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Collagen mimetic peptide discs promote assembly of a broad range of natural protein fibers through hydrophobic interactions

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Abstract

Collagen mimetic peptides that alone formed two-dimensional nanoscale discs driven by hydrophobic interactions were shown in electron microscopy studies to also co-assemble with natural fibrous proteins to produce discs-on-a-string (DoS) nanostructures. In most cases, peptide discs also facilitated bundling of the protein fibers. This provides insight into how synthetic and natural proteins may be combined to develop multicomponent, multi-dimensional architectures at the nanoscale.

Graphical Abstract

The process of biomacromolecular self-assembly of multicomponent complexes is central to life – with astounding examples such as the combination of histone proteins, DNA and a multitude of chromatin-associated molecules controlling the 3D organization of chromosomes in the nucleus¹, or the structured networks of matrix proteins and proteoglycans that make-up basement membranes surrounding cells². Proteins have evolved surfaces that are capable of interacting with nucleic acids, lipids, carbohydrates, proteins and endogenous peptides either as scaffold or substrate agents. How nature controls assembly, structure, and dynamics of such complex multicomponent assemblies is poorly understood.

Designing protein scaffolds that mimic nature or display novel surfaces capable of targeting a specific substrate is of considerable interest to biotechnology and biomedicine³. Inspired by nature, tremendous progress has been made designing protein and peptides that self-assemble into nanostructures using fundamental structural units such as α -helices, amyloids, collagen mimetic peptides, or amphipathic peptide folded units. These materials can scaffold small molecules⁴, interact with the immune system², inhibit bacterial growth⁵, promote

three-dimensional cell growth⁶ and advance numerous medical⁷ and technological⁸⁻¹⁰ innovations.

Collagen mimetic peptides (CMPs) are one such class of molecules that have been used to study the structure and function of natural collagen, and explore synthetic modes of self-assembly – for excellent reviews of this field see ¹¹⁻¹⁵. CMPs are approximately thirty-residues in length and consist of repeating three-residue glycine-*X*-*Y* triplets where glycine is critical for formation of a hydrogen bonded core between three peptide strands, and the amino acids at *X* and *Y* determine their physiochemical properties¹⁴.

In previous studies, several CMPs were designed with hydrophobic amino acids at *X* and *Y* positions that self-assembled into one-dimensional fibers or two-dimensional discs depending on the length of the hydrophobic domain¹⁶. The peptide H4: (POG)₄(LIG)₂(POG)₄ (P = proline, O = 4R-hydroxyproline, G = glycine, L = leucine, I = isoleucine), assembled into predominately oblong, single-peptide thick discs (~8 nm) on the order of 100 nm in diameter, but sometimes as large as several microns. We hypothesized that disc assembly was driven by hydrophobic interactions between the central (LIG)₂ domains of H4 triple-helices. This was supported by the observation that H4 discs often adhered to carbon-coated EM grids edge-on, driven by the hydrophobic perimeter of the disc. Furthermore, when H4 was combined with synthetic fiber-forming peptides, morphologies such as nanostars or stacked discs on a string were observed.¹⁶ These observations motivated us to explore whether similar types of interactions could be utilized to control self-assembly of H4 with natural proteins.

We utilize self-assembling CMP discs as both scaffolds and substrates to explore how multicomponent structural assemblies could be controlled. An empirical study of these complexes by electron microscopy clearly shows co-assembly in all cases. Despite using different structural building blocks for the natural protein component – α -helix, β -amyloid or collagen triple-helix fibers – common supramolecular morphological features are observed in all systems when natural proteins are combined with H4 or related CMPs (Fig. 1).

Our first observation of H4 disc interactions with natural proteins occurred by accident. While visualizing an old batch of H4 stock solutions by transmission electron microscopy (TEM), we noted they had been contaminated. TEM micrographs of the contaminated peptides revealed H4 discs aligned along filaments of unidentified rod-shaped uni-flagellar bacilli over lengths > 8 μ m (Fig. 2A-D). The edge-on images of discs were seen as bright lines ~10 nm thick, equivalent to the length of the H4 peptide. Although H4 discs had been shown to lie edge-on with respect to the EM grid in the absence of other proteins, the co-localization of disc and fiber in these images suggests a disc-on-string (DoS) morphology. In addition to DoS binding, H4 discs bound to flagella using other modes: a ‘stapling’ of flagella (Fig 2C), and a few cases of discs associating with flagellar fibers in the plane of the EM grid were also observed (Fig. 2B,D,E). Unbound discs were also seen lying flat on the EM grid. In numerous cases, multiple flagella deposited in a parallel manner on the grid with bridging discs. This was suggestive of bundling of the flagella by H4 discs. Many

features of these images were qualitatively similar to previously observed H4-amyloid peptide complexes¹⁶, suggesting the DoS may be a general mode of assembly.

Building on this serendipitous observation and previous characterizations of H4 binding to synthetic substrates, we sought to explore whether disc interactions can be modulated by varying the structure and hydrophobic pattern of the target substrate. Further studies of the flagella-H4 interactions were limited due to the unknown identity of the bacterial species and flagellar proteins. We instead chose to study a series of purified fiber-forming proteins available in our laboratory for which extensive information was already known about structure and self-assembly. The β -structure rich α -Synuclein (α -Syn), the α -helical coiled coil tropomyosin (Tpm) and the triple-helical bovine Type I collagen COL1, represent a broad range of structural forms that all self-assemble into supramolecular fibers. Based on existing structural information, all three systems were expected to provide hydrophobic attachment sites for discs on their surface, and we predicted that the DoS binding mode would be observed in each case.

As had been previously observed for the synthetic amyloid-like peptide dNQ¹⁶, we expected that α -Syn, a neuronal synapse-associated protein that plays a central role in the pathogenesis of Parkinson's disease, would also bind H4 discs. α -Syn is proposed to target synaptic vesicles through a central hydrophobic domain¹⁷. It can be induced *in-vitro* to aggregate and form amyloid-like fibrils composed of β -sheets^{18, 19}. We anticipated that H4 might interact with hydrophobic surfaces along the α -Syn amyloid fibril, leading to DoS co-assembly morphology. α -Syn was provided in aggregated amyloid form by the Baum laboratory²⁰. TEM micrographs confirmed extended amyloid-like aggregates (Fig. S2). In the presence of H4 discs, DoS binding along α -Syn fibers was clearly observed (Fig. 3). Similar to flagella, multiple observations of H4 associating with bundles of α -Syn were noted (Fig. S3). A recent solid state NMR structure of the central hydrophobic domain of α -Syn shows a continuous exposed hydrophobic surface along the fiber²¹, consistent with the lack of observed regular spacing of H4 discs along the α -Syn fiber axis.

The α -helical dimeric coiled-coil Tpm is approximately 40 nm in length and assembles in a head-tail manner to produce long cables that associate with actin filaments in the cytoskeleton and muscle^{22, 23}. Shrimp Tpm was extracted and purified from frozen *Penaeus aztecus* (brown shrimp) and shown to be α -helical by circular dichroism spectroscopy (Fig. S4). When Tpm and H4 were combined, the fiber assemblies of variable length and width were clearly observed in TEM images (Fig 4A). Periodic banding patterns of 15 nm along the supramolecular assembly were observed (Fig 4B, E), suggesting highly regular stacking of discs along the fiber axis. Although Tpm self-assembles into extended filaments on its own^{24, 25}, this banded fiber morphology is only observed in the presence of H4. This appears to proceed in the manner of a zipper, where the frayed ends are brought together peptide discs (Fig 4A, C). In areas of the EM grid where sample deposition densities were lower, isolated H4 discs were observed binding edge-on in a clear DoS manner (Fig 4D).

The origin of the 15 nm period of banding is not evident from our understanding of Tpm structure. Individual Tpm proteins are approximately 40 nm long, indicating two or three H4 discs are attached per unit of Tpm length. H4 discs themselves can range in thickness

between 7–10 nm as observed by AFM, suggesting that Tpm is maintaining a consistent several nm spacing between adjacent discs. Unlike α -Syn, which presents a continuous hydrophobic surface, Tpm may have distinct, periodic hydrophobic sites that direct interactions with H4 discs.

The fiber-forming extracellular matrix protein Type 1 collagen (COL1) provides structure and mechanical strength to bone, skin and tissue. COL1 is formed by the association of three chains, each with over three-hundred and thirty repeats of the Glycine-*X*-*Y* triplet motif. The resulting triple-helix is approximately 300 nm long. Unlike Tpm, individual triple-helices can be induced to self-assemble *in vitro* to extended banded fibrils under the appropriate temperature and solution conditions. It has been proposed that hydrophobic amino acids on COL1 may facilitate this self-assembly process²⁶; we hypothesized that H4 discs could also interact with these sites.

Upon incubating H4 with bovine COL1 maintained at 4°C to prevent fibril formation, TEM micrographs showed clear evidence of DoS structures, and instances of multiple collagen chains bundled by H4 discs (Fig. 5A, S5). In multiple cases, H4 discs were seen to bind periodically every 67 nm (Fig. 5B), which corresponds to the spacing of exposed hydrophobic groups on collagen that are proposed to mediate self-assembly^{27, 28}.

Above 20°C, COL1 rapidly undergoes fibrillogenesis. When COL1 was allowed to self-assemble into mature fibers prior to adding H4, the DoS morphology was not observed (Fig. 5C, S5). COL1 fibers themselves exhibited canonical 67 nm D-periodic banding, but no clear discs could be discerned along the fiber axes. Instead, round electron-dense particles were observed adjacent to the fibers, perhaps due to nonspecific aggregation. The existence of condensed particles adjacent to banded Tpm fibers were also observed (Fig 4A, C, D). Aggregates of this morphology have not been seen for H4 alone in this or in previous work.

COL1 fibrillogenesis appeared to preclude interactions with H4 discs, suggesting the two processes might compete for the same hydrophobic sites. Natural collagen interacting proteins such as decorins and matrix metalloproteases have different affinities for dissociated COL1 triple-helices versus assembled collagen fibers²⁹. Changes in availability of hydrophobic sites upon assembly may contribute to interactions between COL1 and its native binding partners as well.

We explored the contributions of CMP hydrophobic domain size and pattern on their interactions with COL1. Previously examined CMPs - H2, H3 and QH4 (Fig 6A) - were shown to form discs similar to H4¹⁶. Additionally, in this study we tested a split H4 (SH4) peptide where the pattern of hydrophobic groups was changed by moving LIG triplets to each end. SH4 folded into a marginally more stable triple-helix than H4 and was also observed to form discs similar to H4 when assembled alone (Fig. S6). The H2, H3, QH4 and SH4 peptides (Fig 6A) were individually combined with COL1 kept at 4°C to prevent fibril formation. All except H2 bound in a DoS morphology. (Fig 6, S7). In H2-COL1 mixtures, the fields were predominantly flat discs, indicating weak interactions between the two components. SH4, which splits the H4 hydrophobic domain into two H2-like regions, did

bind to COL1 effectively, indicating that total hydrophobicity was a more significant factor than the pattern of hydrophobicity along the CMP sequence.

Conclusions

Fiber-forming proteins were selected initially because of their accessibility to our laboratory; nonetheless, they all exhibited similar modes of association with the synthetic CMPs – the formation of DoS morphologies and the bundling of protein fibers by CMP discs. Even unintended associations between H4 and flagellin showed these properties, suggesting this is a general class of multicomponent interactions.

Previous work on multicomponent assemblies showed H4 interacted with fiber-forming CMPs or the synthetic amyloid fibers through complementary hydrophobic surfaces¹⁶. Similar features on α -Syn, COL1, Tpm and flagellins may be responsible for driving association with H4 and related disc-forming peptides. Such hydrophobic interactions are often biologically relevant to the assembly and function of the natural protein – such as the role of hydrophobic groups in the assembly of COL1²⁶.

Alternatively, as may be the case in Tpm-H4 complexes, CMP discs may be adhering to ‘incipient’ binding sites that randomly manifest during neutral protein evolution³⁰. Such sites would have low enough association potential to mitigate uncontrolled aggregation in a natural context. However, when combined with synthetic peptides with extended exposed hydrophobic domains, interactions with these weak binding sites may be possible. As such, the ability to interface CMP discs with proteins may be generalizable to many other natural proteins³¹ and even polymers³², beyond the specific cases presented here.

An important challenge for furthering multicomponent designs will be achieving specificity of assembly. This may be done by engineering the fiber scaffolds such that weak interaction sites are removed and target sites for disc assembly are optimized to couple with peptides. Alternatively, existing CMP nanostructures can be used or new CMP sequences can be designed to bind to specific sites by engineering complementary polar or shape-dependent features into the anticipated binding interface. It is hoped that strategies such as binding and bundling can be optimized to precisely control supramolecular protein material architectures in multiple dimensions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig 1. Schematic of supramolecular assembly of synthetic peptide discs with natural proteins. (A) Self-assembly of synthetic discs and natural fibrous proteins. (B) Co-assembly of discs with fiber proteins to form a larger fiber in two dimensions.

Fig 2.

H4 interactions with bacteria flagella. (A) TEM micrograph of bacteria and H4 (white box) close-up of H4 binding two flagella, (B) H4 binding edge- and side-on along entire flagella (~8 μm) and corresponding schematic drawing (C) H4 discs stapling flagella, (D) H4 discs bundling multiple flagella, (E) H4 disc binding side-on to flagella. Additional images are provided in Fig. S1.

Fig 3.
TEM micrograph of H4 interacting along α -Syn fibers. Additional images are provided in Fig. S3.

Fig 4.

TEM micrographs of H4 and shrimp Tpm interactions. (A) Field highlighting size of H4-Tpm fibers, where the arrows (1) and (2) display the periodic banding patterns (B). (C) Stages of fiber bundling are observed, yellow arrows show “zipping-up” of fiber. (D) Field where H4 discs bind edge-on to H4-Tpm fibers. (E) Image analysis using ImageJ showed a clear 15 nm periodicity along the supramolecular banded fiber axis (C-yellow box). Further examples of H4-Tpm interactions are shown in Fig. S4.

Fig 5.

(A) TEM micrograph of H4 interacting (yellow boxes) along collagen type monomers. (B) Image intensity trace along fiber axis reveals correlation between H4 bands and 67 nm D-period of COL1. (C) In the presence of assembled COL1 fibers, H4 appears to aggregate into spheres. Additional images are provided in Fig. S5.

Figure 6. CMP disc interactions with collagen type-I (COL1) monomer triple-helices. (A) Disc forming sequences, where polar and nonpolar are colored black and orange, respectively. (B) SH4 edge-wise with COL1, (D) QH4 discs bundling discs COL1, (C) H3 bundling of COL1, (E) TEM micrographs of large H2 discs side-on along COL1. Scale bars are 100 nm.