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The *de novo* design of α-helical peptides for supramolecular self-assembly

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Abstract

One approach to designing *de novo* proteinaceous assemblies and materials is to develop simple, standardised building blocks and then to combine these symmetrically to construct more-complex higher-order structures. This has been done extensively using β-structured peptides to produce peptide fibres and hydrogels. Here we focus on building with *de novo* α-helical peptides. Because of their self-contained, well-defined structures and clear sequence-to-structure relationships, α helices are highly programmable making them robust building blocks for biomolecular construction. The progress made with this approach over the past two decades is astonishing and has led to a variety of *de novo* assemblies, including discrete nanoscale objects, and fibrous, nanotube, sheet and colloidal materials. This body of work provides an exceptionally strong foundation for advancing the field beyond *in vitro* design and into *in vivo* applications including what we call *protein design in cells*.

Introduction

De novo peptide and protein design refers to the programming of amino acid sequences to adopt predefined three-dimensional structures. This can be guided by studies of natural proteins, but the aim is to achieve minimal non-natural sequences to realise existing or completely new protein folds [1]. Originally, design approaches were referred to as rational design as they were founded on bioinformatics and empiricism. Increasingly however, powerful computational tools are emerging that allow *in silico* scoring and optimisation of huge numbers of sequences compatible with the target structure [2,3]. A key area within this maturing field is the design of polypeptide-based systems programmed to self-assemble non-covalently into defined supramolecular structures. This tests our understanding of peptide–peptide and protein–protein interactions and also has potential to produce biocompatible materials for applications in biotechnology and nanomedicine.

Thus far, a variety of ordered assemblies have been achieved with *de novo* polypeptides, including discrete particles, linear assemblies of fibres or nanotubes, and multi-dimensional arrays such as 2D lattices and crystals (Figure 1) [4]. A founding tenet of the field is the programmed assembly of simple components with rotational symmetry (C_n) [5]. Assembly *via* a single interface leads to closed rings or filaments, while using two interfaces enables more-complex supramolecular systems [6]. This approach was pioneered by Yeates who demonstrated that natural proteins of defined oligomeric states (*i.e.* symmetries) can be combined to produce fusion proteins that assemble into a variety of architectures [7]. Others have advanced this top-down approach to supramolecular assembly by redesigning symmetric proteins to associate *via* new interfaces or metal coordination [8-11].

De novo peptide self-assembly is a broad field [12]. Currently, much of the work centres on the fibres and hydrogels formed by small β-strand peptides, alternating D- and L-residue cyclic peptides, Fmoc-dipeptides and collagens; this subfield is reviewed elsewhere [13,14]. Assembly of more-ordered and discrete systems from the bottom-up is increasingly being achieved with *de novo* α-helical peptides [15]. This is due to several important features of

the α helix: 1) its predictable geometry defined by the narrow range of energetically favourable combinations of torsion angles in Ramachandran space; 2) straightforward sequence patterns of hydrophobic and polar residues that lead to amphipathic helices, which then readily associate; and 3) further well-understood sequence-to-structure relationships that allow this association to be directed to form specified oligomeric states and topologies. Here, we discuss recent progress in the design of supramolecular structures that use *de novo* α-helical peptides as building blocks.

Directing α-helical association to make coiled-coil building blocks

α-Helical oligomerisation can be programmed precisely through the formation of coiled coils, which are common structural motifs in which two or more helices supercoil into rope-like assemblies [16]. The component helices are amphipathic as defined by heptad sequence repeats *HPPHPPP* (where *H* and *P* represent hydrophobic and polar residues, respectively), often annotated ***abcdefg***. Coiled coils form primarily to bury the hydrophobic residues at ***a*** and ***d*** positions, which are brought together on one face of the helix. Moreover, the helix-helix interactions are characterised by intimate “knobs-into-holes” (KIH) packing of these residues [17]. The ***e*** and ***g*** positions that flank the hydrophobic seam are occupied typically by charged residues leading to complementary electrostatic steering and salt bridging between the helices.

In a seminal study, Harbury and coworkers were the first to demonstrate the close relationship between KIH packing and structure, showing that the nature of the hydrophobic residues at ***a*** and ***d*** directs coiled-coil oligomeric state [18]. Detailed study of natural proteins and rational design attempts have further established reliable sequence-to-structure relationships [16]. Furthermore, the fold can be geometrically described by a small number of structural parameters, first conceived by Crick, which can be used to build and score

sequences *in silico* rapidly [19,20]. This parameterisable, inherently symmetric and relatively fixed geometry of coiled coils make them ideal modules for supramolecular self-assembly.

A basis set of *de novo* coiled coils for self-assembly

Towards such building blocks, we have developed a "basis set" of orthogonal coiled-coil assemblies that robustly adopt specific oligomeric states and C_n symmetries (Figure 2a). Building on Harbury's work, Fletcher *et al.* present completely *de novo* peptide sequences that homo-oligomerise into parallel dimer (CC-Di), trimer (CC-Tri) and tetramer (CC-Tet) arrangements, with all designs confirmed by high-resolution X-ray crystallography [21]. Thomas *et al.* demonstrate charge patterning at the ***e*** and ***g*** positions to design heterodimeric coiled coils (CC-Di-AB) with a range of dissociation constants [22]. Indeed, using rational and computational methods, a swathe of coiled-coil heterodimers are now available [23-27]. Combining rational and computational design, Thomson *et al.* describe parallel, blunt-ended coiled coils with five (CC-Pent), six (CC-Hex2) and seven (CC-Hept) helical chains [28]. These are α-helical barrels as they have contiguous central channels and are able to bind and discriminate between small molecules [29]. Recently, a thoroughly characterised antiparallel homotetramer with D_2 symmetry has been added to the toolkit [30]. From this *de novo* basis set of characterised components, a range of supramolecular structures have been designed and functionalised towards various applications as follows.

Self-assembling cages (SAGEs) have been constructed by linking CC-Tri- and CC-Di-AB-based components into hubs, which then assemble into extended hexagonal networks and close to yield particles (Figure 2b) [31]. The structure and assembly of the SAGEs have been modelled by both coarse-grained and all-atom simulations [32,33]. Modification of the component peptides can alter particle diameter and address the surface with small molecules, peptides and proteins to different densities [34,35]. When added to epithelial cells, SAGE particles show no cytotoxicity and are endocytosed at rates that can be

controlled by varying surface electrostatics [36]. Furthermore, antigenic peptides can be extended from the homotrimer component to drive selective and assembly-dependent immune responses *in vitro* and *in vivo* [37]. Thus, the SAGE system is highly modular, comprises fully *de novo* peptides assembling into a unique non-natural architecture and shows promise for cell-biology and biomedical applications.

The CC-Tri through CC-Hept components have all been adapted to assemble into highly ordered fibres or nanotubes through end-on-end association driven by complementary charge interactions or native chemical ligation *via* the peptide termini (Figure 2c) [38,39]. Crucially, these studies show that the superstructure and persistence of the tubular assemblies is a direct product of the individual coiled-coil geometry and electrostatics. As with the discrete coiled coils, nanotubes built from the α-helical barrels are capable of binding small molecules. Moreover, amphipathic helices of this type can be adapted to bind carbon nanotubes [40,41]. Collectively, this work demonstrates the potential biotechnological value of these proteinaceous fibres in materials and sensing applications.

Outside of the Woolfson group, the Marsh laboratory have fused CC-Tri, -Tet and -Pent to the outer face of a natural trimeric protein to produce closed, small and monodisperse particles with tetrahedral, octahedral and icosahedral geometries, respectively (Figure 2d) [42-45]. Single particle analysis using electron microscopy has generated low-resolution structures that match the design target for all three systems. In addition, the octahedral particles have been decorated with maltose-binding protein through fusion to the coiled-coil domain, further confirming the intended peptide orientation and providing the foundation for application development [44]. To complement these studies, the group have also tested the robustness of the CC-Di to CC-Pent components by fusing these sequences to GFP and investigating the linker length and component orientation to tolerate this large protein [46]. Such studies are critically important in the development of reliable “off-the-shelf” components.

Accessing discrete nanoparticles more generally

Beyond the basis set, α helices can be programmed to assemble into discrete, particulate architectures. Ryadnov *et al.* have designed a homodimeric coiled coil (with C_2 symmetry) with two additional polar "facets" that are each able to interact with facets on two neighbouring coiled coils (with C_3 symmetry) to give a hexagonal array that closes [47]. The resulting assemblies are relatively monodisperse and a low-resolution particle reconstruction from cryo-electron microscopy provides evidence of a hollow core, though the designed three-fold association between the homodimer subunits has not been verified. The assemblies are able to encapsulate and deliver nucleic acids to cultured mammalian cells.

The Burkhard group have developed self-assembling peptide nanoparticles (SAPNs). This is designed through the fusion of a *de novo* trimeric coiled coil with tetramer- or pentamer-forming sequences adapted from natural proteins to generate particles with octahedral or icosahedral symmetry, respectively (Figure 3a) [48,49]. These assemblies often display a broad size distribution due to the flexibility in the component peptides and consequently no high-resolution structural data have been obtained. The system has been successfully decorated with peptide epitopes and whole proteins to develop novel vaccines against influenza virus, HIV and *Plasmodium falciparum* that are highly immunogenic and protective in model murine systems [49-51].

The Jerala group have pioneered a different approach where α-helices form the edges, rather than the vertices, of polyhedral nanoparticles (Figure 3b). In their first study, six orthogonal dimeric coiled-coil sequences are concatenated into a single polypeptide that folds as intended into a discrete tetrahedron [52]. The design strategy has been extended to afford four-sided pyramid and triangular prism geometries [53]. Importantly, a new tetrahedron design folds correctly *in vivo* within the cytosol of murine hepatocytes. Similar approaches using heterodimeric coiled coils to produce discrete 2D polygons have also been reported by the Woolfson and Keating groups [54,55].

Assembling linear filaments and nanotubes

Fibres and nanotubes have also remained key targets for peptide self-assembly. In particular, the Conticello group have contributed two distinct fibrillar materials. A non-blunt-ended heptameric coiled coil was modified to associate longitudinally through terminal electrostatic interactions and shown to encapsulate the small molecule Prodan [56]. The group also describe bifaceted coiled-coil peptides that assemble into spiralling sheets and stack to form wide nanotubes stretching for many microns (Figure 3c) [57]. Two structurally distinct packing modes are observed by cryo-electron microscopy and one or two mutations are sufficient to induce the large structural rearrangement between these forms. The medical potential of this system has been investigated by extending a variety of epitopes from one of the peptide designs to produce immunogenic nanofibers [58]. When injected into mice, the fibres are internalised by antigen-presenting cells and the epitope triggers a specific immune response without the need for adjuvant. These studies, and the related work of Burgess *et al*. [38], demonstrate the versatility of nanotube assembly through non-covalent coiled-coil stacking.

An entirely new form of α-helical filament has been reported by the DeGrado group [59]. Described as a “cross-α amyloid-like fibril” due its similarity to the *Staphylococcus aureus* PSMα3 peptide [60], the structure is composed of two twisted sheets of antiparallel helices that form parallel dimers across the superhelical axis. Mutation to a single interface position impacts assembly kinetics and leads to different structural geometries by X-ray crystallography. As with the SAGEs, these fibres are inherently highly modular, and their functionalisation could be tuned for precise molecular positioning.

Towards multi-dimensional systems

a-Helical building blocks that self-assemble in two or three dimensions will form lattices or crystals, respectively, and may offer new materials for industrial process or therapeutics (Figure 1). The Conticello laboratory describe an 18-residue repeat α-helix using just five amino acids that presents three orthogonal interfaces with pseudo-C_3 symmetry [61]. Due to the octadecad repeat, the helix-helix interactions display no superhelical twist and consequently the peptides assemble laterally to form large, highly uniform hexagonal arrays. Lattice parameters, such as interhelical distance and height, are consistent across multiple techniques, supporting the designed structural model and demonstrating a full understanding of the system that will be essential for downstream applications.

The inherent symmetry of coiled coils can also be used to tesselate lateral self-assembly. The Pochan and Saven groups have collaborated to demonstrate that the exterior surface of a D_2 symmetric antiparallel homotetramer can be designed computationally to form lattices with targeted space-group symmetries (Figure 3d) [62]. Many of these lattices stretch for over a micron with exceptional uniformity and their morphology and size can be controlled through sequence mutations and assembly conditions. A subsequent study investigates the effect of solution conditions on self-assembly to reveal that the system forms tubes, plates or needles depending on pH [63].

Finally, the computational design of a crystal-forming peptide has been accomplished by the DeGrado and Saven laboratories [64]. The C_3 symmetric coiled-coil homotrimer arrays laterally with P_6 symmetry and stacks through *N*-to-*C*-termini interactions. As well as material applications, the approach may aid the crystallisation of natural proteins.

Conclusions and future directions: peptide design in the cells

The α helix has proven to be a reliable module for assembling particles, tubes and lattices from the bottom up. However, only a handful of these *de novo* platforms have gone from design to function, and those that have mostly focus on vaccine development. Moreover, the

high cost and low scale of chemical peptide synthesis can hinder translation of peptide-based materials into real-life applications. A young and exciting area of *de novo* polypeptide design, where sufficient levels of production can be achieved, is within living cells. Furthermore, designed intracellular proteins could control endogenous pathways or augment them with entirely new and orthogonal functionality. Minimal, *de novo* scaffolds that do not interfere with natural infrastructure will be critical for the positioning and spatiotemporal control of such functional proteins. Engineering biological systems in this way is at the core of synthetic biology and could offer organisms to produce fine chemicals, act as biosensors, or perform bioremediation.

Towards these goals, it has been shown that redesigned proteins can fold and self-assembled as prescribed within the cytoplasm [65-67]. Alongside this, a number of *de novo* α-helical peptides and small proteins have been presented that operate in cells [26]. From the Woolfson basis set, homo- and heterodimer peptides can replace the protein–protein interaction domains in transcription regulation machinery, while CC-Di-AB can direct the intracellular localisation of a synthetic “cytoscaffold”, as well as tether enzymes to its surface [68-70]. The Baker laboratory has designed specific hydrogen-bonding networks within four-helix bundles to generate a suite of heterodimers that assemble orthogonally in *E. coli* [71]. Furthermore, several groups have engineered protein-based logic circuits within mammalian cells that can rapidly modulate cell behaviour without the need for gene regulation [72,73]. In particular, the system developed by the Jerala group uses *de novo* coiled-coil dimers as orthogonal protein–protein interaction domains [73]. Using these fast logic networks to control the association of *de novo* protein assemblies temporally could create highly responsive platforms to present functional biomolecules. We see this emerging area of *protein design in cells* as one of the next challenges in protein design, which may contribute engineered organisms for multiple applications in basic and applied science.

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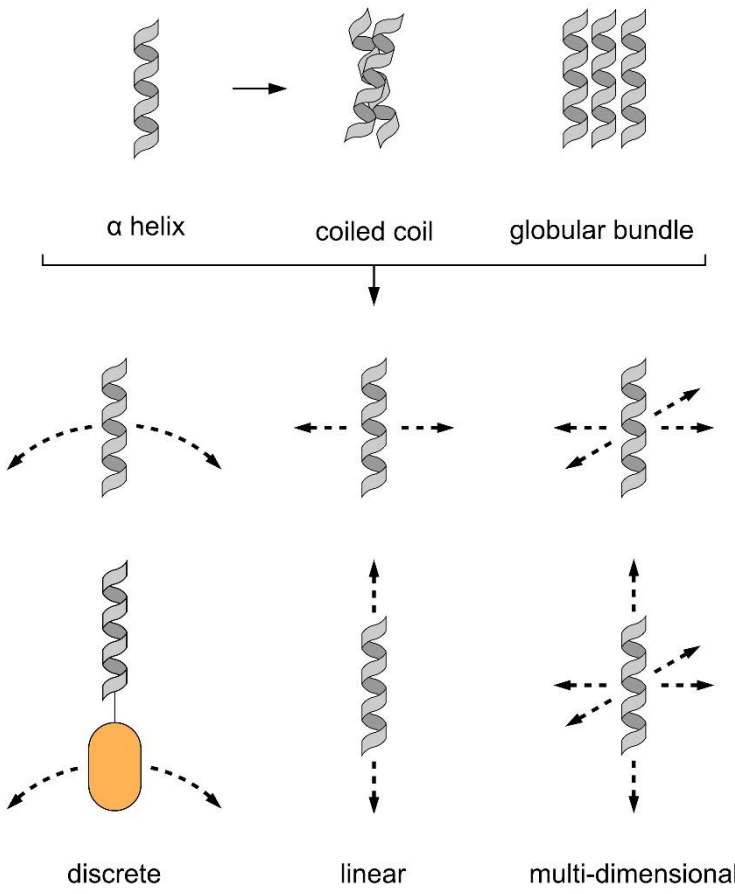

Figure 1. General modes of α-helical peptide self-assembly. α Helices can be programmed to form coiled coils or globular bundles. These structures can be designed to adopt a range of supramolecular architectures including discrete objects (sometimes in concert with natural proteins), linear fibrils and nanotubes, and multi-dimensional arrays and crystals.

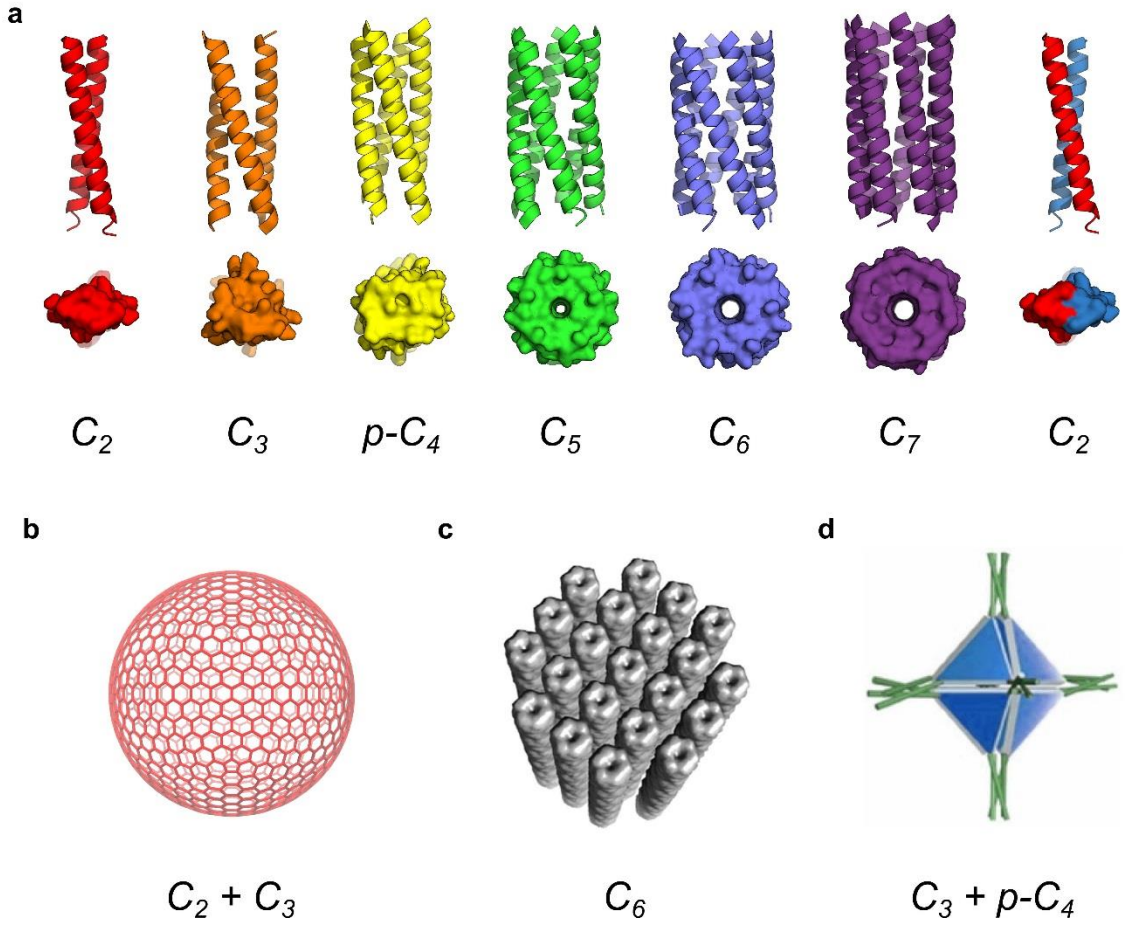

Figure 2. The Woolfson coiled-coil basis set and constructed assemblies. **a** X-ray crystal structures and symmetry groups of CC-Di (red, PDB ID 4DZM), CC-Tri (orange, PDB ID 4DZL), CC-Tet (yellow, *p*- denotes *pseudo*-, PDB ID 3R4A), CC-Pent (green, PDB ID 4PN8), CC-Hex2 (blue, PDB ID 4PN9), CC-Hept (purple, PDB ID 4PNA) and CC-Di-AB (red & blue; model) [21,22,28]. Examples of supramolecular structures created with the basis set include SAGEs (**b** [31]), nanotubes (**c** [38]) and protein-containing polyhedra (**d** [42]).

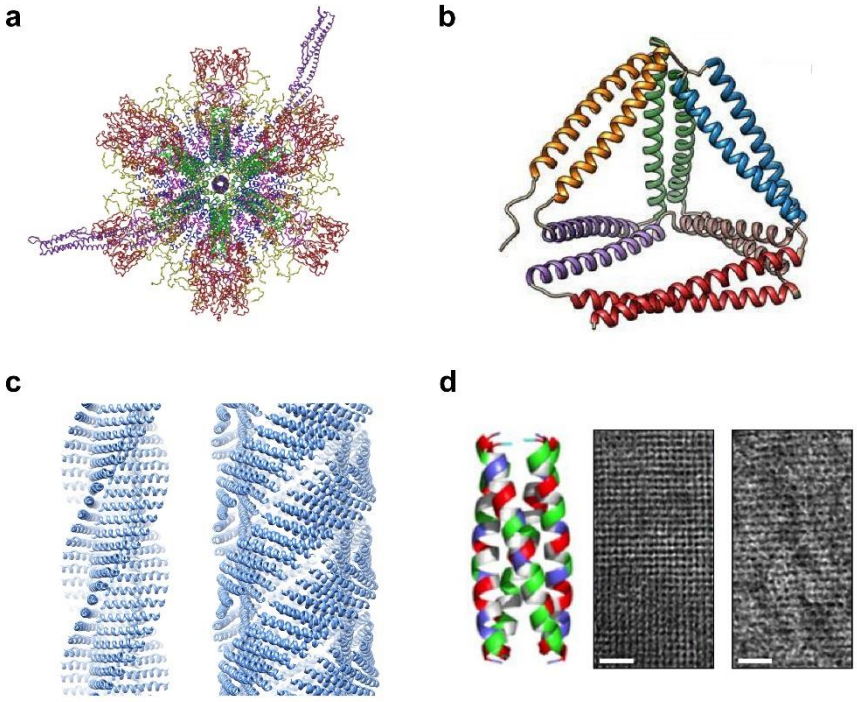

Figure 3. Coiled coil-based supramolecular systems. **a** Computational model of an icosahedral self-assembling peptide nanoparticle (SAPN) constructed with a pentameric coiled coil (green) and a *de novo* trimeric coiled coil (blue) presenting a variety of epitopes from *P. falciparum* (yellow, red and purple) (adapted from [49]). **b** Computational model of TET12SN, a single-chain tetrahedron composed of orthogonal dimeric coiled coils (adapted from [53]). **c** Atomic models fitted to cryo-electron microscopy reconstructions for two fibrillar packing modes accessed by bifaceted coiled coils (PDB ID 3J89 (left), adapted from [57]). **d** Computational model of a homotetrameric coiled coil (left) designed to adopt P422 (centre) and P622 (right) space groups as shown by transmission electron microscopy (scale bars: 20 nm) (adapted from [62]).

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●● Here the basis set *de novo* coiled-coil peptides are adapted to drive the formation of fibres and nanotubes. The geometry of the building blocks impacts on fibre/nanotube formation and assembly. For one peptide, the nanotubes are highly ordered allowing a high-resolution cryo-electron microscopy structure to be determined. The central channels of these nanotubes bind small molecules.

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