, even after mature fibril formation.

We suggest that lower environmental stress results in the formation of several polymorphs of amyloid fibrils because it could allow a higher number of multiple aggregation pathways compared to the higher environmental stress, which result in the formation of homogeneous and more thermodynamically stable rod-like fibrils (Figure 4d). Furthermore, the correlation between amyloid polymorphism and environmental stress could also be discussed in terms of the energy landscape of protein aggregation. From an energetic point of view, the environmental-stress-dependent transition from twisted and helical fibril polymorphs to rod-like fibrils suggests that amyloid polymorphs in this experiment may hold certain levels of free energy and structural stability, as shown in Figure 4e. The twisted and helical fibrils are the metastable and transformable species that occupy a higher level on the energy landscape and form under low environmental stress. Under high environmental stress, the fibrils are able to transit to rod-like polymorphs that occupy a lower energy level. Thus, in our conditions, the rod-like fibrils are the thermodynamically most stable form with minimum free energy in the energy landscape.^{8,18,29,59,60} In addition, parallel stacked rod-like fibrils, also termed multistranded ribbon, were observed occasionally in the high-environmental-stress conditions after long-term (70 days) incubation (Figure S10 and Supplementary Text 3), which might also maintain a similar or even lower energy level compared to rod-like fibrils.^{9,59}

CONCLUSIONS

In summary, our results demonstrate that amyloid fibril polymorphism can be modulated by the environmental hydrodynamic stress in solution, both in-pathway and after mature amyloid fibril formation. We observed that polymorphic twisted and rod-like amyloid fibrils were formed in low hydrodynamic environmental stress conditions, while homogeneous rod-like fibrils were yielded in the high hydrodynamic environmental stress conditions. The results suggest that twisted, helical, and rod-like fibrils may occupy distinct free-energy levels on the energy landscape. Twisted and helical fibrils are metastable states that transit to the more thermodynamically stable and energetic-favorable rod-like polymorphs with a lower energy level. The environmental control in solution of the order-order transition from heterogeneous (twisted, helical, rod-like polymorphs) to homogeneous (rod-like) fibrils may facilitate the production of homogeneous protein aggregates from heterogeneous amyloid polymorphs. This approach shows great potential for the development of future functional amyloid-template

biomaterials, with homogeneous conformation for materials science and biomedical applications.

METHODS AND MATERIALS

Sample Preparation. Full-length recombinant human α -synuclein (AnaSpec, USA) was dissolved in buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) and filtered with a 100 kDa filter. The concentration of the α -Syn solution was measured by ultraviolet absorbance (Nanodrop 2000). An aliquot (1 mL) of α -Syn solution was sealed in sterilized low protein binding wall syringes and placed in different incubation conditions (Figure S1) at a concentration of 30 μ M at 37 °C. Three syringes were incubated in the absence and presence of a large or small bubble in the RPM conditions, and another syringe with a small bubble was statically incubated. In the large (L-bubble) and small bubbles (S-bubble) the ratio of the volume of protein solution was maintained at 2:1 and 4:1, respectively, throughout the experiment. The size of the surface of the air bubble in the L-bubble condition is roughly 2–3 times larger than that in the S-bubble condition.

Rotating Bioreactor System. The three oscillating syringes with different sizes of bubbles were incubated on the platform of an in-house-developed random positioning machine at 37 °C. The rotation of the platform was regulated by two independent motors in three dimensions, which enables the rotation in random directions. The samples were fixed at the center of the RPM, and a rotation speed of RPM of \sim 5 rpm was used to reduce the gravity effects of sedimentation and to maintain a gentle bubble oscillation in the syringe.

CFD Simulation. To quantify the hydrodynamics generated by bubble motion, we performed a CFD simulation using the finite elements method. The oscillating bubble generates a hydrodynamic flow in the capillary, and its flow field is mainly contributed by the strain rate (Figure S2), referring to the extensional flow field. This strain rate (elongational) is known to be crucial for protein aggregation due to its ability to stretch protein molecules and to increase protein kinetics compared with the shear rate.³⁶ In Figure 1b, the strain rate maximized, on the order of 10 s⁻¹, at the rear and at the front side of the bubble, due to an intermediate Reynolds number ($Re = 60$) of the protein solution. Meanwhile, no significant differences of flow field and strain rate values were observed between the oscillating L-bubble and the oscillating S-bubble conditions (Figure S2).

The CFD simulations were carried out by using the laminar flow module of the finite element method (FEM) solver Comsol (COMSOL Inc., MA, USA). To account for the flow created by the buoyancy-driven bubble oscillation in the experiments, we performed the simulation in the frame of the bubble. The dimensions of the capillary were 1 × 16 cm, while the height of the bubble was 7 mm. The no-slip boundary condition was applied to the walls of the capillary, and the interface of this air bubble was considered to be shear-free (full slip) as a reasonable assumption for air–liquid interfaces. A flow of constant flow rate $q = Vd$ was injected from the left of the capillary, where d is the capillary width and V is the mean velocity of the flow. Here we took $V = 10$ mm/s to meet the velocity of bubble motion in the experiment, which corresponds to a Reynolds number:

$$Re = \rho Vd / \mu = 60$$

where ρ and μ are the density and viscosity of the protein solution, respectively. We neglected the orientation variation of the oscillatory flow, as the hydrodynamic flow can be represented in each cycle and the strain rate distributes mainly due to the expansion of the flow independent from bubble oscillation. Navier–Stokes equations were solved in the bidimensional domain composed of 18 433 mesh elements and 801 boundary elements (corresponding to 28 854 degrees of freedom solved). The simulations converged, and doubling the number of mesh elements led to the same results. The strain rate (st) is defined in the following as

