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A Clear View of Polymorphism, Twist, and Chirality in Amyloid Fibril Formation

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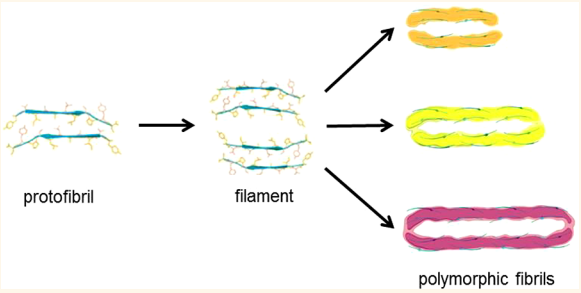
ABSTRACT The self-assembly of protein molecules into highly ordered linear aggregates, known as amyloid fibrils, is a phenomenon receiving increasing attention because of its biological roles in health and disease and the potential of these structures to form artificial proteinaceous scaffolds for biomaterials applications. A particularly powerful approach to probe the key physical properties of fibrillar structures is atomic force microscopy, which was used by Usov *et al.* in this issue of *ACS Nano* to reveal the polymorphic transitions and chirality inversions of amyloid fibrils in unprecedented detail. Starting from this study, this Perspective highlights recent progress in understanding the dynamic polymorphism, twisting behavior, and handedness of amyloid fibrils and discusses the promising future of these self-assembling structures as advanced functional materials with applications in nanotechnology and related fields.



Amyloid and amyloid-like fibrils are elongated nanostructures that self-assemble from a diverse range of peptides and proteins. They are characterized by the cross-β motif that composes their core and consists of an array of β-strands oriented perpendicular to the long axis of the fibril and stabilized by a dense network of hydrogen bonds. They represent a generic class of highly ordered nanomaterials that have been found in a variety of functional and pathological roles in nature.[1] It has been well-established that the phenomenon of amyloid formation is associated with several debilitating disorders including Alzheimer's, Parkinson's, and Creutzfeldt–Jakob diseases and type II diabetes.[1−3] At the same time, amyloid forms of proteins have also been found in several physiologically beneficial roles such as algal adhesives,[4] bacterial coatings,[5] and templates for melanin polymerization in mammalian melanosomes.[6]

The robustness of amyloid structures as fibrillar materials, which is comparable to collagen and dragline silk,[7] in conjunction with the discovery of functional amyloid, has inspired several research groups to explore the use of protein self-assembly in the synthesis of artificial materials.[8] For example, amyloid fibrils have been used as biotemplates in the formation of silver nanowires[9] and light-harvesting nanomaterials[10] as well as storage depots for the controlled release of biologically active peptides.[11] In this context, understanding the fundamental properties of amyloid structures, such as their atomistic assembly, polymorphism, and chirality inversion, is crucial not only to gain insight into disease states but also to elucidate further applications of these structures in nanotechnology.

In this issue of *ACS Nano*, Usov *et al.* present key steps towards understanding the physical basis of amyloid fibril self-assembly using high-resolution atomic force microscopy studies.[12] This work reveals the hierarchical assembly and polymorphism of such structures in unprecedented detail. In particular, the authors demonstrate the existence of six different classes of bovine serum albumin (BSA) fibrils and discover polymorphic transitions and chirality inversions that occur as a function of time.[12] By performing a statistical analysis on atomic force microscopy (AFM) images, they determine the handedness inversion of globular protein fibrils in response to increasing structural complexity. Initially, they detect flexible

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left-handed structures that undergo one of two observed transitions to form rigid fibrils. In the first mechanism, a single left-handed fibril transforms from a twisted ribbon into a helical ribbon that subsequently evolves into a nanotube-like structure[12] similar in nature to the end products of other types of peptide self-assembly phenomena.[9,13] In the second pathway described in this study, two left-handed twisted ribbons combine to form a right-handed twisted ribbon, which finally transforms into a rigid right-handed helical ribbon.[12]

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These results open up the possibility of using the control of chirality to access new forms of protein and peptide nanostructures and show how the twist of such structures can control their final morphology. Insights into these mechanisms can have significant implications into the growing field of peptide nanotechnology. One of the most promising routes toward the scalable manufacture of high-performance nanomaterials relies on "bottom-up" approaches, in which a small number of simple precursor molecules spontaneously assemble into hierarchical nanostructures. To this extent, protein nanofibers displaying characteristics of amyloid fibrils have been produced from crude mixtures of crystalline proteins.[14] Gaining a deeper understanding of the mechanisms of chiral self-assembly has the potential to extend significantly the palette of nanostructures that can be fabricated through such approaches.

Polymorphism. Morphological variations in amyloid fibrils, of the type described by Usov *et al.* for fragments of BSA and characterized by visually striking AFM images,[12] are increasingly emerging as a general feature of the self-assembly of proteins into fibrillar structures. This characteristic has been of particular interest in the context of the involvement of protein aggregation in disease and is thought to represent the basis of the propagation of prion strains.[15] In this phenomenon, a prion protein can support the propagation of multiple strains, each of which results in a distinct pathology in the host, but without variations in the sequence of the prion protein itself. The molecular basis of this process has been ascribed to a polymorphism in the structures of the prion aggregates, and in agreement with this view, AFM studies have been able to highlight distinct nanoscale differences in the morphologies of an *in vitro* model of the propagation of mammalian prions.[16] Interestingly, the morphological differences that were identified as key factors in the prion strain phenomenon influence the twisting behavior of the resulting structures in a manner closely analogous to the twist documented by Usov *et al.*[12]

Such polymorphic behavior has also been observed in the assembly of other pathological protein aggregates. Investigations of the main component of inclusions related to Parkinson's disease, α-synuclein, revealed that fibrils exhibit polymorphism in response to changes in the solution conditions during self-assembly; aggregates formed at pH 7.0 and 6.0 were fibrillar, while those formed at pH 5.0 and 4.0 were amorphous.[17] Moreover, a study of the 40-residue amyloid β peptide (Aβ40), which is associated with Alzheimer's disease, showed that fibrils formed under quiescent or agitated conditions exhibited distinct morphologies, which led to differing cytotoxicities in a neuronal cell culture model.[18] The molecular basis for these different morphologies was attributed to the variation of molecular structure at the protofilament level. Crucially, when seeded with a parent fibril of a specific strain, both the molecular structure and its resultant morphology are self-propagated.[18]

Polymorphic variations of amyloid fibrils have also been studied to gain structural insight about the packing mechanisms of their constituent protofilaments. For instance, an investigation of fibrils composed of the peptide hormone insulin that contained 2, 4, or 6 protofilaments revealed that these protofilaments exhibited a compact shape and consistent size.[20] Recently, atomic-scale structures of entire amyloid fibrils have become available, covering length scales associated with atomic distances within the β-sheet core structure to their defining nanoscale morphology of protofilament assembly. In particular, a study of the 11-residue fragment of transthyretin, TTR(105−115), has exemplified in atomic detail the hierarchical nature of the amyloid assembly phenomenon and shown that significant polymorphism can emerge through variations in the manner in which protofilaments come together to form mature fibrils; the protofilaments were found to self-assemble into three distinct morphologies that differ in their twisting behavior with helical pitches of 84, 121, and 154 Å.[21] While these fibrils are polymorphic in terms of their cross sections, number of constituent protofilaments, and twist, they share the structural similarities of two-fold electron density symmetry and left-handedness, and each consist of a double-layered helical ribbon with a hollow core.[21]

A common feature emerging from the phenomenon of polymorphism in amyloid fibril formation is the accurate propagation of the morphology through the ability of monomers to adopt the structure

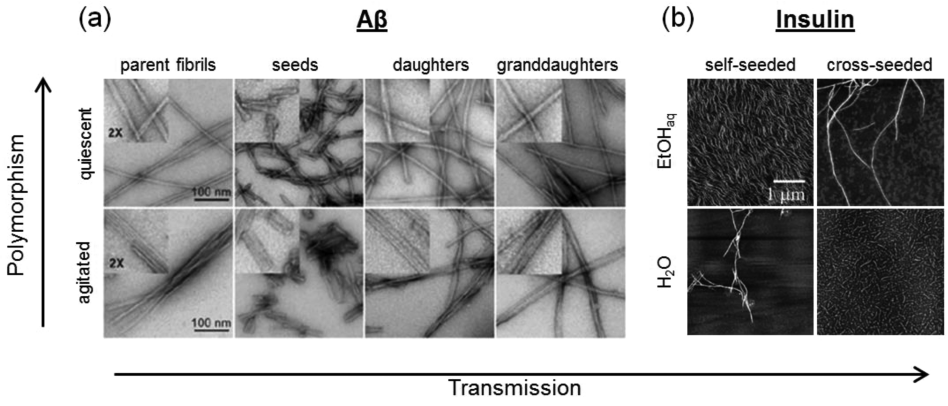

Figure 1. Polymorphism in natural amyloid fibrils. (a) Transmission electron micrographs of amyloid fibrils formed by the Aβ40 peptide. Parent fibrils were prepared under either quiescent or agitated conditions, and daughter and granddaughter fibrils were seeded with fragments of parent and daughter fibrils, respectively.[18] Reproduced with permission from ref 18. Copyright 2005 American Association for the Advancement of Science. (b) Atomic force microscopy (AFM) images of insulin fibrils formed in ethanol or water; when fibrils are cross-seeded with fragments of the other polymorph, the morphology of the parent fibril is propagated.[19] Reproduced with permission from ref 19. Copyright 2005 Physical Chemistry Chemical Physics Owner Societies.

imposed by the seed aggregate onto which they become attached, a phenomenon often called "templating". Furthermore, it has become apparent that fibrils can multiply in number through secondary processes such as fragmentation.[22] These features suggest that the preferential propagation of specific structures within a filament population could form the basis for the fabrication of highly uniform amyloid-based nanomaterials.

> With a highly stable and ordered molecular structure, amyloid fibrils are promising candidates as uniform self-assembled scaffolds in biomaterials.

Twist. Since different fibril polymorphs can exhibit a range of helical pitches, filament twist emerges as a key marker of strains within a polymorphic filament population. A study of insulin, SH3, and TTR-(105−115) demonstrated that amyloid fibrils maintain their torsional fluctuations, and hence their twist, over length scales corresponding to several thousands of molecules along the fibrils' axes.[23] This high level of order suggests that the molecular structure of a fibril is propagated to all new molecules added to the fibril and that this structure is accurately reflected in nanoscale characteristics such as the fibril twist. Thus, with a highly stable and ordered molecular structure, amyloid fibrils are promising candidates as uniform self-assembled scaffolds in biomaterials.

The helical pitch of fibrils has generally been found to increase with the width of the filament. In particular, a detailed investigation of four polymorphic structures formed by β-lactoglobulin revealed that an increase in periodicity (35, 75, 100, and 135 nm) correlated very strongly with a linear increase in fibril diameter (4, 6, 8, and 10 nm).[24] Furthermore, it has been shown that it is possible to modify the periodic twist of amyloid fibrils at the postsynthetic stage in response to changes in the ionic strength of the environment, indicative of the ability of amyloid fibrils to act as adaptive materials capable of responding to stimuli from environmental changes.[25] The ribbon-like cross section, periodicity, and height distributions suggest hierarchical multistranded self-assembly of filaments into helical aggregates that arises from the competition between the intrinsic chirality of the peptide building blocks that favors twisted structures and a mechanical strain that suppresses twisting.[26]

The competition between these two opposing driving forces leads to the finite level of twist observed in the majority of amyloid fibrils. A prediction following from these arguments is that, as filaments become thicker, the penalty for twisting increases, and that it may be energetically more favorable to adopt an untwisted microcrystalline arrangement at larger widths. Consistent with this idea, untwisted amyloid-like microcrystals have been observed for a wide variety of different sequences of short peptides,[27,28] whereas at the small diameters observed in fibrils, the twisted morphology prevails. In this sense, it is the chiral nature of the filaments that controls their width and establishes a connection between the atomistic forces in the core of amyloid fibrils and their nanoscale properties.[26,29] Similar findings have been reported in the context of sickle hemoglobin fibers suggesting universality in the role of twist in stabilizing protein aggregates.[30]

Chirality. As discussed in relation to the work of Usov *et al.*, high-resolution AFM can also be used to observe the handedness of fibrils,

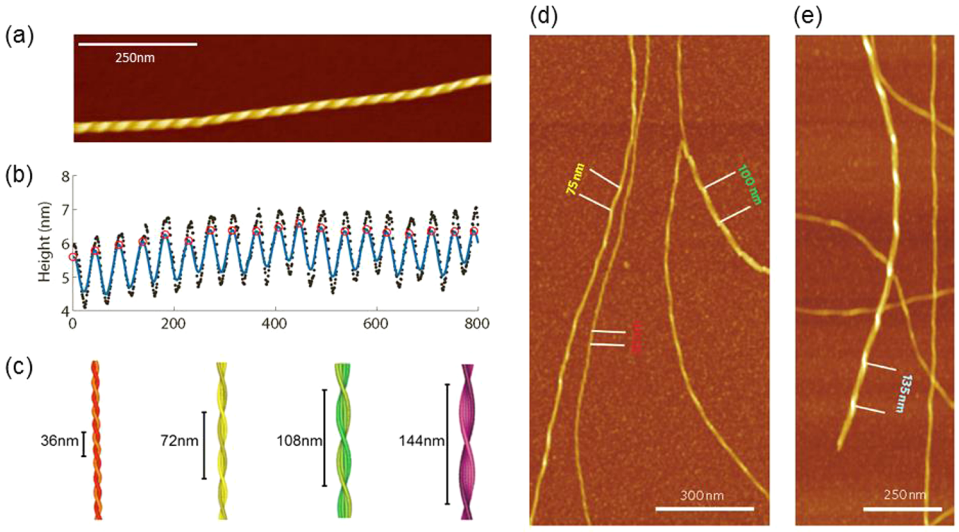

Figure 2. Variations in twist among distinct polymorphs of amyloid fibrils. (a) Atomic force microscopy (AFM) topographic image of a twisted two-filament insulin fibril.[23] (b) Height profile along the fibril backbone depicting its periodic twist.[23] (a,b) Reproduced with permission from ref 23. Copyright 2006 American Physical Society. (c) Coarse-grained molecular dynamics reconstructions of left-handed fibrils formed by the twisting of two to five filaments, corresponding to the fibrils shown in (d) and (e).[24] (d,e) Helical pitches of β-lactoglobulin fibrils as shown by AFM height images.[24] (c–e) Reproduced with permission from ref 24. Copyright 2010 Macmillan Publishers Ltd.

another important parameter in determining mechanisms of self-assembly.[12] As all biologically relevant proteins are composed of L-amino acids, it might be expected that amyloid fibrils would exhibit a fixed handedness in all cases, in a manner analogous to that in which L-amino acids form almost exclusively right-handed α-helices. Indeed, since β-sheets are most commonly left-handed, we expect amyloid fibrils, the core of which is composed of β-strands oriented perpendicularly to the fibril axis, to have a left-handed twist. Consistent with this idea, many fibrils are indeed left-handed.[26] A particularly striking verification of this connection between the chirality of the building blocks and the twist of the resulting filament was provided by studying the assembly of amyloid fibrils formed from the conventional Aβ40 peptide sequence composed of L-amino acids and the analogous peptide consisting of D-amino acids. It was found that while the conventional L-amino acid peptides formed left-handed fibrils, the inversion of the chirality of the building blocks in peptides containing D-amino acids led to the inversion of the handedness of the resulting fibrils.[31]

Examples are increasingly appearing, however, that suggest that the connection between the chirality of the molecular building blocks and the resulting nanostructures is likely to be more complex. For instance, a range of serum amyloid A (SAA) truncated peptides, all composed of L-amino acids, were found to exhibit differing suprastructure chirality; the helicity of SAA_{1-11} and SAA_{2-6} amyloid fibrils was exclusively left-handed, while the SAA_{2-9} structures consist of a mixture of both left- and right-handed fibrils, and SAA_{1-12} and SAA_{2-12} fibrils were exclusively right-handed.[32] Fibrils of α-synuclein have also been found to coexist as both left- and right-handed fibrils with helical pitches of 95 and 45 nm, respectively.[17] These differences in chirality suggest variations in the protofilament molecular structure, indicating that the β-sheet structural motif is structurally versatile and can allow filaments of either handedness to be generated.

Other studies have reported the spontaneous inversion of handedness caused by small changes in the conditions used to form the fibrils. An analysis of the competition between self-propagating polymorphism and flow-driven (*i.e.*, vortex-induced) self-assembly of insulin fibrils showed that the suprastructure chirality was dependent on the concentration of seeds.[33] When the concentration of fibril seeds is high enough, native insulin self-assembles to form helical suprastructures with the opposite chirality of those formed in the absence of seeds.[33] This study provides further evidence that the material properties of amyloid and amyloid-like fibrils can be tuned in a controlled manner to create novel synthetic protein-based nanomaterials.

There are also indications that even small changes in solution pH can trigger chirality inversion in amyloid fibril formation.[34] When the pH is above 2.4, insulin forms left-handed fibrils in solution; at lower pH, however, insulin aggregates into right-handed fibrils. Then, on increasing the pH from 1.5 to 2.5, the right-handed helices spontaneously reconfigure into the normal left-handed fibrils as revealed by vibrational circular dichroism (VCD).[34] The high-resolution AFM spectroscopy of Usov *et al.*[12] provides an alternate methodology that could supplement other techniques such as transmission electron microscopy,[17,20] scanning electron microscopy,[32]

and VCD spectroscopy[34,35] to characterize chirality inversion unequivocally in several classes of amyloid fibrils.

The high-resolution AFM spectroscopy of Usov *et al.* provides an alternate methodology that could supplement other techniques such as transmission electron microscopy, scanning electron microscopy, and VCD spectroscopy to characterize chirality inversion unequivocally in several classes of amyloid fibrils.

OUTLOOK AND FUTURE CHALLENGES

While amyloid aggregates have historically been associated with pathological conditions, they have recently increasingly been studied in the context of nanotechnology and functional biomaterials, and research in this field is currently undergoing an expansion from molecular biology to polymer physics approaches. This development is in part motivated by the remarkable mechanical properties of amyloid fibrils whose robustness is comparable to the theoretical limit of material performance that can be achieved in proteins with the maximal density of intermolecular hydrogen bonds.[22] Additional beneficial properties of amyloid assemblies include the relative ease and cost of production since they self-assemble in aqueous solutions at room temperature, multiplicity of properties such as charge and hydrophobicity caused by changes in peptide sequence, and inherent biocompatibility. Indeed, although it may seem paradoxical that these pathogenic aggregates can be biocompatible, the controlled and rapid polymerization of functional amyloid can bypass the formation of toxic oligomeric intermediates and result in mature, stable fibrils.[36]

The combination of these desirable material and biological properties has led to the use of amyloid forms of peptides and proteins in many synthetic applications, including the slow-release of anticancer drugs,[11] cell culture scaffolds,[37] and biosensors.[38,39] Since amyloid fibrils are formed from a vast library of peptides and proteins, many researchers have exploited the universality of their hierarchical self-assembly by altering the amino acid sequence of existing amyloidogenic peptides or synthesizing *de novo* self-assembling peptides.[8] These designer peptides can be readily modified to include a variety of functionalities depending on the desired application. The key challenge in this context is understanding the manner in which the structure and properties of the final material can be encoded into its component self-assembling elements. Here, AFM approaches of the type described by Usov *et al.*[12] provide novel opportunities to perform a systematic analysis on multiple sequences that may help define the amino acid code that controls the morphology and self-assembly process of amyloid fibrils, thus creating the possibility of rationally designing their material properties.

More generally, studies of the physical basis of this assembly phenomenon are increasingly providing key information on the connections between the molecular interactions that drive the assembly process and the nanoscale properties of the final structures. The twist and handedness of fibrils are notable properties that are only recently being characterized in quantitative detail, as exemplified by the article of Usov *et al.* in this issue of *ACS Nano*.[12] The increasing recognition of the amyloid state as a general protein fold that can possess functionality rather than being exclusively pathogenic opens up the possibility of the discovery of amyloid-based solutions to key questions in the fields of nanotechnology, materials science, biomedicine, and food science.

Conflict of Interest: The authors declare no competing financial interest.

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