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Peptide-based nanomaterials: Building back better & beyond

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ABSTRACT

The exquisite structure–function correlations observed for native protein filaments have prompted research into the design of simpler peptide-based analogues that can be tailored for specific applications as synthetic filamentous nanomaterials. Sequence-structure correlations that have been established from analysis of native proteins have been previously adapted to create a supramolecular folding code based on simple design principles. While successful, the supramolecular folding code has not been critically examined in terms of the relationship between the proposed models and experimentally determined structures. Recent cryo-EM analyses of peptide-based filaments at near-atomic resolution offers the opportunity to compare the predictions of the supramolecular folding code to the resultant atomic models. The results provide insight into the limitations of the folding code and suggest an approach to refine the design of peptide-based filaments.

1. Introduction

Peptide self-assembly has been explored for at least three decades as a method for selective fabrication of functional nanomaterials [1,2]. The primary focus of this research effort has been development of more effective biomaterials for medical applications, e.g., as drug delivery agents, tissue engineering matrices, and vaccine scaffolds [3]. This goal requires the design of peptide assemblies that can effectively and predictably engage in interactions with biological interfaces to direct a desired functional response. The appeal of peptide-based biomaterials for these applications derives from the analogy to native, i.e., biologically sourced, protein-based assemblies in living systems, in which control of supramolecular structure enables the evolution of complex biological function under physiological conditions. Peptide-based filaments have been the primary target of these design efforts since they can be elaborated into functional biomaterials, e.g., hydrogels, fibers, films, etc., in which the biological, chemical, and mechanical properties may be controlled through sequence selection. Native protein-based filaments, e.g., pili, flagella, cytoskeletal proteins, and extracellular matrix, are involved in a variety of cellularly important functional roles including mechano-transduction, substrate transport, locomotion, electronic conduction, cellular adhesion, and regulation of enzymatic catalysis [4–7]. Many of these biological functions would be desirable to capture in synthetic systems designed for targeted applications. However native protein-based filaments are challenging to re-purpose toward non-native function due to the complexity of their *in vivo* assembly. This situation has motivated for the design of more synthetically tractable peptide-based assemblies in which structural control could enable development of functional materials that emulate and potentially expand upon the properties of the native protein filaments [8].

The development of peptide-based nanomaterials has been predicated on the assumption that empirical rules derived from structural analysis of native proteins can be applied to establish sequence-structure correlations that could guide the design of synthetic self-assembling peptides. These empirical rules have often been discussed in terms of a supramolecular folding code in which sequence information encoded at the molecular level would selectively and predictably direct the folding of synthetic peptides into structurally defined assemblies [9]. Initially, this supramolecular folding code encompassed simple design principles, e.g., amino acid conformational preferences of amino acid residues, pairwise residue correlations, and polar sequence patterning, to selectively direct the formation of higher order structure (Figs. 1 and 2). As in conventional protein design efforts, stabilizing or destabilizing interactions between amino acid side chains within a given structural context can be introduced to bias the sequence toward formation of the desired structure (positive design) and against alternative structures (negative design). However, while successful in affording self-assembled peptide-based materials, the reliability of these design principles and, more generally, the validity of the concept of a supramolecular folding code has not been rigorously tested as regards the correlation between structural predictions and experimental outcomes.

2. Challenges in the design of peptide-based filaments

Recently, near-atomic resolution structural analysis, primarily derived from single-particle reconstruction of cryo-EM images obtained with direct electron detection [10], has enabled insight into the relationship between the peptide design and the corresponding structures of

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the resultant filamentous assemblies [11–16]. While the total number of structures solved to near-atomic resolution remains limited at present, the reliable atomic models that have emerged from these structural analyses have provided significant insight into the limitations of the supramolecular folding code. A necessary corollary to this discussion is that is important to distinguish between structural information derived from lower resolution experimental methods and that derived from near-atomic resolution methods (i.e., cryo-EM, X-ray crystallography, and NMR spectroscopy). Atomic models based on structural information from lower resolution methods must be regarded as speculative in the absence of independent confirmation from structural analysis using near-atomic resolution methods [8]. For filamentous nanomaterials, the most facile and widely applicable approach for structural analysis is cryo-EM helical reconstruction [10]. That being stated, lower resolution analytical methods are not without value in experimental studies of peptide-based filaments, e.g., in screening conditions for self-assembly as well as in thermodynamic and kinetic analysis of the assembly pathway. However, even in the latter case, these mechanistic studies would certainly be aided with prior knowledge of the structure of the corresponding assemblies.

Before proceeding to discussion of specific examples, it should be noted that several factors confound the discussion of higher-order structure in peptide- and protein-based assemblies. Synthetic peptide-based filaments usually display a significant degree of structural polymorphism, in which the type and multiplicity of observed structures often depends on the experimental conditions employed for the self-assembly [11,13,14,17]. In addition, remnant structure within the peptide sample prior to self-assembly, i.e., arising from the synthetic procedure, may bias the system towards formation of a specific set of supramolecular structures. These considerations appear to hold not only for synthetic, i.e., designed, peptides, but also for native, i.e., biologically derived, peptides and proteins that have been assembled *in vitro*. This phenomenon is particularly pronounced for amyloidogenic peptides [18–20], in which *in vitro* assembly often results in a high degree of structural polymorphism in the resultant filaments. In contrast, *ex vivo* isolated amyloid filaments often display limited polymorphism in which only one or two distinct structures are observed. Cryo-EM analyses of these *ex vivo* amyloid assemblies have provided evidence for the formation of different structural polymorphs that appear to be specifically associated with different pathologies, e.g., in the case of tau protein filaments associated with a range of neuro-degenerative diseases [21]. Nevertheless, the folding landscape for self-assembly of specific amyloidogenic peptide sequences may not be unbounded. It is possible that only a limited range of structural interfaces may be accessible for these filaments, even though it would be difficult to ascertain *a priori* the scope of accessible structures. Lovestam et al. [20], have demonstrated that experimental conditions can be selected for *in vitro* self-assembly of tau protein isoforms such that the resultant structures of the amyloid filaments replicate the structures of the different classes of *ex vivo* isolated filaments. In the latter case, the experimental selection of self-assembly conditions benefited from prior knowledge of the structures of the respective *ex vivo* filaments. This precedent suggests that structural information derived from previous near-atomic resolution analyses of synthetic peptide filaments could facilitate the rational design of peptide sequences [13] or identify experimental conditions that would favor formation of a specific structural polymorph among the currently known ensemble of possible variants [14].

An additional complicating factor in the structural analysis of peptide-based filaments relates to the experimental determination of helical symmetry [10]. Most peptide- and protein-based filaments arise from non-covalent interaction between subunits (protomers) to form a pseudo-infinite polymer. The arrangement of protomers within the polymer can be understood in terms of its helical symmetry. Successive protomers in a helically symmetric filament are related to each other through an axial translation (Z) and angular rotation (ϕ)-often with superimposed rotational point group symmetry (C_n). However, structurally related peptide-based filaments, resulting from minor modifications of peptide sequence or variations in experimental assembly conditions, can display differences in helical symmetry [11,22,23]. This lability in helical symmetry necessitates that the cryo-EM structural analysis be performed *ab initio* for any peptide filament, i.e., without an assumption of a relationship in symmetry to a previously characterized structure. Helical symmetry is currently assigned through a trial-and-error approach in which Bessel orders are determined for two independent layer lines in the averaged power spectrum. All possible helical symmetries that are compatible with the spacing of layer lines in the averaged power spectrum need to be independently tested as solutions in the cryo-EM structural analysis. Reliable prediction of the symmetry of a helical filament from sequence information is beyond the scope of our current capabilities, even for peptide sequences that are closely

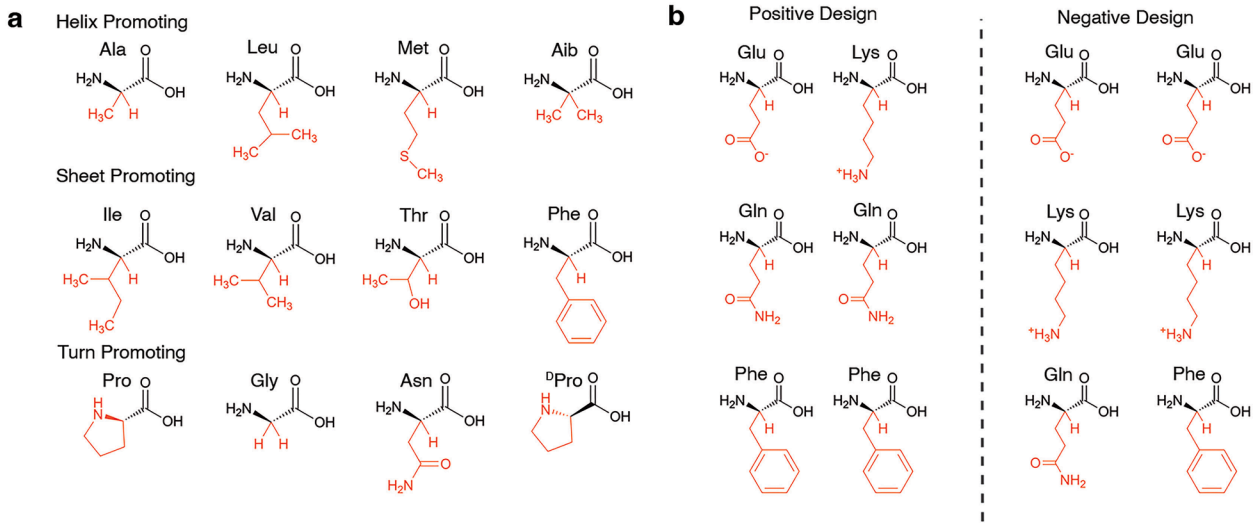

Fig. 1. Residue-based determinants of peptide conformation as design elements. (a) Structures of different classes of amino acids with secondary structure preferences. Chemical peptide synthesis enables incorporation of non-canonical amino acids, e.g., Aib and ^{D}Pro, that have structures that differ from the canonical amino acids in order to enhance the formation of a desired chain conformation. (b) Representative examples of sidechain pairings that can be employed for positive and negative design in peptide sequence-space. Pairwise residue correlations are usually considered from the perspective of interactions that would occur within a specific peptide chain conformation or in terms of interfacial interactions between protomers in the assembly.

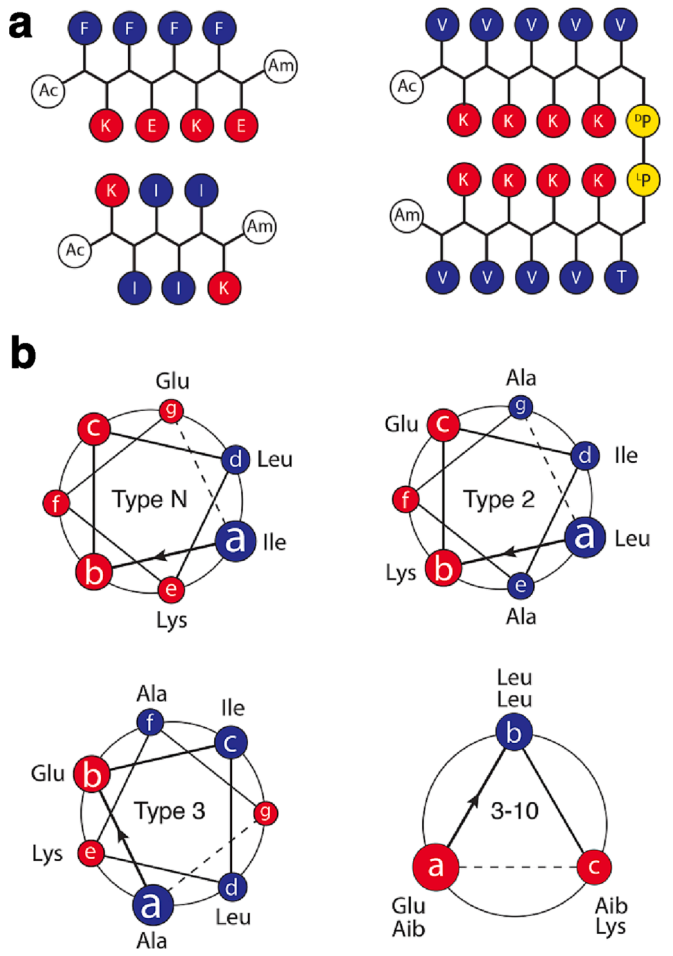

Fig. 2. Polar patterning in the sequence design of synthetic, self-assembling peptides. (a) Polar patterns based on an alternating sequence of polar and hydrophobic residues favor the formation of facially amphipathic β-sheet assemblies as observed for peptides KFE8 (upper left) and MAX1 (right) [30,54]. In certain cases, the introduction of contour-length amphipathic character can also favor the formation of β-sheet assemblies as observed for surfactant-like peptides (lower left) [55]. (b) Helical wheel diagrams depicting position-specific polar patterning preferences in the sequences of designed coiled-coils and a 3–10 helix. In the cases of coiled-coil peptides, polar patterns can be introduced into the peptide sequences that result in the formation of a single hydrophobic face (Type N) or two different offset hydrophobic faces (Types 2 and 3) [37]. These different polar patterns usually result in different modes of assembly. Similar considerations can be extended to alternative helical conformations such as a 3–10 helix based on a triad repeat [56]. (Color code: blue, hydrophobic face; red, hydrophilic face; yellow, reverse turn; white, acetylated (Ac) and amidated (Am) peptide termini). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

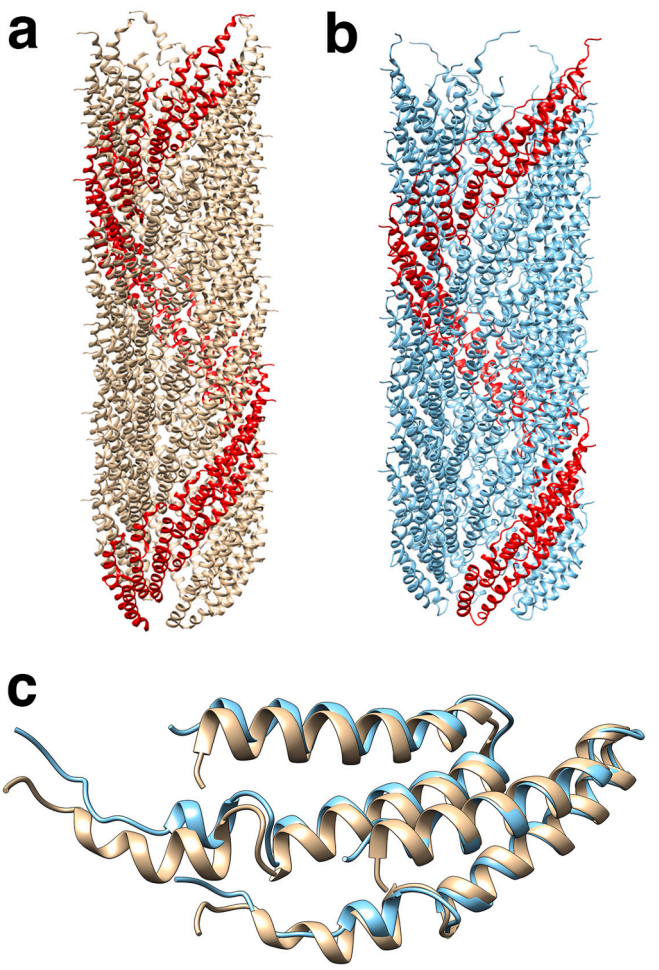

Fig. 3. Structural comparison between bacterial conjugative pili as representative examples of homologous biological derived protein assemblies. (a) Atomic model of the pKpQIL conjugative pilus (PDB ID: 7JSV) displaying C_1 symmetry (pitch = 12.5 Å) at 3.9 Å resolution [57]. (b) Atomic model of the pED208 conjugative pilus (PDB ID: 5LEG) displaying C_5 symmetry (rise = 12.1 Å) at 3.6 Å resolution [58]. In each case, a single protofilament is highlighted in red, which corresponds to protomer interactions along one of the right-handed 5-start helices. (c) Structural alignment of the mainchain atoms between a pair of protomers for the pKpQIL (tan) and pED208 (cyan) conjugative pili. The protomers from the respective structures are oriented along the 5-start helices. Despite the structural similarity of the inter-protomer interfaces, the structurally homologous assemblies differ in helical symmetry (C_1 versus C_5) and the helical pitch of the 5-start protofilaments (~174 Å and ~ 155 Å for the pKpQIL and pED208 pili, respectively). The ribbon models in Fig. 3 were rendered in UCSF Chimera. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

related to those derived from experimentally determined structures [10]. Nevertheless, despite differences in helical symmetry, the interfacial packing of protomers is often strongly conserved between structurally homologous assemblies (Fig. 3). Therefore, sequence-based control of interfacial interactions may represent a potentially productive approach to the design of peptide filaments. This approach assumes that sequence-structure correlations are sufficiently reliable to enable predictive design of interfacial interactions (*vide infra*). In this scenario, self-assembly of a designed peptide sequence under a given set of experimental conditions would preserve the structurally critical interfaces within the filament but would not require *a priori* imposition of a specific helical symmetry as an explicit design criterion.

Two obvious questions arise from the previous discussion. What have we learned thus far from near-atomic resolution structural analyses of designed peptide filaments? How can we best employ this information to inform the future design of self-assembled peptide filaments? The former question requires a discussion of the structures of peptide filaments that have been solved thus far and the latter question necessitates a reanalysis of these results in the context of the supramolecular folding code that was employed as a basis for the peptide design. In this discussion, we focus primarily on insights into peptide design that can be drawn from analysis of the relatively few structures of synthetic peptide-based filaments that have been solved to near-atomic resolution. The vast and rapidly expanding field of amyloid filament structural analysis can provide additional information on the sequence-based determinants of peptide self-assembly. However, in general, amyloidogenic peptide sequences are more complex than designed peptides in that they are

derived from native proteins. In addition, the sequences of amyloidogenic peptides have been subjected to the filter of evolutionary selection. Although this process does not necessarily select for self-assembly behavior, the sequences of amyloidogenic peptides are not naïve in the sense that they do not derive from simple, i.e., uncritically examined, design rules such as the supramolecular folding code (such as it has been and is currently implemented).

3. Structural analysis of a synthetic cross-β assemblies

Historically, the most common structural motif for the design of synthetic peptide assemblies has been the β-sheet fold. The self-assembly of amyloidogenic peptides provided a well-established precedent for the formation of β-sheet filaments [24,25]. The earliest structural studies of amyloid assembly date back nearly sixty years, although reliable atomic models derived from cryo-EM analysis of isolated filament structures first became available in 2017 [26]. The importance of the cross-β architecture was well established at that point and served as the basis for the *de novo* design of numerous self-assembling peptides [8]. The cross-β structural motif has several significant advantages for *de novo* design of self-assembling peptides. The cohesive interactions within a β-sheet involve directional hydrogen bonding between main-chain carbonyl and amide groups on structurally adjacent β-strands, which provides a strong driving force for self-assembly into filaments [27]. Consequently, β-sheet peptides need not be very long, usually ≤ 10 residues, in order to reliably self-assemble and are therefore easily amenable to preparative scale synthesis. The conformational repeat of a β-strand comprises a pair of amino acids (i.e., a diad) such that polar patterning can easily encode the formation of an amphipathic β-sheet through an alternating sequence of polar and nonpolar amino acids (Fig. 2) [28]. Self-association between the hydrophobic faces of such amphipathic β-sheets can provide an additional driving force for self-assembly. In addition, side-chain interactions, e.g., electrostatic complementation and hydrogen-bonding, can be introduced between amino acids to direct the alignment and orientation of β-strands through a combination of positive and negative design. Despite the numerous examples of designed β-sheet peptides, few of these assemblies have been structurally characterized at near-atomic resolution. One complicating factor in the structural analysis, in analogy to amyloid-derived filaments, is the presence of extensive structural polymorphism. In the absence of analysis at near-atomic resolution, the structural relationship between the different filamentous polymorphs cannot be completely understood [29].

Recently, we reported the cryo-EM structural analysis [14] of peptide-based filaments derived from self-assembly of a previously reported peptide KFE8, Ac-FKFEFKFE-NH_2 [30,31]. The KFE8 sequence encompasses several design elements that can be related to the supramolecular folding code derived for filamentous peptide assemblies (Figs. 1 and 2). The polar patterning of the diad repeats should favor the formation of amphipathic β-sheet, which in turn should promote mating of the hydrophobic faces to form a bilayer filament [28]. The alternating sequence of oppositely charged lysine and glutamic acid at the polar positions of successive diad repeats in KFE8 was designed to introduce attractive electrostatic interactions between the side chains in an antiparallel orientation (positive design) and repulsive electrostatic interactions in a parallel orientation (negative design). An initial AFM analysis indicated that the peptide spontaneously self-assembled to form helical ribbons at ambient temperature. Based on this information, the authors suggested that the ribbon structure of the KFE8 assemblies derived from self-association of a pair of amphipathic β-sheets. However, the proposed antiparallel orientation of strands within the β-sheets could not account for the observed helical coiling of the bilayer ribbons [30,31].

In contrast, our structural investigation of KFE8 self-assembly revealed the presence of multiple species in which the relative population and corresponding structures of the filaments depended on the preparative conditions (Fig. 4) [14]. At ambient temperature, the KFE8 peptide initially formed the previously observed helical ribbons. The atomic model derived from cryo-EM analysis of the helical ribbon was consistent with a bilayer β-sheet structure. However, the arrangement of protomers within the two β-sheet leaflets was significantly different than the initially proposed model and could explain the structural origin of the helical coiling of the ribbon *(vide infra)*. Over a period of 24 h, pairs of helical ribbons self-associated to form closed nanotubes based on four-bilayer β-sheets. Helical reconstruction of single protofilaments from the nanotube resulted in an atomic model that was identical within experimental error to the independently determined atomic model of the helical ribbons. However, if the sample of KFE8 peptide at the same concentration and in the same buffer was thermally annealed, i.e., heated to 95 °C and slowly cooled, a mixed population of filaments was observed that could be individually characterized. The minor population (~30 %) of nanotubes corresponded to the four-bilayer β-sheet structure observed as the primary species resulting from self-assembly of KFE8 at ambient temperature. The major population (~70 %) filaments corresponded to a nanotube derived from self-association of five-bilayer β-sheets.

While structural polymorphism has been previously observed for β-sheet filaments assembled from the same peptide under different preparative conditions [29], the availability of reliable atomic models enables a detailed structural comparison between the various self-assembled species of KFE8, which can be also be evaluated with respect to the supramolecular folding code that served as the basis for the peptide sequence design. The major difference between the two different nanotubes is that the asymmetric units, i.e., the fundamental structural repeats, are based on eight peptides and four peptides for the four-bilayer and five-bilayer β-sheet structures, respectively. The initial structural model for the KFE8 assemblies was proposed to be a bilayer of antiparallel β-sheet based on the supramolecular folding code (*vide supra*). Instead, cryo-EM structural analysis of ribbons and nanotubes was consistent with atomic models based on packing of an interior parallel β-sheet against an exterior antiparallel β-sheet. This packing arrangement at the bilayer interface could not have been predicted *a priori* for the KFE8 assemblies because simple design rules based on polar sequence patterning should have disfavored the formation of parallel β-sheet due to electrostatic repulsion between similarly charged side-chains at the cross-strand interface. This unusual packing arrangement of antiparallel sheet on parallel sheet provides an explanation for the observed helical curvature as well as the size of the asymmetric unit. In addition, the doubling in size of the asymmetric unit of the smaller diameter nanotube and helical ribbon vis-à-vis the larger diameter nanotube results from a presumably weak lateral interaction between hydrophobic phenylalanine residues on two KFE8 peptides across the inner and outer layers on adjacent β-sheets. The presence of this local interaction seems dependent upon the experimental conditions employed for peptide assembly. These results highlight the current scope and limitations of the supramolecular folding code, which was successful for prediction of the β-sheet secondary structure and bilayer arrangement of the KFE8 assemblies. However, the design principles could not specify the observed differences in β-strand orientation within the inner and outer β-sheets and were unable to account for the lateral interactions between bilayer sheets that guided higher-order assembly into ribbons and nanotubes.

4. Structural analysis of a synthetic cross-α assemblies

While the design and structural analysis of the KFE8 peptide filaments provides a useful context to evaluate the predictions of the supramolecular peptide folding code, β-sheet peptide assemblies may not be the best substrates for a proper evaluation of these design principles. One could argue that the formation of β-sheet assemblies may be particularly susceptible to chaotic processes during self-assembly since individual β-strands are not conformationally stable in the absence of

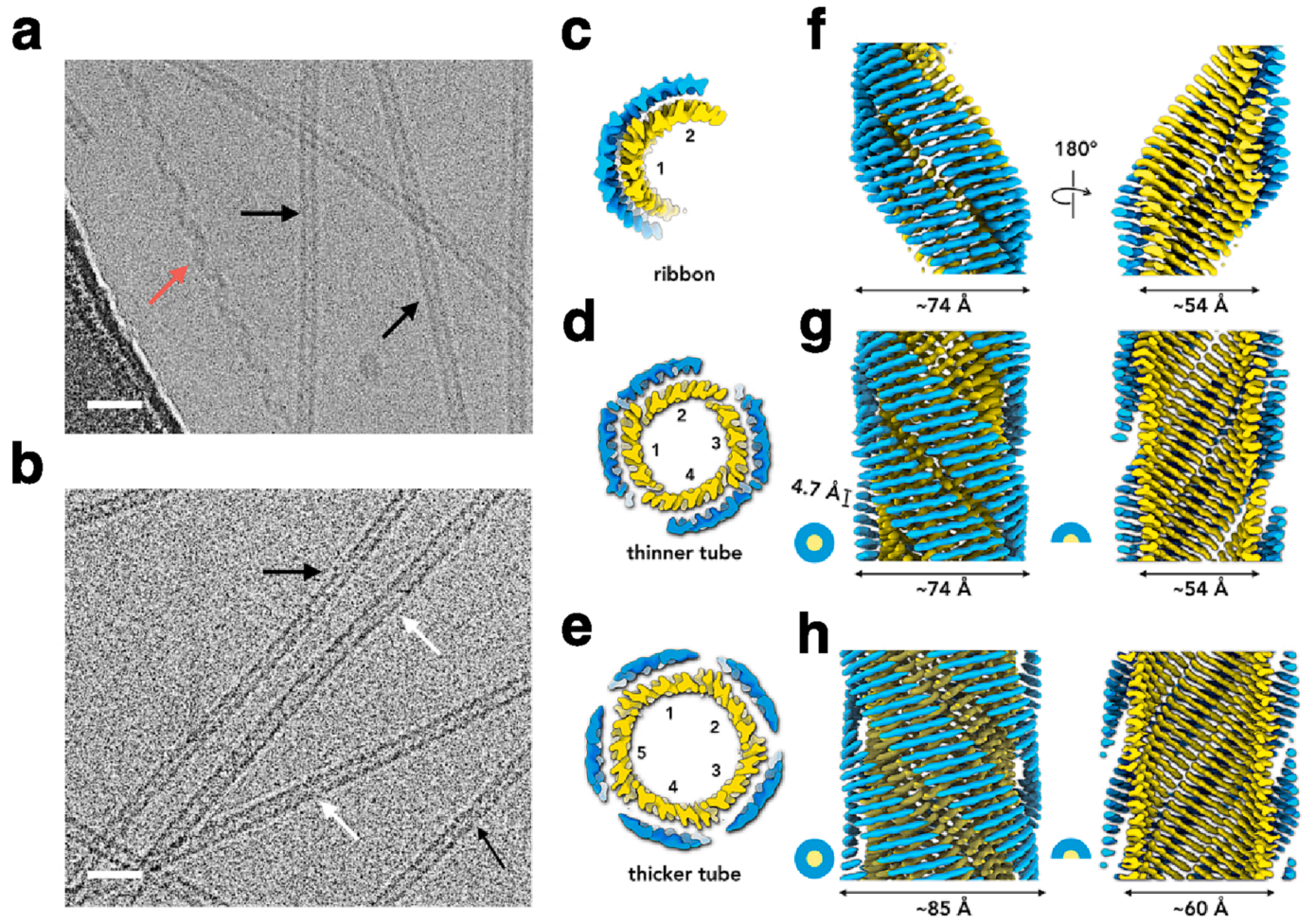

Fig. 4. Cryo-EM structural analysis of peptide KFE8 (Ac-FKFEFKFE-Am) [14]. Representative cryo-EM images of KFE8 assemblies prepared at ambient temperature (a) and after thermal annealing (b). Arrows indicate the presence of different self-assembled species: bilayer ribbon (red), thinner nanotubes (black) and thicker nanotubes (white). The corresponding 3D reconstructions are presented in three different views for the bilayer ribbon (c,f), thinner nanotube (d,g), and thicker nanotube (e,h). The inner and outer β-sheets are depicted in gold and cyan, respectively, for all three structures. Note that in all three cases the protofilaments display left-handed helical symmetry. The atomic models have been deposited in the PDB for the bilayer ribbon (PDB ID: 7LQE and 7LQF), the thinner tube (PDB ID: 7LQG and 7LQH), and the thicker tube (PDB ID: 7LQI). Reproduced with permission from Wang F, Gnewou O, Wang S, Osinski T, Zuo X, Egelman EH, Conticello VP: Deterministic chaos in the self-assembly of β-sheet nanotubes from an amphipathic oligopeptide. Matter 2021, 4:3217–3231. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

intermolecular association [14]. Consequently, β-sheet filaments typically display long lag times for self-assembly that often involve liquid–liquid phase separation into peptide-rich droplets [32]. These condensates may provide an environment that favors the formation of nuclei that can seed the growth of filaments. In contrast, α-helical peptides can fold intramolecularly, which can pre-organize the peptide into a conformation that can promote self-assembly into filaments in the absence of micro-phase separation. Therefore, the self-assembly of α-helical filaments commonly proceeds with a negligible or minimal lag phase [33,34]. In addition, the interfacial interactions between protomers that drive the formation of α-helical filaments usually involve amino acid sidechain interactions rather than the mainchain hydrogen bonding interactions that promote β-sheet formation. These hydrophobic interactions in α-helical assemblies are generally weaker and lack the directional specificity of the mainchain β-sheet interactions. However, in analogy to conventional protein design, the identity of the residues at structurally critical sites in the sequence can be rationally varied to enhance specific modes of α-helical peptide self-association [35,36]. Notably, many native protein-based filamentous assemblies, including filamentous phage capsids, flagella, type IV pili, conjugative pili, type III secretion system needle complexes, etc., are composed of protomers that adopt α-helical conformations in the context of the assembly, e.g., as in Fig. 3.

The design rules for α-helical coiled-coil oligomers have been thoroughly investigated. Sequence-structure correlations have been established that enable design of specific oligomerization states [35]. This knowledge can be effectively leveraged for the design of synthetic coiled-coil protomers that can self-assemble into open-ended filaments rather than closed circular oligomers. As for β-sheet peptides, a simple supramolecular folding code can be elaborated for the design of α-helical peptides [11,13,37,38]. However, a wealth of structural information is available from native and synthetic helical proteins that can further refine design efforts. Generally, synthetic helical peptides require longer sequences to promote the formation of a stable conformation and inhibit misfolding into β-sheet structures. The preparative synthesis of helical peptides often proceeds in high yield and the peptides usually have excellent solubility. One significant challenge to the design of α-helical filaments is that the peptides require a pair of appropriately oriented hydrophobic interfaces to provide the driving force for self-assembly into extended structures [37]. Within an α-helical conformation, the hydrophobic residues should be periodically interspersed with polar residues in the peptide sequence, which can provide additional structural specificity through positive and negative design considerations (Fig. 2). In comparison to β-sheet peptides, self-assembling α-helical

peptides have been far less studied due to the greater challenge of peptide design, the higher cost associated with synthesis of longer peptide sequences, and the absence of a general model for self-association. The recent characterization of the cross-α peptide architecture in biologically derived peptide assemblies [16,33] provided a structural model for the design of synthetic helical peptides that could be directly compared to the cross-β architecture of β-sheet filaments. Fortunately, the structures of several designed cross-α peptide filaments have been solved at near-atomic resolution using cryo-EM helical reconstruction [11,13]. These structures can be interpreted in terms of the design rules associated with the supramolecular folding code to provide further insights into the scope of this approach.

The designs of these synthetic cross-α peptides were based on a type 3 coiled-coil sequence in which the helical protomer displayed two offset hydrophobic faces; each displaying the typical 3–4 heptad periodicity of the coiled-coil motif. The two-residue offset of the hydrophobic faces should promote self-assembly into a filament since they would occur on opposite sides of the helical protomer (Fig. 2). Four peptides were designed ranging in length from 15 to 36 amino acids such that the sequences of successive peptides in the series differed by an additional heptad repeat (Fig. 5) [13]. The residues at the hydrophobic faces were conserved in all designs and the pattern of charged residues was selected to stabilize the interaction between helices (positive design) and inhibit β-sheet formation (negative design) [11]. Since the designs were based on the well-studied coiled-coil structural motif, position-specific amino acid preferences could be introduced into the respective sequences to refine the designs and provide further structural specificity. Cryo-EM structural analysis provided atomic models for these peptide filaments at near-atomic resolution (Fig. 5). In each case, the filament structures were based on interactions of cross-α protofilaments that displayed a right-handed helical twist. The stacking interfaces within the cross-α protofilaments were highly conserved between the four structures and consistent with the helical interfaces observed in the structures of discrete coiled-coil oligomers. Computational analysis of the helical interfaces within the respective assemblies indicated that the helix-helix orientation was representative of packing interfaces within comparable protein structures in the Protein Data Bank (PDB). The helix-helix stacking interactions within these cross-α assemblies could be considered to arise from a natively designable interface [13,39]. From this perspective, the supramolecular folding code, enhanced through application of the known residue preferences for coiled-coil packing interfaces, must be regarded as affording a successful outcome in terms of predictive design of individual cross-α protofilaments.

While the design of individual cross-α protofilaments was demonstrated, the structures of these designed peptide assemblies displayed significant differences with respect to the arrangement of the protofilaments within corresponding nanotubes. The most notable distinction lies in the number of protofilaments that comprise the structure of the corresponding filaments, which was observed to depend on the sequence

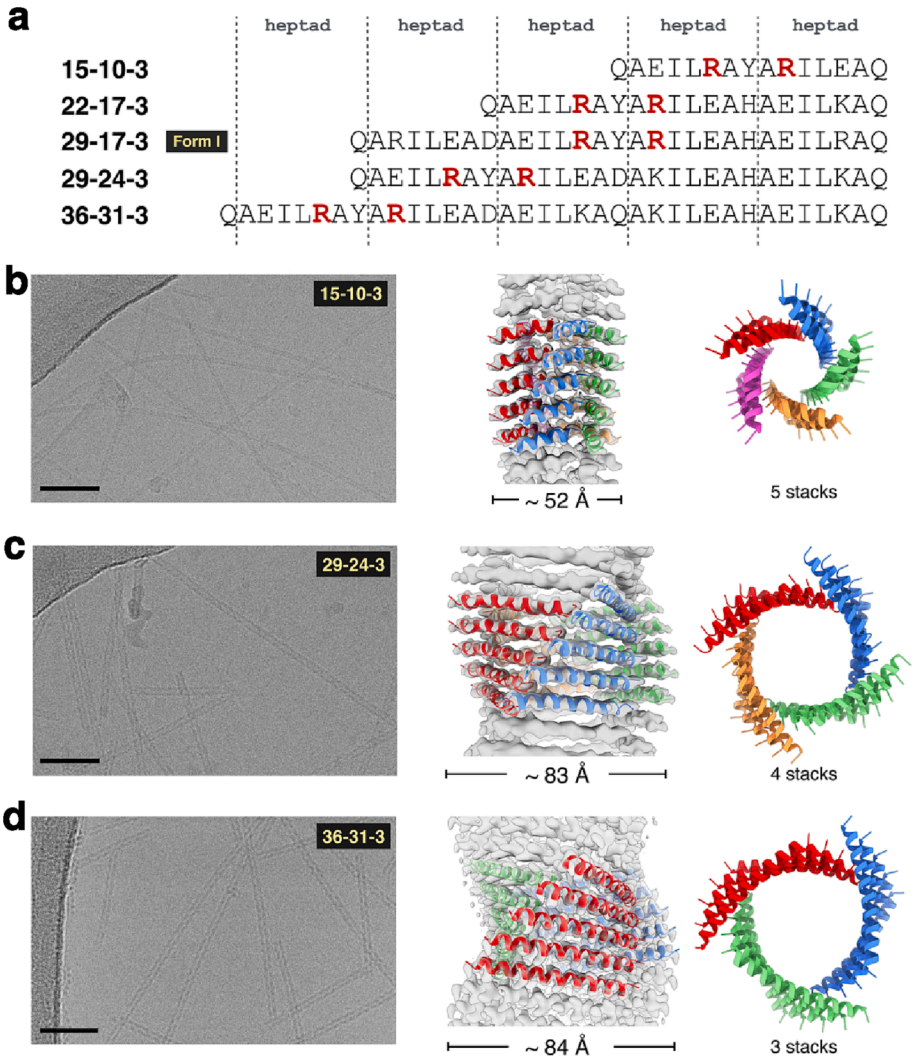

Fig. 5. Cryo-EM structural analysis of designed cross-α nanotubes [13]. (a) Peptide sequences are arranged according to increasing length. The boundaries between heptad repeats are indicated in the respective peptide sequences. The arginine residues that participate in the lateral interactions between protofilaments are highlighted in red (RxxxR motif). Representative cryo-EM images, three-dimensional reconstructions and atomic models for peptides 15-10-3 (b), 29-24-3 (c), and 36-31-3 (d). Cross-α protofilaments within the respective assemblies are highlighted in different colors. The protofilaments propagate along right-handed 5-start, 4-start, and 3-start helices for the 15-10-3, 29-24-3, and 36-31-3 assemblies, respectively. The atomic models have been deposited in the PDB for peptide assemblies derived from 15-10-3 (PDB ID: 6WKX) 29-17-3 (PDB ID: 3J89), 29-24-3 (PDB ID: 6WKY), and 36-31-3 (PDB ID: 6WL0 and 6WL1). Reproduced under the Creative Commons License from Wang F, Gnewou O, Modlin C, Beltran LC, Xu C, Su Z, Juneja P, Grigoryan G, Egelman EH, Conticello VP: Structural analysis of cross alpha-helical nanotubes provides insight into the designability of filamentous peptide nanomaterials. *Nat Commun* 2021, 12:407. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

length of the peptide (Fig. 5). The shortest peptide assembled into a nanotube based on five protofilaments, the medium length peptides assembled into nanotubes based on four protofilaments, and the longest peptide assembled into a nanotube based on three protofilaments. In each case, the lateral interactions between protofilaments were mediated through a pair of appropriately spaced (*i*,*i* + *4*) arginine residues, RxxxR, that interacted with the C-terminus of an α-helical protomer in an adjacent protofilament through a network of electrostatic and hydrogen-bonding interactions. Neither the nature of this "arginine clasp" interaction nor the differing arrangements of the laterally interacting peptides within the respective assemblies could have been predicted from the simple design principles embodied within the supramolecular folding code. The arginine clasp interaction is not observed for similarly oriented helical interfaces in protein structures in the PDB, which suggests that it has not been evolutionarily sampled and may not be designable in the structural context of native protein interfaces. In addition, semi-conservative mutagenesis of either arginine residue within the RxxxR motif to lysine resulted in loss of the original structure and formation of a double-walled nanotube in which three bilayer protofilaments laterally associated through weak terminal interactions between α-helical protomers [11]. Higher order structure, especially local interactions that occur between protofilaments, may be difficult to predict from simple design rules due to the limited stability of such interactions in isolation and their lability in sequence space. However, these weak interactions may profoundly influence the final structure of the assembly, as was also observed for KFE8, as well as the evolution of functional properties that would influence their ability to effectively serve as a nanomaterial in a particular application.

Despite the differences in structure between these designed cross-α filaments, the assemblies derived from individual peptides were observed to display limited structural polymorphism. This situation contrasts with that typically encountered for cross-β filaments in which *in vitro* assembly usually results in extensive structural polymorphism. The question arose as to whether structural polymorphism might be a less common occurrence for α-helical assemblies. However, the design of these self-assembling coiled-coil peptides (Fig. 5) benefitted from prior knowledge of sequence-structure correlations for coiled-coil peptides. The reliability of these empirical guidelines for sequence design was reflected in the native designability of the helix-helix stacking interface within the protofilaments [13]. In addition, the adventitious presence of the RxxxR motif introduced a robust local interaction between structurally adjacent helices that mediated lateral association between protofilaments. The specific combination of these two interfacial interactions may have limited the range of supramolecular structures that these designed peptide sequences could be adopt under the experimental conditions for self-assembly.

To assess the potential for cross-α assemblies to display structural polymorphism, the self-assembly behavior of the phenol-soluble modulin PSMα3 was investigated (Fig. 6). PSMα3, an amphipathic peptide-based virulence factor secreted by *S. aureus*, was previously demonstrated to self-assemble into cross-α filaments *in vitro* [33,40]. TEM imaging was employed to monitor PSMα3 self-assembly as a function of incubation time. The population of self-assembled species progressed from twisted filaments to helically coiled ribbons to nanotubes over twenty-four hours [16]. Moreover, the equilibrium population could be shifted through changes in temperature or pH, which suggested that the transition between different morphic forms of PSMα3 was reversible. STEM imaging of the PSMα3 provided further evidence for the relationship between different self-assembled structures as twisted filaments could be occasionally observed to emerge from the ends of the nanotubes (Fig. 6). Cryo-EM was employed to investigate the structure of the PSMα3 nanotubes. Two major classes of assemblies could be identified based on the observed diameter of the nanotubes. Independent three-dimensional reconstructions of these two populations were performed after 2D classification. The resultant atomic models displayed differences in helical symmetry as well as the number of protofilaments in the

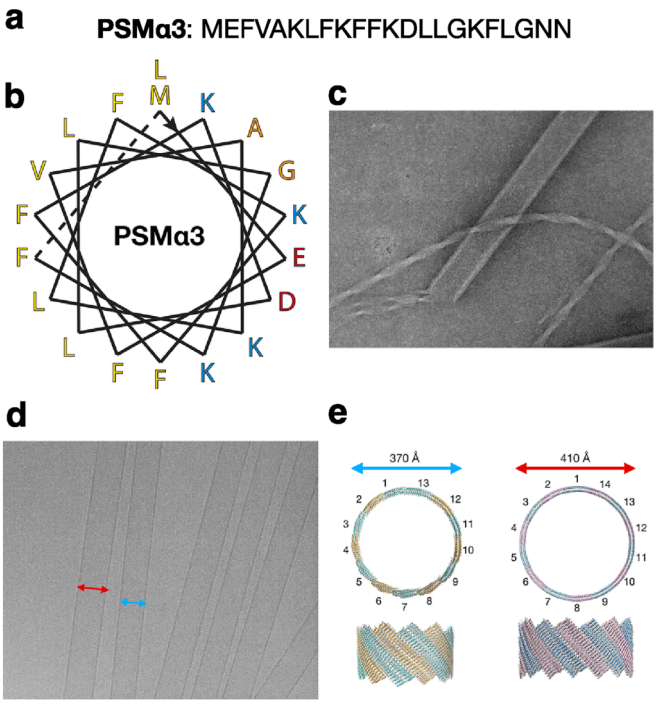

Fig. 6. Polymorphism of cross-α nanotubes derived from self-assembly of the biologically derived phenol-soluble modulin peptide PSMα3 [16]. Sequence (a) and helical wheel projection (b) highlighting the amphipathic character of PSMα3. (c) Dark-field STEM image of unstained PSMα3 nanotubes in which twisted protofilaments can be observed emerging from the ends of the assemblies. (d) Representative cryo-EM image from a preparation of PSMα3 nanotubes in which at least two different populations of nanotubes can be observed having narrower (blue) and wider (red) diameters. (e) Three-dimensional reconstructions of the narrower and wider diameter PSMα3 nanotubes with the corresponding atomic models. The narrower and wider nanotubes are composed of thirteen (C1 symmetry) and fourteen protofilaments (C7 symmetry), respectively. The atomic models have been deposited in the PDB for the narrower nanotube (PDB ID: 7SZZ), and the wider nanotube (PDB ID: 7T0X). Reproduced with permission from Kreutzberger MAB, Wang S, Beltran LC, Tuachi A, Zuo X, Egelman EH, Conticello VP: Phenol-soluble modulins PSMalpha3 and PSMbeta2 form nanotubes that are cross-alpha amyloids. *Proc Natl Acad Sci U S A* 2022, 119:e2121586119. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

respective nanotubes. The narrower diameter nanotubes were based on C_1 symmetry and consisted of thirteen cross-α protofilaments, while the wider diameter nanotubes displayed C_7 symmetry and consisted of fourteen protofilaments. The structure of the protofilaments in both nanotubes was based on an asymmetric unit comprising four peptides arranged in a bilayer composed of an inner and an outer pair of amphipathic PSMα3 helices (Fig. 6). The structural differences between the two nanotubes resulted from slight differences in interfacial interactions between peptides within and between protofilaments. These results suggested that structural polymorphism is a common phenomenon among self-assembled filamentous nanomaterials, as has been discussed previously [10], and that the observation of uniform populations of filaments may be the exception rather than the rule. Therefore, the consequences of structural polymorphism need to be explicitly considered in the design of peptide-based filaments.

5. Outlook and perspectives

What new insights can be taken from these structural studies that might advance the future design of synthetic peptide-based assemblies and lead to a further refinement of the supramolecular folding code? If

one compares the experimentally derived atomic models of the cross-β and the cross-α assemblies (Figs. 4–6), a common structural feature is the presence of protofilaments that display strong internal interactions and weak lateral interactions. In general, the relative strength of interactions within synthetic peptide-based filaments can be estimated from calculation of the buried surface area at specific inter-protomer interfaces [12,13,16]. Alternatively, computational evaluation of the native designability of specific inter-protomer interfaces may also serve as proxy for the reliability of the design. However, these methods are typically employed for retrospective analysis of the atomic models resulting from structure determinations rather than to guide the design of new self-assembling peptides [13]. In terms of structural prediction from sequence information, deep learning methods, such as multimer prediction with AlphaFold2, may be able to estimate the feasibility of different sequence designs in terms of predicted local different distance test (pLDDT) and predicted aligned error (PAE) associated with interfacial interactions between protomers [41]. However, these methods are not necessarily optimized for computational prediction of open-ended polymers rather than the more commonly observed structures of closed structures of discrete oligomers. In addition, at present, the PDB may not contain sufficient structural information on filamentous protein assemblies to enable successful structural predictions using data-driven, knowledge-based computational algorithms [42,43]. Coarse-grained molecular dynamics simulations [44], and, more recently, machine learning approaches [45], have been employed to identify short peptide sequences that are competent for self-assembly into higher-order structures although structural prediction with atomistic accuracy lies outside the scope of these methods. Currently, no slam-dunk solution exists for the reliable and predictable design of peptide-based filaments with near-atomic level accuracy with respect to supramolecular structure. This situation may not always be the case as our knowledge of the protein structure evolves concurrently with the development of better computational tools and methods for high throughput structural analysis at near-atomic resolution [10]. Certainly, the widespread availability of advanced cryo-EM imaging and the development of more facile methods for computational image analysis offer the promise that the number of high-resolution structures of peptide-based filaments will continue to increase at a rapid rate.

In the meantime, how can peptide design methods be improved, in a practical sense, to build better nanomaterials? For the materials scientist, peptide-based assemblies offer the opportunity to control nanoscale structure through specifically designed intermolecular interactions that can be rationally programmed into the sequence through synthesis [1,2]. Ultimately, interfacial structural control at the nanoscale could be employed to encode functional properties at the micro- and meso-scale, e.g., mechanical response and ligand display, that are important for the development of materials for specific applications. The previously described cryo-EM analyses suggest that the design of peptide-based filaments with precise structural control is beyond our current capabilities. However, the peptide-based filaments in Figs. 4–6 display some commonalities, namely, the presence of protofilaments that associate into higher-order structures. Protofilaments can often be discerned in the structures of biologically derived protein-based filaments, e.g., as in Fig. 3. These substructures may not be mechanistically related to the assembly of the filaments but can provide insight into the interfacial interactions that are critical to maintain their structural integrity. In the case of the designed peptide assemblies described here, the lateral interactions between protofilaments in a filament were estimated to be significantly weaker than those observed within a protofilament. This situation may have arisen as an unintended consequence of the application of the supramolecular folding code to the peptide design. Protofilament formation in either the cross-α or cross-β architecture resulted from interfacial interactions across the contour length of the peptides in the respective asymmetric units, which provided a driving force for self-assembly in a direction orthogonal to the peptide backbone. In contrast, the interactions between protofilaments often involve a few amino acids on the interacting peptides in structurally adjacent asymmetric units across the lateral interface. In this scenario, individual lateral interactions will be quite weak, but the impact of these interactions is magnified due to their occurrence many times along the contour length of the respective protofilaments.

This protofilament model may offer an alternative approach to the design of peptide-based filaments (Fig. 7). The conformational anisotropy of common peptide secondary structures, e.g., α-helix and β-strand, should promote self-assembly in a direction oriented perpendicular to the net direction of the peptide backbone. Furthermore, peptide sequences with appropriate polar patterning can be designed that adopt facially amphipathic conformations (Fig. 2), which would promote longitudinal self-assembly into cross-α and cross-β architectures. This supramolecular architecture would constitute the structural basis for the formation of individual protofilaments. The supramolecular folding code was based on the concept of such polar sequence patterns. However, as the cryo-EM structural analyses have demonstrated, these relatively simple design considerations are not sufficient to uniquely specify a protofilament structure. Positive and negative design strategies need be further amended to discriminate in favor of a desired interfacial orientation and against alternative arrangements. The strategy had been previously employed for the design of self-assembling peptides, e.g., KFE8, however the full scope of possible structural interactions was not fully comprehended. Fortunately, the scope of structural information on protein–protein interfacial interactions in extended assemblies has expanded exponentially over the last two decades, particularly from crystallographic and cryo-EM structural analyses of cross-β amyloids [24]. The structural landscape of cross-β assemblies has been described in terms of the four basic sheet geometries and ten symmetry classes, which can be employed as starting points in peptide design as well as the structural basis for a more refined application of positive and negative design principles. In theory, a similar approach can be employed to classify the potential modes of interfacial interactions and higher-order packing in cross-α assemblies. In addition, computational methods are available to predict local interaction interfaces and analyze their designability [39]. For the relatively strong stacking interfaces in cross-α and cross-β arrays, computational optimization of the peptide sequence may be possible especially if one has a reliable interfacial geometry as a starting point, as has been demonstrated for a cross-α filament [13].

If indeed it becomes possible to design peptides that can reliably and predictably form structurally defined protofilaments, control of the lateral association into filaments is a substantially more significant challenge. The interacting interfaces between protofilaments usually involve only a few residues per peptide and the position of these interacting surfaces may be difficult to predict. Side-to-end and end-to-end interactions between surface-accessible residues on adjacent peptides have been observed to mediate lateral association between protofilaments (Figs. 4–6). This potential promiscuity in the number and type of interacting residues is a critical feature that underlies the frequently observed forms of polymorphism in peptide-based filaments. However, within a defined structural context, e.g., an α-helix or β-strand, it may be possible to use positive and negative design principles to engineer local cross-protofilament interactions. Sticky patches, usually derived from small clusters of spatially proximal hydrophobic residues, have been incorporated at the periphery of oligomeric protein complexes to drive association into filaments [46]. In fact, evolution appears to select against incorporation of surface hydrophobic patches to prevent aggregation or agglomeration of soluble, oligomeric protein complexes [47]. One or more hydrophobic residues could be placed at specific positions within a peptide sequence such that, upon self-assembly into a protofilament, formation of a sticky patch would occur in an otherwise hydrophilic surface (Fig. 7). While these hydrophobic interactions may be conceptually the most straightforward to design and implement, other positive design elements, e.g., salt bridge formation, hydrogen-bonding, cation-pi interactions, etc., could also be employed as weak, non-covalent interactions, either alone or in combination with the

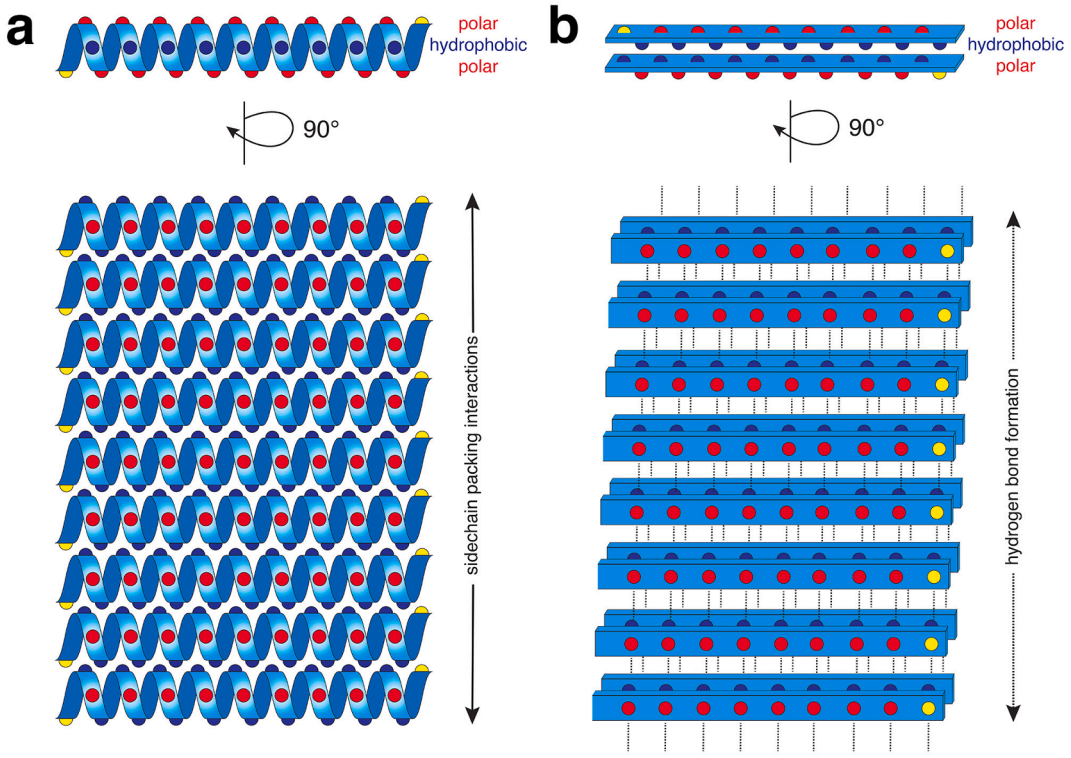

Fig. 7. General model for design of self-assembled protofilaments based on cross-α (a) and cross-β (b) structures. The cross-α protofilament results from hydrophobic packing of bi-faceted type 3 coiled-coil assemblies as depicted in Fig. 5. The cross-β protofilament model results from formation of an amphipathic sheet structure in which hydrophobic residues interact across a mating interface. Hydrogen bonding occurs between pairs of β-strands to generate the protofilament as depicted in Fig. 4. Terminal residues (yellow) can be introduced into the peptide sequence to promote lateral interactions between protofilaments in this model. While these models are based on characterized structures, the general principles of protomer design may in theory be extended to other peptides, peptide-mimetic foldamers or non-peptide amphiphiles. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

hydrophobic interactions, to more selectively direct lateral association between protofilaments. Computational design has also been employed to introduce hydrogen-bonding networks into oligomeric protein complexes to provide specificity to the interfacial interactions. This approach could potentially be employed to more precisely introduce lateral interactions at inter-protofilament interfaces [48].

The protofilament model of assembly does have some significant drawbacks. The most sanguine appraisal of this approach must recognize the persistent potential for polymorphism in the resultant filaments due to differences in interfacial interactions within and between protofilaments. As stated earlier, the extent and structural nature of the polymorphism observed for designed peptide-based filaments is impossible to predict *a priori*. This situation most likely results from a shallow potential energy landscape for the self-assembly of naively designed peptide sequences, in which the energetic differences between different structural polymorphs would be expected to be minor. Consequently, it has been suggested that the formation of peptide-based filaments from designed sequences may be understood to a first approximation as a chaotic process, in which the observed ensemble of structures may be strongly dependent on the initial experimental conditions [14]. While the range of polymorphs may be bounded for any given sequence, it probably represents a known unknown in design space. However, as recent cryo-EM analyses have demonstrated, once experimental structures have been determined, the solution conditions can be varied to bias the self-assembly process toward a particular polymorph [20]. In addition, design principles can be employed to narrow down the range of accessible structures. In this process, near-atomic resolution structural information will be critical to understand the relationship of the proposed structural model to the experimentally determined structures and to identify the mechanisms through which polymorphism may be manifested. Cryo-EM is the method of choice for the analysis of peptide-based filaments in that different filaments in a single specimen can be independently classified and structurally analyzed using very small sample sizes with automated data collection [10]. While the lability of helical symmetry in structural space remains a challenge in the cryo-EM analysis, it also reinforces the idea that design efforts should focus on controlling interfacial interactions rather than the *de novo* design of peptide filaments in which initial constraints on helical symmetry are applied.

An attractive feature of the protofilament model for the design of helical filaments is that it is not limited to protomers derived from peptide sequences. Self-assembly processes often involve selective association of amphiphiles into higher-order structures that is driven by the hydrophobic effect in aqueous media. The shape anisotropy of the amphiphilic protomers strongly influences the supramolecular structure of the assembly with the formation of filamentous nanomaterials being a commonly observed occurrence. As described above for peptide-based assemblies, the anisotropic shape of amphipathic peptides drives self-association into protofilaments that can laterally associate into higher-order structures. Peptides are convenient substrates for the design of filamentous nanomaterials due not only to sequence specificity and shape anisotropy but also to the vast amount of prior knowledge of protein structure available in the PDB.

Perhaps a truer test of the protofilament approach would be the design of filamentous assemblies from protomers in which much less structural knowledge is currently available to guide sequence design. Peptidomimetic foldamers represent potential candidates for such studies since they adopt a limited range of stable conformations that depend on the sequence of the monomer [49]. The conformations of synthetic foldamers are distinct yet structurally related to the periodic secondary structures of peptides and proteins. Therefore, in analogy to peptides, foldamers should adopt conformations that display shape

anisotropy. Synthetic sequence control enables the introduction of polar sequence patterns that reinforce the periodicity of the conformation and that can drive self-association into protofilaments. This strategy is directly analogous to the design principles embodied in the original version of the supramolecular folding code for peptide sequence (Figs. 1 and 2). Several research groups have employed this approach to design filamentous assemblies from a small number of sequence-specific promoters derived from β-peptides, peptoids, and oligo-ureas [8]. However, thus far, the structures of only a few of these filaments have been characterized at near-atomic resolution and these studies have been based on crystallographic analyses or fiber diffraction rather than from cryo-EM helical reconstruction of isolated filaments. In these reported structures, the presence of protofilaments can be discerned in the corresponding peptidomimetic filaments although the mechanism of self-assembly remains relatively unexplored.

The rational design of synthetic non-peptidomimetic protomers that are capable of selective assembly into filamentous nanomaterials represents an even greater challenge. However, extension of these design efforts toward protomers derived from different structural subunits promises access to supramolecular assemblies that can extend the functional properties of these materials well beyond the limitations associated with peptide and peptidomimetic assemblies. However, general principles are lacking for the design of these abiotic filamentous nanomaterials and, given the structurally diverse range of anisotropic amphiphiles that can be chemically synthesized, design rules may need to be specifically tailored for different classes of protomer structures. The most attractive substrates for these design efforts would derive from aromatic compounds since the corresponding structures often display limited conformational flexibility and, therefore, the entropic cost associated with self-assembly is often less than that which would be expected for self-assembly of more flexible protomers. The conformational rigidity of aromatic structures limits the structural space for implementation of positive and negative design elements, which may be an advantage for cases in which little initial structural information is available to guide protomer design. In addition, π-π stacking interactions between the aromatic subunits, often in conjunction with the hydrophobic effect, provide a strong driving force for association into protofilaments and, ultimately, into filaments and nanotubes. It should be noted that such an approach has been employed by many investigators for the design of organic filaments and nanotubes [50], so it is hardly an original insight. However, cryo-EM analysis at near-atomic resolution has been applied to few of these structures. In these cases, the protofilament model appears to be conserved, although it remains challenging at this early stage to deconvolute the interfacial interactions that are structurally critical to self-assembly of the corresponding filaments and therefore might be amenable to synthetic control through rational design [51,52]. A protofilament-based model may be a useful concept to rationalize the design of such systems, which has otherwise been idiosyncratic and usually application-oriented, as is usually the situation for the discovery driven phase of research. A potentially under-utilized source of structural information, particularly for the design of interprotomer interfaces, is the Cambridge Structural Database (CSD). The CSD, currently (2022) contains 1.1 million accurate 3D structures of small molecules, could potentially play a similar role in design of interfaces between chemosynthetic protomers that the PDB provides for the design of protein–protein interfaces. If effectively mined, crystallographic data from the CSD could be employed to identify designable interfaces between small molecules, which would represent suitable substrates for fabrication of synthetic filamentous assemblies.

In summary, the objective of building better filamentous nanomaterials requires refinement of our current approaches to peptide and, more generally, protomer design. Near-atomic resolution structural analyses, primarily using cryo-EM helical reconstruction, have provided insights into the limitations of the supramolecular folding code, which, while successful, could not accurately predict interfacial interactions across different levels of structural hierarchy (*vide supra*). Deep-learning methods have demonstrated that protein structural prediction and, in some cases, *de novo* protein design can be successfully accomplished for individual proteins and discrete oligomers [53]. This *ab initio* approach may become possible in the future for the design of peptide-based filaments and, more generally, for synthetic filaments and nanotubes derived from self-assembly of designed small molecules. In the meantime, retrospective analysis of the atomic models of self-assembled peptide filaments suggests an alternative approach. Amphipathic protomers displaying anisotropic molecular structures or conformations can be designed to self-associate into hierarchical structures in which the filaments are composed of laterally associated protofilaments. While this structural model does not represent the sole approach to filament design, the protofilament paradigm effectively captures a common element among filamentous supramolecular assemblies across different supramolecular structures. In this scenario, the design problem reduces to a question of how to best introduce interfacial interactions into the protomer structure that would selectively drive self-assembly of the protofilaments and guide their lateral association. The supramolecular folding code provided initial structural principles to inform rational design of filamentous peptide assemblies. While several ensuing decades of protein structural analyses at near-atomic resolution have provided further insight into sequence-structure correlations for protein assemblies, insufficient structural information is available at present to unambiguously specify the higher order structure of a helical filament through sequence selection. Therefore, the success of this approach will require that the structures of the corresponding filaments are verified through high-resolution cryo-EM analysis. These structural data can not only provide further refinement of the design principles that underlie the supramolecular folding code but also provide insight into the associated issues of structural polymorphism and the lability of helical symmetry.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Vincent P. Conticello reports financial support was provided by National Science Foundation.

Data availability

No data was used for the research described in the article.

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