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## **Extending the self-assembly of coiled-coil hybrids**

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### **Citation**

Robson, M. H. (2009, December 9). *Extending the self-assembly of coiled-coil hybrids*. Retrieved from <https://hdl.handle.net/1887/14498>

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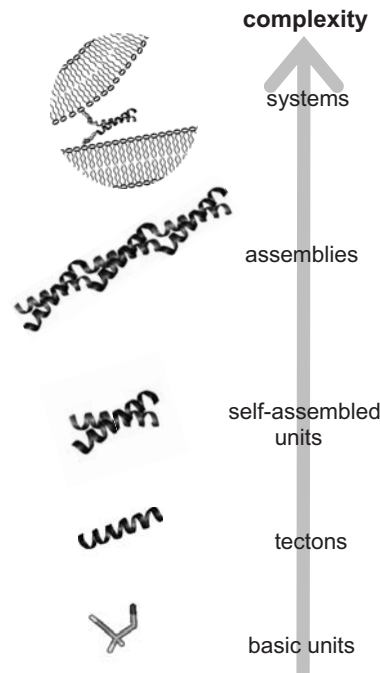
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## **COILED-COIL SELF-ASSEMBLY IN SYNTHETIC BIOLOGY: INSPIRATION AND PROGRESS**

Biological self-assembly is very complex, and results in highly functional materials. In effect it uses a bottom-up approach using well-defined biomolecular building blocks of precisely-defined shapes, sizes, hydrophobicity, and spatial distribution of functionality. Inspired by and drawing lessons from the self-assembly processes in nature, scientists are learning how to control the balance of many small forces to increase the complexity and functionality of self-assembled nanomaterials. This thesis takes the coiled-coil peptide motif, which plays a variety of roles in natural self-assembly, and connects it to other molecules to push the boundaries of synthetic self-assembly using this motif.

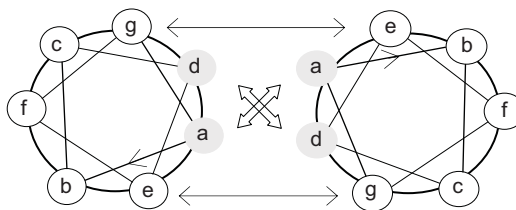
### INTRODUCTION

Synthetic biology aims to understand and harness the emergent properties of complex biological systems. As discussed here, one approach towards this is the use of biological, or biologically inspired modules, for the prescriptive self-assembly of functional synthetic systems. This Chapter draws attention to the versatility in nature of one of these biological modules, the simple coiled-coil peptide structure, and then highlights recent efforts towards meeting the synthetic-biological challenge this presents: attempts to use coiled-coil forming peptides to assemble functional units, assemblies, and systems of increasing complexity (Figure 1).



*Figure 1.* An overview of the use of the coiled-coil peptide motif in prescriptive self-assembly. In synthetic biology there are a range of natural and synthetic basic units, and for each there is a progression from basic units, to tectons, to self-assembled units, to assemblies. As a final goal multiple assemblies combine to yield functional systems.<sup>1</sup>

Both in nature and in the laboratory,  $\alpha$ -helical coiled coils are formed by the binding of two or more  $\alpha$ -helical peptides in a specific manner producing a stable complex in aqueous solution. The specificity of binding results from the amino acid sequences: the majority of coiled-coil forming peptides are characterized by a heptad repeat, denoted abcdefg, with apolar amino acids at most of the a and d positions, resulting in an amphipathic helix (figure 2). The packing of the hydrophobic a,d face against that of another coiled-coil forming peptide produces the majority of the binding energy. The apolar face of the helix is not parallel to the helical axis, but winds around the helix once every  $\sim 15$  nm, such that the packing of the hydrophobic strips against one another leads to the coiling of individual  $\alpha$ -helical ‘coils’. Amino acids with charged side chains are often located at positions e and g, which border the hydrophobic core when the peptides are in the coiled-coil conformation, and contribute to the specificity of binding. Coiled coils have a rope-like structure, with each heptad extending the length of the complex by  $\sim 1$  nm. Many aspects of coiled-coil binding are determined by the amino acid sequence: the oligomerization state (two or more peptides), size ( $\sim 2$  nm – 200 nm long), direction of binding (parallel or antiparallel), homo- or heterobinding, stability, and rigidity. The non-covalent association of these peptides is sensitive to changes in the environment, for example pH, temperature, ionic strength and metal ions, which affect the electrostatic or hydrophobic interactions. This versatility arising from a simple helix has resulted in many functions of coiled coils in nature, and has inspired many advances in synthetic biology.<sup>2</sup>



*Figure 2.* Helical wheel representation of a parallel dimer with a heptad repeat of amino acids. The heptad repeat positions are labeled from a to g and the  $\alpha$ -helices propagate into the page. The a,d surface (shaded grey) is predominantly hydrophobic, and residues at positions e and g are often charged.

## COILED COILS IN NATURE

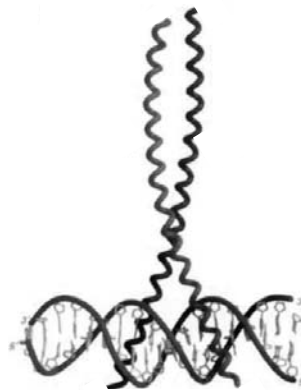
Predictions based on analyses of primary sequences suggest that the  $\alpha$ -helical coiled-coil motif makes up approximately 2.5% of all protein residues.<sup>3</sup> An analysis of a yeast proteome using two coiled-coil predicting programs identified 1,316 proteins that are thought to contain at least one potential coiled-coil domain, which corresponds to approximately 20% of the proteome of *S. cerevisiae*.<sup>4</sup>

$\alpha$ -Helical coiled coils are remarkable not only for their quantity, but for the range of functions that they exhibit *in vivo*. The very definition of coiled coils – two or more  $\alpha$ -helices binding together in a specific manner – means that wherever they are found – in every compartment of plant cells, in every eukaryote and prokaryote cell, they all have one role in common: the molecular recognition between two or more  $\alpha$ -helices causes the peptide segments to function as ‘cellular velcro’ – holding together the molecules and sub-cellular structures to which they are covalently attached.<sup>5</sup> The specific amino acid sequences modulate the velcro binding properties, and can also give rise to the other, more specific functions of coiled coils. Shorter coiled coils function primarily as highly specific cellular velcro, whereas longer coiled coils act as binding domains and simultaneously take on a wider variety of tasks in the cell.<sup>6</sup> *In vivo* many coiled-coils domains are long, containing several hundred amino acids, and the proteins are often composed of a long coiled-coil domain flanked at one or both ends by a globular domain. In contrast to short coiled-coil domains, where binding leads to lateral positioning of protein segments, the binding of long coiled-coil domains results in rod-like supramolecular structures. Only few long coiled-coil proteins have been characterized in prokaryotes (organisms without a cell nucleus, usually single-celled). In contrast eukaryotic organisms (containing a nucleus, multiple cells) contain more types of long coiled-coil proteins, such as motor proteins, membrane tethering and vesicle transport proteins, many of which are eukaryote-specific, suggesting that coiled-coil proteins have gained functions in the increasingly complex processes of the eukaryotic cell.<sup>5</sup> Although thousands of proteins have been acknowledged to contain coiled-coil domains, in the majority of cases the function of these coiled coils is not known. The functions of coiled-coil domains that have been elucidated to date have predominantly been binding, structural and dynamic. All of the

identified functions are summarized in the following sections, with one or two proteins that fulfill each role chosen as illustrative examples.

### ***Protein binding***

Short coiled-coil domains are most commonly found as oligomerization segments, where by means of molecular recognition they bring together proteins or protein segments, mediating a large number of specific protein interactions.<sup>6</sup> These coiled-coil domains can contain as little as two heptad repeats (~ 2 nm long),<sup>7</sup> but often have six or seven heptad repeats (~ 6 – 7 nm long). The folding of these domains into a stable complex can result in intramolecular binding, such as contributing to the assembly of the hydrophobic core of globular proteins,<sup>8</sup> or intermolecular binding, examples being in the assembly of ion channel signaling complexes and transcription factors (proteins that bind to specific sequences of DNA to either activate or repress gene transcription).<sup>6, 9</sup> The most widely studied coiled-coil containing proteins are the bZIP transcription factors. Proteins in this family consist of a ‘basic region leucine zipper’ (bZIP) domain, and an activation domain, which modifies the gene transcription. The protein complexes are formed by coiled-coil dimerization of the leucine zipper, and are anchored in position by a basic DNA-binding sequence (Figure 3). Homo- or hetero-dimerization of coiled-coil forming domains on different bZIP containing proteins determines which activation domains are in the protein complex, and hence precisely modulates the transcription of genes. An example of how sensitive the coiled-coil function is to amino acid sequence is the large extent to which a single amino acid modification can modulate the level of transcription. A serine in the position of a 31 residue coiled-coil domain of a bZIP transcription factor was phosphorylated, leading to additional intra- and interhelical electrostatic interactions. This resulted in the protein dimer becoming more stable, and as a consequence the phosphorylated protein bound to DNA with a 15-fold higher affinity.<sup>10</sup> Although the binding even of short coiled coils is specific it is not necessarily exclusive, and it is thought that the coiled-coil sequence of some signaling complexes allows for different coiled-coil partners at different stages of the signaling process.<sup>11</sup>



*Figure 3.* Crystal structure of the heterodimeric bZIP domain of the transcription factor c-Fos–c-Jun. The upper eleven helix turns constitute the coiled coil, and pincer the proteins onto the DNA strand. The activation domains are not shown.<sup>12</sup>

### ***Structural functions***

As coiled coils have a rod-like morphology an unsurprising role is as a structural component of the cell.

In some proteins the function of the long coiled-coil domain is to serve as a rod, which connects, spaces, and positions functional head and tail domains,<sup>6</sup> leading to the assembly of multi-unit complexes, bringing together bioactive components with defined stoichiometries and orientations at set distances with nanometer precision.<sup>1</sup> Examples of long coiled-coil spacer rods are the 8.3 nm long parallel homotrimer that separates the outer membrane from the bacterial cell wall in *Escherichia coli*,<sup>13</sup> and in the yeast spindle pole body where the distance between the plaques is determined by the length of a parallel homodimer in the connecting proteins.<sup>5, 14</sup> The amino acid sequence of spacer rods varies considerably between species, with positions a and d showing the least variation.<sup>15</sup> The sequence divergence is constrained only by the need to maintain the coiled-coil structure, which is predominantly driven by positions a and d. Coiled-coil rods are often homooligomers, with maximal apolar and/or ionic interactions<sup>16</sup> accounting for their rigidity.

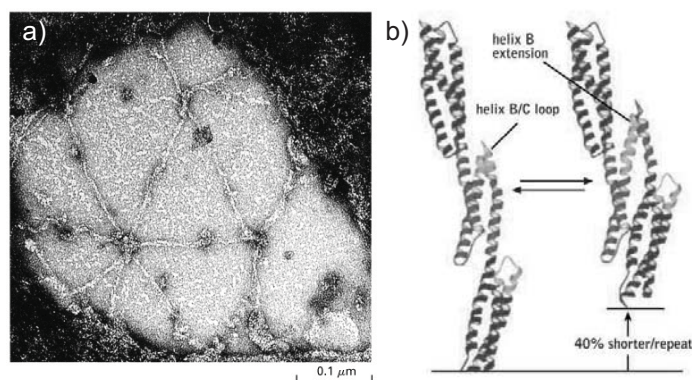
A remarkably stable coiled-coil stalk forms a structural edifice at the cell surface of the bacterium *Staphylothermus marinus*, which inhabits geothermally heated marine environments, and has an optimum growth temperature of 92 °C.<sup>17</sup> The bacterium is coated by umbrella-like tetrabrachion proteins consisting of four identical subunits that form a 70 nm long coiled-coil stalk that is anchored to the cell membrane at its C-terminus and branches into four  $\beta$ -sheet arms each 24 nm long at its N-terminus (Figure 4).<sup>18</sup> The arms form a canopy-like meshwork by end-to-end contacts that creates a semi-isolated sheath around the bacterium.<sup>19</sup> The coiled-coil domain sequence is such that the tetramer is remarkably stable, remaining folded at temperatures of 130 °C and in the presence of strong denaturants such as 6 M guanidinium hydrochloride.<sup>18</sup> The core positions contain an almost flawless pattern of aliphatic residues, mainly leucine and isoleucine, which contributes to its extreme stability.<sup>20</sup> This surface meshwork presumably has a cytoskeleton-like structural function,<sup>21</sup> and acts as a stabilization structure for the lipids and proteins of the cytoplasmic membrane.<sup>22</sup>



*Figure 4.* Negative stained TEM image of the tetrabrachion protein.<sup>17</sup> The 70 nm long coiled-coil stalk is stable to 130 °C and 6 M guanidine hydrochloride.<sup>18</sup> There are four  $\beta$ -sheet arms at the top of the coiled coil and two proteases noncovalently bound around the center of the stalk.<sup>19</sup>

The protein family of intermediate filaments has high sequence divergence, but all contain a  $\sim 45$  nm coiled-coil rod.<sup>23</sup> Intermediate filaments dimerize via homo or hetero coiled-coil formation. These parallel coiled-coil dimers pack together into filaments that are  $\sim 10$  nm wide and micrometers long.<sup>24</sup> The filaments have a persistence length of  $\sim 1$   $\mu\text{m}$ , and can be stretched to 350% of their original length. Both the properties of the coiled-coil dimers<sup>23</sup> and axial slipping between dimers<sup>25</sup> lead to the flexibility of intermediate filaments, and they are thought to function as stress absorbers in animal cells, which lack a cell wall.<sup>23</sup>

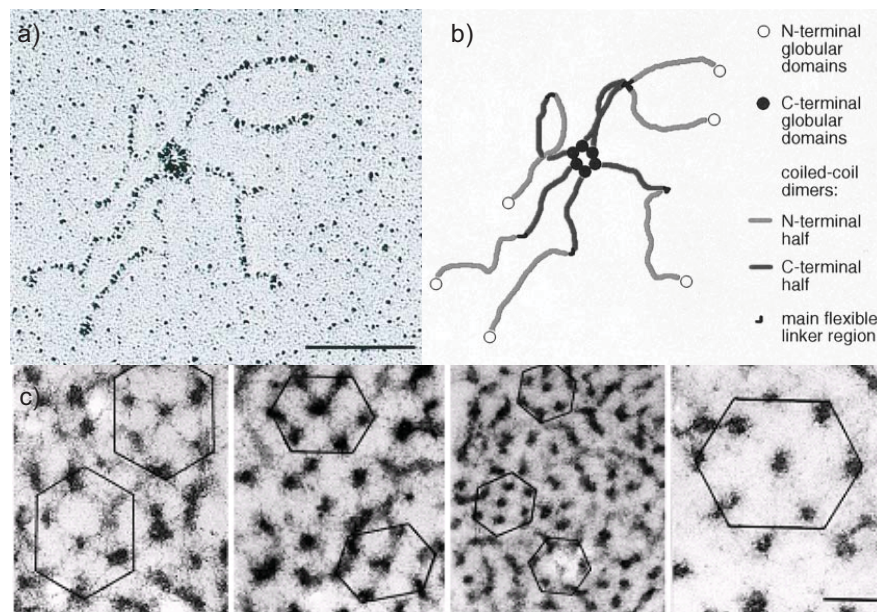
Many coiled-coil proteins utilize long coiled coils to create ordered two-dimensional networks and three-dimensional scaffolds that support the cell.<sup>26</sup> Like the intermediate filaments, these two and three dimensional structures can span microns. One such protein is spectrin, a cytoskeletal protein that forms a planar layer on the inner surface of the cell membrane of all animal cells (Figure 5a).<sup>27</sup> Spectrin is a fibrous protein largely made up of multiple 106-residue coiled-coil domains that fold into repeats of intramolecular coiled-coil trimers (Figure 5b). Four folded spectrin proteins self-associate end-on-end and side-to-side in a manner that is not fully elucidated but does not seem to be through coiled-coil interactions.<sup>28</sup> Multiple spectrin tetramers bind at actin junctions such that a membrane skeleton composed of ordered mosaics is formed (Figure 5a). These mosaics link to both membrane proteins, and to proteins in the cytoplasm.<sup>29</sup> The coiled-coil binding is dynamic, and coiled-coil rearrangements (the switching of one section of the protein between a loop and an  $\alpha$ -helix, Figure 5b) and variations in binding between two spectrin chains can rapidly vary the length and flexibility of the molecule, which controls the organization of proteins that are bound to each mosaic, and the membrane shape and mechanical resilience.<sup>29, 30</sup> An equivalent coiled-coil protein has also been found in a bacterial cell, and was found to be essential for the shape of the cell.<sup>31</sup>



*Figure 5.* a) TEM image of a membrane skeletal network showing the actin hubs and linking spectrin network. The darker spots along the spectrin spokes are where spectrin is cross-linked to membrane proteins.<sup>32</sup> b) Spectrin mosaics are largely composed of intramolecular antiparallel heterotrimers. The coiled-coil trimer repeats are depicted in dark grey, and the section that switches between loop and  $\alpha$ -helical conformations is depicted in light grey.<sup>29</sup>



The protein NuMa contains the longest known coiled-coil domain, (1485 residues, 207 nm long) which forms the major component of this fibrous nucleoskeletal protein. *In vitro* it self-assembles into multi-arm oligomers, and when overexpressed *in vivo* it induces a three-dimensional nuclear scaffold with a quasi-hexagonal organization that can fill the nuclei (Figure 6), indicating that its function is related to building up the architecture of the nuclear matrix.<sup>33</sup>



*Figure 6.* a) TEM image and b) schematic of NuMa multi-armed oligomers *in vitro* in which each arm is a homodimeric coiled coil. Scale bar 100 nm. The globular N-terminus domains (rings) can bind to the centers of neighboring oligomers resulting in a coiled-coil scaffold. c) When NuMa is overexpressed *in vivo* it forms a 3D scaffold with the mesh size determined by the engineered coiled-coil domain length. TEM images, scale bar 200 nm.

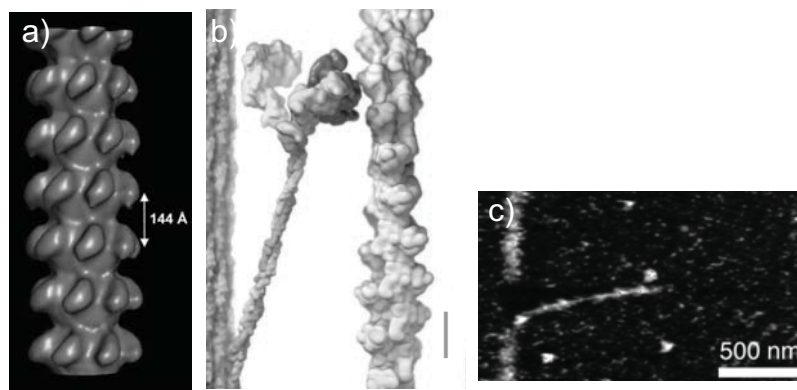
### ***Dynamic functions***

Directly interacting with the cytoskeleton are the cytoskeletal motor proteins. Three classes of cytoskeletal motor proteins have been identified – myosins, kinesins and dyneins, all of which contain coiled-coil domains.<sup>6</sup> These ‘movement’ proteins undergo large conformational changes in which the dynamic nature of the coiled-coil domains plays a key role. During each movement cycle of the proteins, which lasts tens of milliseconds,<sup>34-37</sup> the coiled-coil packing changes in response to applied force.

In muscle cells, myosin II is responsible for producing the contractile force by pulling along actin filaments. Myosin has a globular head domain and a ~150 nm long coiled-coil forming tail.<sup>[38]</sup> Parallel homodimers lead to two globular head domains, the motor units, being positioned adjacent to one another. Multiple coiled-coil tail domains associate laterally and longitudinally, in a very precise manner, forming thick filaments. The force-producing head domains protrude from the side of the thick filament, arranged helically around the filament with a repeat distance of 43.0 Å (Figure 7a). The packing of many



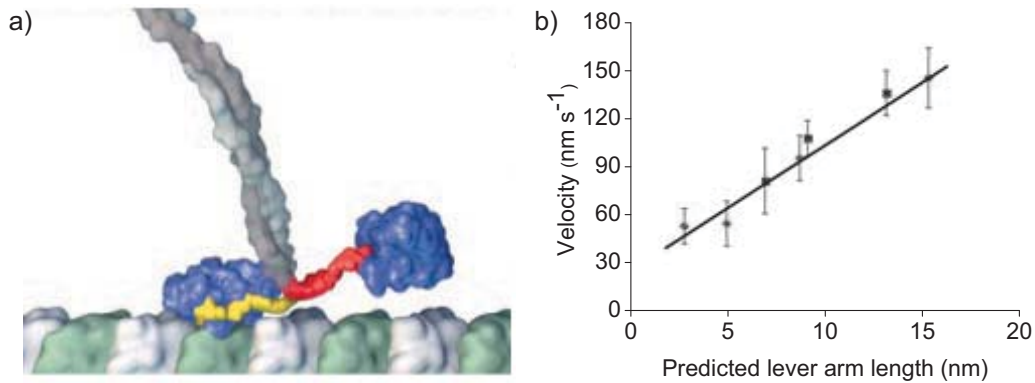
coiled-coil domains together means that not only the a d interface directs the packing, but the outer residues as well, and in fact in myosin the positions b, c, e, f, and g are more constrained between species than are residues in positions a and d.<sup>15</sup> The amino acid sequences of myosin coiled-coil domains are such that the N-terminus of the coiled-coil dimers extend out from the filament (Figure 7b). Thus, the packing of the coiled-coil domains keeps the myosin heads in the required orientation and spacing along the thick filament,<sup>21</sup> and the flexibility of coiled-coil domain allows movement of the head groups along the adjacent actin filaments, creating tension.<sup>6</sup> In vitro the myosin thick filaments have been shown to bend and to reversibly and quickly extend to more than 350% of their original length (Figure 7c).<sup>38, 39</sup> Bending is dominated by shearing between the coiled-coil dimers within the thick filament, whereas the stretching behavior is explained by shearing between coiled-coil dimers and coiled coil and  $\alpha$ -helix unfolding.<sup>39</sup> Elastic energy storage has been proposed as an important mechanism for minimizing the energetic cost of insect flight, and these elastic properties of myosin thick filaments in muscle may constitute part of this mechanism.<sup>39</sup>



*Figure 7.* a) 3D reconstruction from single particle EM analysis of relaxed muscle myosin thick filament illustrating the regular configuration of the myosin headgroups brought about by the packing of coiled-coil dimers.<sup>40</sup> b) Model of myosin dimer flexing out from the thick filament and binding to an actin filament on the right of the image. The elasticity of the coiled-coil domain allows the motor head group to ‘walk’ along actin filaments. Scale bar 60 nm.<sup>41</sup> c) AFM image of a myosin thick filament that has been stretched and broken by lateral pushing by the AFM tip.<sup>39</sup>

In myosin the coiled-coil dimer must be flexible in order to bend out from the thick filament to allow the head domains to ‘walk’ along actin filaments. In another motor protein, kinesin, the coiled coil plays a more direct mechanical role in the ‘foot over foot’ movement of the molecular motor along microtubules. Kinesin contains a central coiled coil with a motor domain at one end and a cargo binding domain at the other, and forms a dimer via the coiled-coil domain (Figure 8a). A small conformational change at the forward most ‘foot’ is conveyed and amplified by the coiled-coil ‘lever’ to the trailing motor domain, thrusting it forward, and pulling the cargo 8 nm along the microtubule.<sup>41</sup> The length of the coiled-coil ‘lever’ determines the velocity of the gliding motion (Figure 8b).<sup>42</sup> In order for the motor domains to walk along the microtubules it is essential that the

strands of the coiled-coil dimer adjacent to the motor domains can unwind. To demonstrate this the dynamic native domain was replaced with a more stable coiled coil, and the motility of the protein was effectively eliminated.<sup>43</sup>



*Figure 8.* a) Conformational changes in the kinesin motor domain are amplified by the coiled-coil lever, causing the second head to swing forward (cargo not shown).<sup>41</sup> b) Kinesin velocity along microtubules depends on the coiled-coil domain length.<sup>42</sup>

Dynein, the third class of motor proteins, comprises a  $\sim 12$  nm<sup>44</sup> antiparallel coiled-coil stalk domain that binds to microtubules via a small globular domain, a central globular head, and a cargo-binding stem (Figure 9a). As with kinesin, dynein also moves along microtubules in 8 nm steps.<sup>45</sup> The movement of dynein is not as well understood as for the other cytoskeletal motor proteins, but the microtubule-binding domain at one end of the coiled coil changes its affinity for microtubules depending on events at the headgroup, which is at the other end of the coiled coil (and vice versa), therefore structural changes must be transmitted along the length of the coiled coil. This implies a requirement for dynamic changes to helix–helix interactions.<sup>46</sup> It has recently been found that sliding the strands in the coiled-coil stalk by four amino acids couples the microtubule binding and headgroup activity.<sup>47</sup> It has also been observed that before the movement phase of each cycle, when dynein is tightly bound to the microtubule, the coiled-coil stalk is more flexible than after the powerstroke, when the coiled coil is straighter and has a lower standard deviation in its relative position, and is therefore thought to be more stable (Figure 9b). It is proposed that this flexibility may render the coiled coil capable of storing elastic energy when the molecule develops force against a load.<sup>48</sup> The length of the coiled-coil domain is highly conserved and is thought to be optimal for its force transduction role.<sup>21</sup>

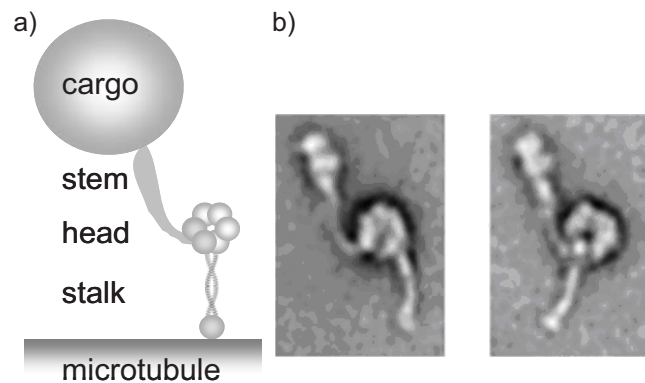
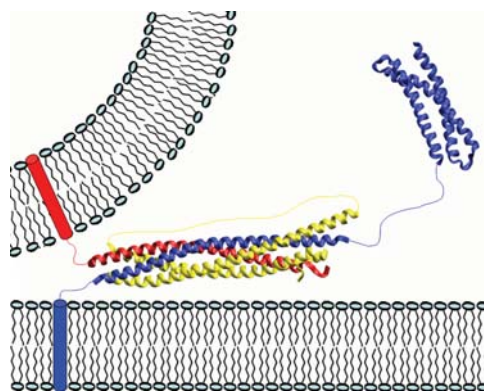


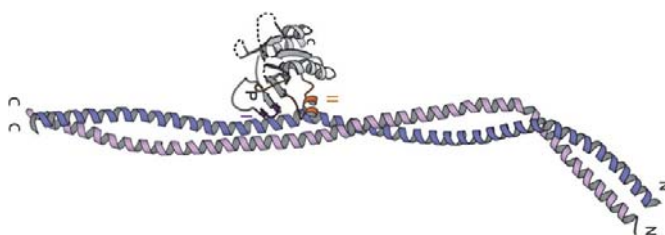
Figure 9. a) Dynein carries cargo along microtubules. b) Composite images of dynein from negative stained TEM. The coiled-coil stalk pre-power stroke (left) is more flexible than post-power stroke (right).<sup>48</sup>

The motor proteins discussed above all transport cargo along intracellular cables. Another method for intracellular transport that takes place in all eukaryotic cells is via transport vesicles. SNARE proteins are key components of this form of transport, as the dynamic coiled coil that forms between different SNARE proteins facilitates the docking and fusion of transport vesicles with organelles or the cell membrane. The SNARE proteins are a large family, with 27 SNARE proteins identified in a single unicellular parasite.<sup>49</sup> Although there is considerable variation in their structure and size, the coiled-coil domains are highly conserved, and it is thought that they all operate by way of the same mechanism. The SNARE proteins that are involved in the exocytosis of neurotransmitters from neurons are the best characterized. There is one type of SNARE protein connected to the transport vesicle membrane, another to the target membrane (in this case the neuronal membrane), and a third SNARE protein in the cytoplasm. A very stable coiled-coil complex forms between these three proteins, converging the membranes (Figure 10).<sup>50</sup> Assembly proceeds spontaneously from less structured monomers and results in a 6.5 nm coiled-coil heterotetramer.<sup>16, 51, 52</sup> The energy released by the formation of the stable four helix bundle overcomes the free energy barrier for fusion, producing enough force to disrupt the lipid bilayers, leading to membrane fusion, although the exact mechanism is unknown.<sup>16, 52</sup> The bundle is then ‘unzipped’ with the aid of four proteins and energy from ATP-hydrolysis so that the proteins can be used again.<sup>53</sup>



*Figure 10.* Structure of a SNARE protein complex featuring a coiled-coil tetramer that docks a transport vesicle to the target membrane and leads to membrane fusion and contents transfer. PDB codes 1sfc and 1br0.

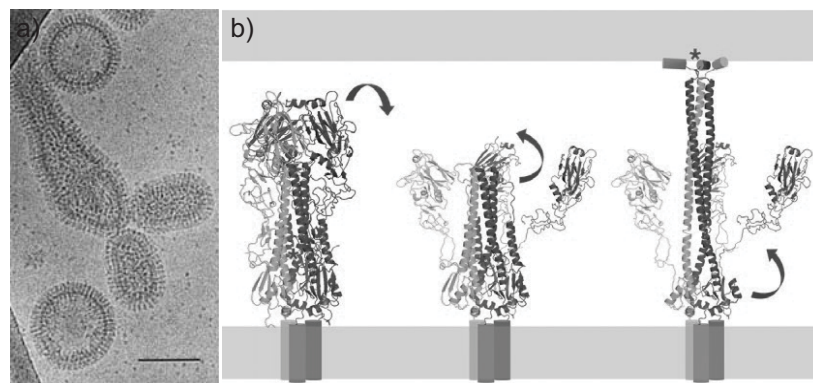
A group of proteins, Rabs, are thought to act upstream of the SNARE coiled-coil complex formation to organize the fusion site.<sup>54</sup> Via conformational changes Rab proteins are switched between active and inactive forms, with this change catalyzed by specific guanine nucleotide exchange factors (GEFs). Coiled-coil proteins have recently been found to function as GEF catalysts, a role which is usually carried out by much more structurally complex proteins. The Sec2p GEF domain forms a 22 nm long parallel coiled-coil homodimer that makes use of the coiled-coil motif for catalysis in a very simple manner. A small mid-section of twenty five amino acids of the coiled-coil hydrophobic core packing is disrupted, and this region binds specifically to a Rab protein (Figure 11). The binding interface is mostly hydrophobic and buries  $\sim 30$  nm<sup>2</sup> of solvent-accessible surface. The binding induces extensive structural rearrangements in the Rab protein, which activates the protein. The amino acids from both helices of Sec2p that are involved in this binding interface are highly conserved in other GEFs whose mode of function is currently unknown, indicating that they also operate similarly to Sec2p.<sup>55</sup>



*Figure 11.* Crystal structure of the homodimeric coiled-coil GEF domain of Sec2p in complex with a Rab protein domain, which catalyzes the Rab's cellular transport modulation activity.<sup>55</sup>

Similarly to the transfer of cargo within and out of cells as discussed above, viruses also employ a dynamic aspect of coiled coils to transfer their contents across membranes; however the mechanism by which coiled-coil formation leads to this is rather dissimilar. Enveloped viruses (i.e. surrounded by a lipid membrane) such as influenza, Ebola or HIV fuse their membrane coats with cellular membranes to import their genomes into cells by

way of pH mediated coiled-coil extension.<sup>56, 57</sup> An extensively studied example is the entry of the influenza virus, which displays a parallel trimer surrounded by globular head domains as an 8 nm long ‘spike’ on the surface of the viral envelope at normal physiological pH (Figure 12a). In the initial steps of cell entry, viruses are internalized by endosomes, where the pH is gradually lowered to ~ 5. The pH change causes the globular head subunits to dissociate from the spike, triggering what was previously a loop region to change into the coiled-coil configuration, irreversibly extending the coiled-coil ‘spike’ to 13.5 nm (Figure 12b).<sup>58-60</sup> The folding of the coiled coil propels a hydrophobic fusion peptide from a buried, basal position 10 nm towards the target membrane,<sup>58</sup> inducing membrane fusion and hence the release of the viral RNA into the cell.<sup>61</sup> In effect the central coiled coil provides a spring-loaded hinge that is set off by a drop in pH. Recent results indicate that the means of membrane entry of non-enveloped viruses also involves a coiled-coil spring-loaded hinge that brings a fusion sequence close to the target membrane, although the stimulus that releases the spring (i.e. that leads to coiled-coil formation) is not clear.<sup>62</sup>



*Figure 12.* a) Cryo-TEM of influenza virions at 30 °C, pH 7.4 with the coiled-coil containing protein complex visible at the surface.<sup>59</sup> b) The drop in pH as a virus is encapsulated in endosomes dissociates globular head domains from the coiled-coil bundle (left), causing a loop domain to fold (center), thereby extending the coiled coil, and projecting a fusion peptide towards the endosome membrane (right). The crystallographically determined components are in ribbon representation.<sup>60</sup>

Whereas the coiled coils in motor proteins are dynamic in response to applied force, and enveloped viruses form a coiled coil in response to a pH drop, some proteins make use of the temperature dependent dissociation of coiled coils. Virulent bacteria are under constant pressure to sense their environment as they advance along their route of invasion experiencing changes in pH, temperature, and osmolarity.<sup>63</sup> As coiled coils respond to changes in the environment they may act as sensors to variations in the intracellular environment. Salmonella contains a protein, TlpA, with an N-terminus DNA-binding region and a coiled-coil domain of 250 amino acids.<sup>63</sup> This is similar to the b-ZIP domains of transcription factors, except that as well as functioning via molecular recognition these coiled-coil domains also function via temperature ‘recognition’. At temperatures below 37 °C TlpA forms a homodimer that can bind to sequence-specific DNA, repressing its



activity. When the bacterium enters warm bodies, i.e. with temperatures above 37 °C, the homodimer is destabilized,<sup>64</sup> releasing the DNA, which is then available for replication.<sup>65</sup> Circular dichroism spectroscopy demonstrates that the temperature induced dimer to monomer transition of TlpA is reversible, and upon cooling both function and full  $\alpha$ -helicity are regained.<sup>65</sup>

It is evident that by variations in the interfaces between  $\alpha$ -helices (through different amino acid sequences), a remarkable assortment of properties emerge, and coiled coils are used in numerous ways in the cell. Coiled-coil structures provide one, two, and three dimensional mechanical stability to the interior and surfaces of cells via rods, mosaics, and scaffolding. The supramolecular structures are also involved in movement processes for which particular degrees of flexibility are essential. Natural coiled coils are utilized for their extremely thermostability in some cases, and their relative lability in others, switching structure in response to temperature or pH. Furthermore, coiled coils act as a molecular recognition system, catalyzing cell activities. The biological function of the coiled-coil motifs in many other proteins is not clear, and it is expected that several other functions will be elucidated in the future.

## COILED COILS IN SYNTHETIC BIOLOGY

The functions of natural coiled coils discussed above took approximately 3.8 billion of years to develop.<sup>66</sup> Since the 1950s scientists have been reverse engineering nature: by studying the form and function of proteins, and tracing these back to amino acid sequences the 'rules' for their self-assembly can be obtained,<sup>67, 68</sup> allowing de novo peptide design, yielding novel form and function. In a synthetic sense this means designing molecules that organize into well-defined structures with specific functions.

Coiled coils are good candidates for the self assembly of smart biosynthetic nanostructures for many reasons: they have precisely defined size and shape (i.e. rods 2 nm in diameter with each heptad  $\sim$  1 nm long) and surface functionality; the intra- and inter helical noncovalent interactions are relatively well understood; they can self-assemble into stable structures at low concentrations (sub-nanomolar<sup>69</sup>); coiled coils can be functionalized at the N- or C terminus or via solvent-exposed amino acids; and the affinity and specificity of the binding of coiled coils are very sensitive to the amino acid sequence. This rich array of controllable properties means that there is a coiled-coil 'building block' to suit many castles in the (supramolecular) sky.

Self-assembly that is inspired by  $\alpha$ -helical coiled-coil peptides is discussed in terms of 'synthetic-biology space', as put forward by the Woolfson group, in which basic units bind covalently to form tectons, which hierarchically self-assemble via units and functional assemblies, and combine with other functional assemblies to culminate in systems.<sup>[1, 70]</sup> In this quadrant of synthetic biology the basic units are amino acids,



sequences of which covalently bind to form the tectons,  $\alpha$ -helices. The  $\alpha$ -helices bind noncovalently to form the units, coiled coils, which organize further into assemblies, and finally aspire to entire systems (Figure 1). The mapping and exploration of coiled-coil synthetic biology up to the current date is reviewed below. First units are discussed, then assemblies, and finally the first uses of coiled coils in systems are charted, and parallels are drawn between these advances and the sophistication of naturally occurring coiled-coil motifs.

### ***Coiled-coil units***

The initial aim of coiled-coil research was to understand the structures and binding of natural coiled coils. Peptides derived from transcription factors and other natural coiled coils have been mutated in order to delve into their binding properties. Once the rules mapping peptide primary sequence to intermolecular interactions had started to emerge the design aspect broadened from changing isolated residues in a natural sequence to designing completely de novo sequences, and the units have become more removed from native form and function as the possibilities of the unit are explored. Many aspects of coiled-coil binding have been tailored in coiled-coil units, namely coiled-coil length, stability, specificity of recognition partners, oligomerization number, strand orientation and conformation, with highlights touched upon in the following section.

To date the majority of the peptide units whose sequence to structure relationships have been investigated and modified have been short, usually with 3-5 heptad repeats. Generally peptides with more heptad repeats form more stable complexes. Very short homodimers with only two heptad repeats have been created by optimizing design criteria, i.e. by enhancing the hydrophobic packing, and intra- and intermolecular salt bridges, utilizing amino acids with high  $\alpha$ -helix propensity, and using suitable capping moieties.<sup>71, 72</sup> The shortest identified coiled coils in nature also contain two heptad repeats,<sup>7</sup> and this appears to be the lower size limit.

A common goal is to design coiled coils with greater binding stability while retaining the other aspects of coiled-coil binding. This feature of unit self-assembly has primarily been targeted by optimizing the primary sequence. As an example, amino acid substitutions in the 37 residue coiled-coil domain of the c-Jun transcription factor<sup>12</sup> caused an increase in the melting temperature the Fos-Jun Heterodimer of 37 °C. By analyzing different amino acid substitutions it was concluded that the substitution of amino acids that increase the buried hydrophobic area and improve helix stability accelerate the formation of a partially-folded dimeric intermediate, and that after this intermediate is formed improved intermolecular coulombic interactions increase the thermodynamic stability of the final coiled-coil structure.<sup>73</sup> In another example the substitution of two amino acids in position a of the 34 residue coiled-coil domain of another DNA binding protein decreased the dissociation constant for homodimers by a factor of 105.<sup>74</sup>

Non-natural fluorinated amino acids, which have a large hydrophobic area, have been incorporated into recombinant coiled-coil peptides leading to increased stability with minimal structural perturbation of the final complex.<sup>75, 76</sup> For instance isoleucine residues in core positions of the bzip domain of peptides derived from the transcription factor GCN4 were substituted with 5,5,5-trifluoroisoleucine, resulting in an increase of 27 °C in the melting temperature while the affinity and specificity for DNA binding was similar to the hydrogenated counterparts.<sup>76</sup>

Another non-natural approach to increasing coiled-coil stability is the modification of amino acids. An example of this approach was the attachment of an azobenzene moiety as an intramolecular crosslinker between two residues in position *f* of a heterodimer, i.e. solvent exposed and parallel to the helix length. Irradiation of the peptide reversibly changed the conformation of the azobenzene cross-linker from *trans* to *cis*, thereby decreasing its length, and bringing it into line with the natural helical repeat length of the peptide, which increased its helicity and promoted coiled-coil folding.<sup>77</sup>

Metal binding to histidine and cysteine residues can also affect the stability of coiled coils. Divalent binding of metal ions to residues at positions *i* and *i* + 4 can be used to stabilize coiled coils, while *i* and *i* + 2 binding destabilizes coiled coils. Coordination of two peptides to a single metal ion can induce the coiled-coil complex, with this effect having been demonstrated with the metal ion at both solvent exposed and internal positions.<sup>78</sup>

Coiled-coil complexes are specific in terms of the sequences of the peptides that will bind, the number of strands that associate, and the orientation of the binding partners. The high degrees of binding specificity that can be designed into the coiled-coil interaction has been exemplified by the formation of three distinct heterodimers in solutions of six peptides.<sup>79,</sup>

<sup>80</sup> In one case the four heptad repeat peptides were based upon natural coiled-coil domains from transcription factors, and the selectivity was introduced by substituting a single amino acid in a core position. As well as substituting natural amino acids, non-natural, urea derived side chains were utilized to improve selectivity.<sup>79</sup>

Amino acids with charged side chains are important determinants of which peptides will form a coiled-coil complex, and controlling inter- or intra molecular coulomb interactions through pH or salt can be used to modulate coiled-coil binding by destabilizing certain complexes. Many hetero coiled coils gain their specificity by having charged strips bordering the hydrophobic core such that one helix is positively charged and the other negatively charged, hence preventing homo coils forming. In this way pH can be used to influence coulomb interactions such that hetero coiled coils form at neutral pH, and homo coiled coils at low<sup>81</sup> and high pH.<sup>82</sup>

This concept of pH controlled strand exchange has been developed further with iterative cycles specifically replacing one, two, or all three initial helices of a coiled-coil trimer.<sup>83</sup> The strand exchange can also be programmed to be accompanied by a switch from a parallel to antiparallel trimer.<sup>84</sup>

Alternatively to the electrostatic destabilization of particular helix combinations, the number of  $\alpha$ -helices in a coiled-coil bundle can be changed by the stabilizing effect of steric packing in the hydrophobic core, which is the major driving force for coiled-coil formation. For example, an engineered form of a native coiled coil is predominantly two stranded, but the coiled-coil trimer becomes the most stable arrangement when one benzene molecule is bound in the hydrophobic core, as the increased buried hydrophobic surface renders the trimer the energetically preferred complex.<sup>85</sup>

The oligomerization state can also be varied by tuning the hydrophobicity by way of substituting amino acids in positions within the coiled-coil hydrophobic core. This was investigated by systematically substituting the 20 natural amino acids in the central a and d positions of a five heptad repeat peptide that forms homo coiled coils. The  $\beta$ -branched residues isoleucine, valine and threonine which have side chains with large hydrophobic areas promote trimer formation whereas amino acids with charged side chains favor two-stranded coiled coils.<sup>86, 87</sup>

Small changes in peptide sequence can also lead to different binding orientations. For instance the five heptad repeat coiled-coil domain from an osmosensory transporter binds as an antiparallel homodimer. When two charged residues in position a of the heptad repeat are replaced with isoleucine the hydrophobic packing is altered, interchain salt bridges are eliminated, and the dimer changes orientation from antiparallel to parallel, rendering the protein inactive *in vivo*.<sup>88</sup>

Considering the distribution and range of functions of coiled coils *in vivo* there are many potential ways in which controlling existing coiled-coil binding can influence *in vivo* function. For example there are research groups investigating coiled-coil forming peptides to specifically bind to the coiled-coil bundles essential to viral entry, with the aim of inhibiting them,<sup>89, 90</sup> or to bind to specific transcription factors to modulate the replication of DNA.<sup>91</sup>

Another aspect of coiled-coil unit self-assembly that has been investigated is to switch the secondary structure of the peptides, which can be programmed to fold into different structures in different environments. The most common conformational switch (other than coiled coil - random coil) is between coiled coils and  $\beta$ -sheets. This is generally achieved by incorporating amino acids with high  $\beta$ -sheet propensity or that are hydrophobic into solvent exposed f positions of coiled coils, and upon heating the  $\alpha$ -helices undergo a rearrangement into  $\beta$ -sheets which aggregate into amyloid-like fibers.<sup>92-94</sup> In another approach, a peptide that forms homodimers at neutral pH was modified such that there was a lysine or glutamic acid face next to the hydrophobic core of the coiled-coil complex. Changing the pH led to these faces being charged, destabilizing the coiled coil, which rearranged into random coil or  $\beta$ -sheet structures.<sup>95</sup>

The final examples in this 'coiled-coil unit' section demonstrate that even without any larger scale assembly the units can be highly functional. Self-replicating complexes have

been developed in which coiled-coil folding catalyzes amide bond formation, producing replicates of the coiled-coil forming peptide. Two peptide fragments fold onto a full length peptide template, and an amide bond is formed between the two fragments via a cysteine thioester intermediate.<sup>96</sup> Subsequent developments included enhanced catalysis at reduced pH,<sup>97</sup> or at high ionic strength,<sup>98</sup> heterodimeric complexes,<sup>99-101</sup> a hypercycle, in which two self-replicating peptides catalyze each other's replication,<sup>102</sup> and a chirality-dependent self-replication cycle. In order for the cycle to continue the peptides must dissociate once the bond is formed, and to speed this up peptides one heptad repeat shorter were used,<sup>103</sup> or alternatively a proline kink was introduced to destabilize the coiled-coil complex.<sup>104, 105</sup> An interesting advance contains two self-assembling groups: peptides with nucleobases introduced as side chains. The complementary nucleobase interaction (via hydrogen bonding) enhanced the peptide self-replication reaction.<sup>106</sup>

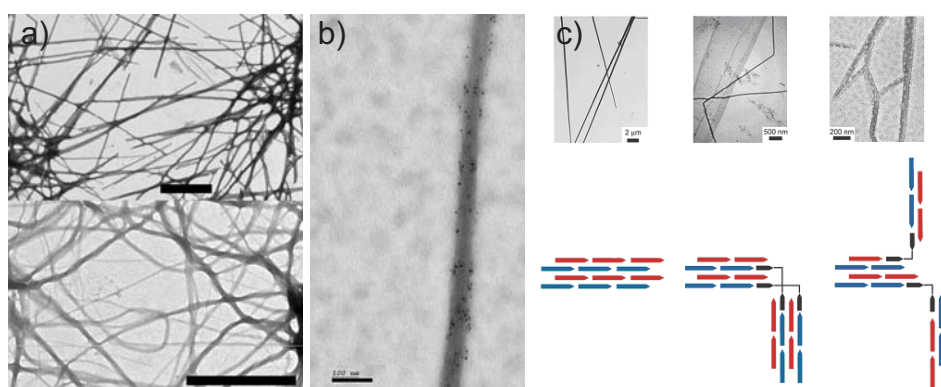
The examples touched upon above demonstrate how researchers have taken the coiled-coil motif as a natural binding unit, deconstructed and rearranged it in many permutations to elucidate the mechanisms and subtleties of the binding, and in the process explored the wide variety of functions that can be chemically programmed into coiled-coil units. From the perspective of synthetic-systems chemists designed peptides have the advantage over natural peptides that the chemical, physical, and biological properties of the complex can be precisely defined over a broader range. For this reason it is predominantly designed peptides that are used to create higher order structures and systems. In the following sections an overview is given of the use of these functional building blocks to create one, two, and three-dimensional assemblies.

### ***Coiled-coil Assemblies***

Since 1997 coiled-coil based synthetic biology has been extended by the self-assembly of coiled-coil units into larger assemblies that contain multiple coiled-coil units.<sup>107</sup> As with the synthetic study of coiled-coil units, all of the coiled-coil assembly examples that follow use peptide lengths that would be considered short in nature (3-6 heptads). For the study of isolated coiled-coil units it is convenient to elucidate the binding properties with small molecules because they can be readily synthesized on solid support and because the effect of, for example, changing a single amino acid can more readily be identified. As the functional possibilities of coiled-coil assemblies are explored it is likely that there will be an expansion (via protein engineering) to longer coiled coils and de novo proteins with coiled-coil domains. In this section an overview is given of coiled-coil assemblies, with the structures categorized into those for which coiled-coil formation is the sole driving force for material organization, and those which contain two self-assembling entities.

### *Materials formed solely by coiled-coil folding*

The first examples are of materials composed entirely of coiled coils and the following examples are assemblies that are formed from coiled-coil hybrids, but which nevertheless derive their higher order structure from coiled-coil folding. A well established mode of assembly is fibers and fibrils.<sup>1, 108-112</sup> This field takes inspiration from nature, in which coiled-coil proteins are often in the form of fibers, such as spacer rods or intermediate filaments. The rod-like structure of long native coiled coils is mimicked by using multiple short homo or hetero coiled-coil forming peptides which associate laterally<sup>113</sup> and in a staggered way such that each peptide is involved in two coiled-coil interactions simultaneously, leading to fibers, some up to hundreds of micrometers long. The fibers are generally composed of a bundle of coiled coils due to interactions between the amino acids on the outside of the coiled coil. To control this higher order structure more thought has to be put into the design of the amino acids in positions b, c, and f of the heptad repeat, an analogue to the decreased sequence variation in buried native coiled coils in comparison to non-buried motifs, e.g. in myosin filaments. Although the native rod structures can be emulated, the functions have by in large not been mimicked yet. With an eye to this current efforts are geared towards controlled design of the fiber morphology and related properties, for instance thinner and more flexible peptide fibers (Figure 13a).<sup>114</sup> The functionality of the fibers has been increased by conjugating additional molecules to the coiled-coil forming peptides, resulting in fibers coated with recruiting agents. These molecules on the surface of the fibers were able to bind to and hence localize proteins from solution (Figure 13b).<sup>115</sup> An additional dimension can be introduced by engineering kinks and branches into the fibers (Figure 13c).<sup>116, 117</sup> These coiled-coil fibers have been used to template silica layers at ambient temperature and physiological pH, which upon removal of the peptide (achieved most effectively by a protease), resulted in hollow silica tubes nanometers wide and microns long that are straight, kinked, or branched depending on the peptide template.<sup>118</sup> Alternatively, the fibrils can be induced to change to spherical objects at neutral pH,<sup>119</sup> or to reversibly dissociate at low pH.<sup>120</sup>



*Figure 13.* a) The rigidity of coiled-coil fibers can be programmed into the amino acid sequence. Negative stained TEM images, scale bars 1  $\mu\text{m}$ .<sup>114</sup> b) TEM micrograph of a peptide fiber coated with recruited proteins. 5 nm gold particles were bound to the protein to enable visualization.<sup>115</sup> c) TEM images of straight, kinked, and branched coiled-coil fibers, with the modes of assembly shown schematically.<sup>112</sup>

Another method of accessing long coiled-coil rods has been to covalently link multiple coiled-coil forming peptides such that larger scale assemblies form upon complex formation. Helix-loop-helix peptides have been linked into four arm dendrimers via a sulfide bridge between cysteine residues in the loop region. These assemble into fibers with diameters of only  $\sim 5$  nm, which are postulated to be one complex wide, and are many microns long. Heterofibers or homofibers can be formed depending on the pH dependent charge of the peptides (Figure 14).<sup>121</sup>

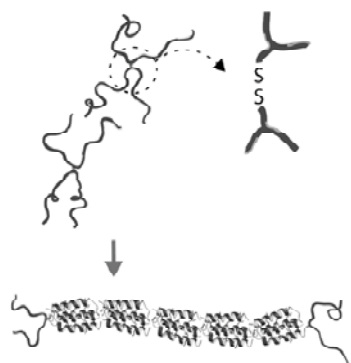


Figure 14. a) Two helix-loop-helix polypeptides are dimerized via cysteine residues and assemble into either homo- or heteroassociated fibers upon folding, depending on the pH value (pH 4.5 = homoassociated fibers, pH 7 = heteroassociated fibers).<sup>121</sup>

The majority of hierarchical coiled-coil structures are fibrous.<sup>122</sup> Dendrimers allow one to branch off from this direction and form three dimensional assemblies. Relatively complex self-assembly has been programmed with coiled-coil dendrimers: each peptide of a three armed dendrimer forms a dimer with a complementary peptide monomer, and the six-helix bundle then binds to three other dendrimer complexes through electrostatic interactions. In this way supramolecular porous sub-micron to micron sized spheres self-assembled. Silver colloids were formed within these ‘nanoreactors’, with diameters matching the pore sizes (Figure 15). As thiols have been shown to have a size-stabilizing effect on metallic colloids a cysteine residue was placed at position f in the coiled coil, such that the cysteine residues were orientated into the cavities.<sup>122</sup> This has certain parallels with the coiled-coil protein NuMa, which also forms dendrimers that self assemble into well defined three dimensional networks, creating a porous structural support (Figure 6),<sup>33</sup> although NuMa contains the longest known coiled coil, and this assembly is built up from the shortest known heterocoil length.



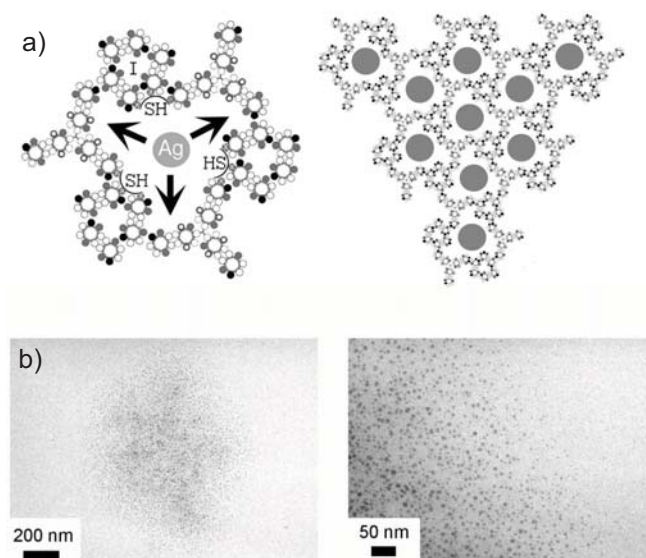


Figure 15. a) Schematics of coiled-coil dendrimers that form mesoscopic spheres with pores serving as reaction sites for nanoscopic silver particles. b) TEM images of the colloidal silver clusters formed in the cavities of the 3D coiled-coil cavities.<sup>122</sup>

Most self-assembly strategies using coiled coils are targeted at controlling the hydrophobic core and the charged residues bordering the core. The previous example varied from this in that the charged residues were designed for interactions between coiled coils. As an extension of this concept a complete departure from the standard coiled-coil binding follows. Amphipathic  $\alpha$ -helical coiled-coil forming peptides were located at water-air interfaces, with the hydrophobic face of the helix oriented towards the air. Intra- and intermolecular cross-links between histidine residues stabilized the helices, which created a film at the interface, strengthening the foams. The films were disrupted by adding a metal chelator or by changing the pH to break the peptide-metal bond.<sup>123</sup> This is the only case in which the self-assembly of coiled-coil forming peptides is utilized not for their specific coiled-coil properties, but for their more general amphipathic property. This parallels the recently discovered coiled-coil GEF catalyst, in which the hydrophobic coiled-coil interface is temporarily disrupted and binds to a hydrophobic patch on a Rab protein.<sup>55</sup> An important difference is that the protein-protein interface remains highly specific, in contrast to this synthetic example.

The remainder of the assemblies in this section are composed of coiled-coil hybrids. The biological role of coiled coils in linking larger molecules and sub-cellular structures has been mimicked in the many instances of coiled-coil induced aggregation of nanoparticles. The first demonstration of this use of coiled coils was the decoration of gold nanoparticles with two different three heptad repeat peptides. Upon introduction of a complementary six heptad repeat peptide to the solution, a coiled coil formed, resulting in reversible networks of gold nanoparticles.<sup>124</sup> Utilizing the environmental sensitivity of coiled coils, conditional nanoparticle aggregation has also been investigated. Gold particles decorated with coiled-

coil forming peptides have been induced to aggregate only at low pH or in the presence of metal ions, both of which reduce the charge on multiple glutamic acid side chains (by protonation or chelation), allowing homocoils to form.<sup>125-127</sup> The same peptide also forms a heterocoil with a complimentary peptide dendrimer, which when added to solution induced gold particle aggregation with well defined spacing (Figure 16). The four armed dendrimer linker has a central disulfide bridge, which could be reduced in solution, re-dispersing the gold particles.<sup>126</sup>

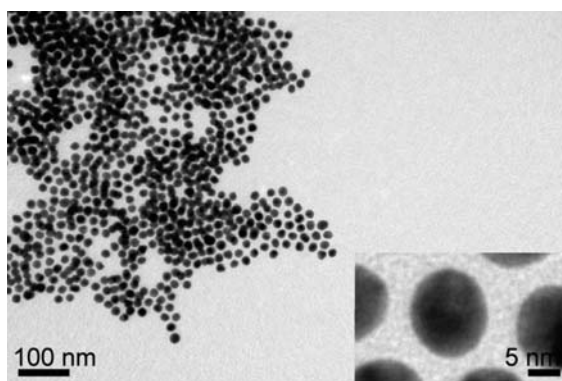


Figure 16. The aggregation of gold nanoparticles can be controlled by coiled-coil association/disassociation.<sup>126</sup>

The responsiveness of coiled-coil based assemblies is most frequently programmed by directly disrupting the binding. A recent example where the binding is indirectly targeted involves a hetero coiled coil (with a  $T_m > 85$  °C) attached to gold nanocapsules which aggregated due to coiled-coil formation. By irradiating the sample with infrared radiation the gold nanoshells, which have a large photothermal response, produced enough heat to denature the coiled-coil complex, separating the nanoshells. When individual nanoshells were decorated in the same way with quantum dots, irradiation caused a large increase in quantum dot fluorescence, but the heat produced by the single nanocapsule did not dissociate the coiled coil (Figure 17).<sup>128</sup> This indirect photothermal control over coiled-coil assembly has no known parallels in nature.

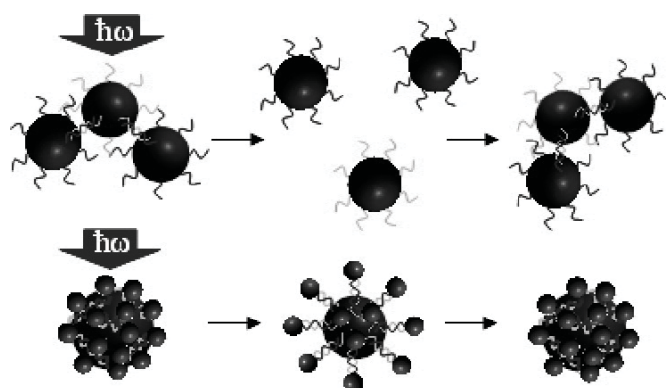


Figure 17. Cartoon of the dissociation of coiled coils by the heat released by illuminated gold nanoshells.<sup>128</sup>

Coiled coils can also be used to link other objects, for example, carbon nanotubes have been linked with gold nanoparticles when each is functionalized with complementary heterodimer forming peptides (Figure 18). In addition, the dimers were able to chelate cobalt via histidine residues.<sup>69</sup> The aim of this is to produce an interface for electrically conducting carbon nanotubes that will sense soluble biomolecular targets.

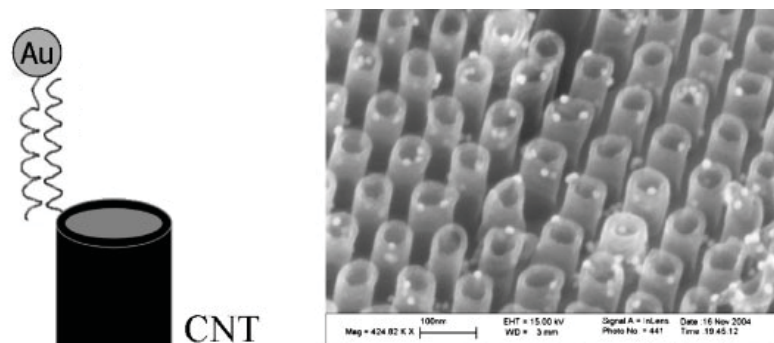


Figure 18. Cartoon and SEM image illustrating the reversible decoration of carbon nanotubes (CNTs) with gold nanoparticles by way of coiled-coil recognition.<sup>69</sup>

Since 1998 assemblies of coiled coils coupled with large water soluble polymer blocks have been investigated.<sup>129</sup> Currently the only materials assembled from coiled-coil hydrophilic hybrids have been hydrogels. As with the assemblies constructed entirely of coiled coils these materials have parallels to the structural function of cyto- and nucleoskeletal coiled-coil networks. In these constructs coiled-coil motifs flank a water soluble protein or polymer segment, and the coiled-coil interaction creates a randomly connected network. In the first example of this kind two coiled-coil forming peptides were linked by a long genetically engineered random coil polypeptide.<sup>129</sup> These artificial proteins form hydrogels via homodimer formation.<sup>129-131</sup> Shortly after this a more synthetic equivalent was demonstrated: a peptide-poly(ethylene glycol)-peptide hybrid that forms a hydrogel via homodimer formation.<sup>132</sup> The response of the coiled coil to temperature, pH, and metal ions allows the triblock hybrids to be switched between solution and gel states. Coiled-coil mediated hydrogels have also been created with the arms consisting of another water soluble polymer, N-(2-hydroxypropyl)methacrylamide, with the coiled-coil binding in a parallel orientation,<sup>133</sup> or an antiparallel orientation, which reduces the steric crowding of the polymer arms.<sup>134</sup> A recent review of peptide directed self-assembly of hydrogels, gives more details on hydrogels via coiled-coil formation.<sup>135</sup>

In contrast to the coiled-coil networks and scaffolds in nature, in which coiled coils constitute the structure, or 'arms' of the network (for example see spectrin in natural coiled coils), in the synthetic examples of networks mentioned above the coiled coils are used to connect the arms of the network together. There is one example, in which synthetic biology is entered close to the biology end of the spectrum, of a hydrogel with coiled-coil arms. A long  $\alpha$ -helix from the intermediate filament keratin (a fibrous coiled-

coil structural protein) was expressed fused to a globular cell binding domain, and this hybrid was co-assembled with extracted keratins that form hydrogels through intermolecular coiled-coil association of  $\alpha$ -helical segments (Figure 19). It was found that neurosphere-forming cells specifically adhered to the modified keratin hydrogel and actively proliferated with a high survival rate.<sup>136</sup>

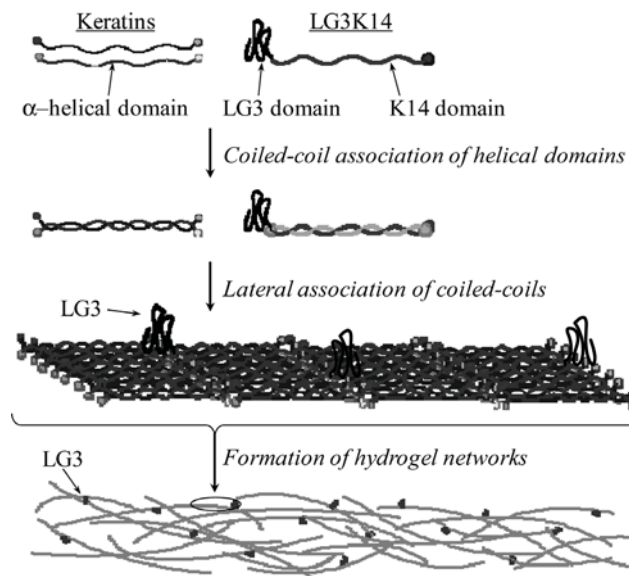


Figure 19. The fusion of a natural keratin peptide with a cell-binding domain modified the properties of the keratin hydrogel.<sup>136</sup>

#### *Coiled-coil assemblies incorporating orthogonal self-assembly*

Proteins, themselves hybrids of many self-assembled units, do not operate in isolation, they are embedded in cells, which are composed of self-assembled lipid compartments, self-assembled nucleotides etc. The complexity in coiled-coil based synthetic biology can be extended by coupling a wide variety of particles or molecules to coiled-coil forming peptides, leading to hierarchical self-assembly of “smart” nanostructures in which both coiled-coil formation plays a role and the properties of the other block/s.

An interesting hydrogel uses star-shaped poly(ethylene glycol) functionalized with a lysine rich peptide that folds into a coiled-coil homodimer which in turn binds to a polysaccharide segment (heparin) on a second star-shaped PEG by electrostatic interactions, hence leading to a hydrogel (Figure 20). This is one of the few examples in which coiled coils mediate self-assembly in a way other than by coiled-coil formation.<sup>137</sup>

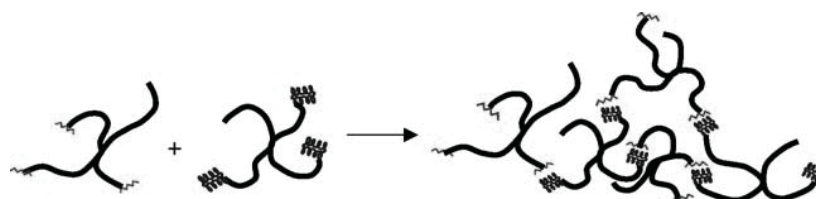


Figure 20. A hydrogels which contains coiled coils, but with the gelation caused by coiled-coil polysaccharide binding.<sup>137</sup>

Hydrogels have been constructed in which random coil polypeptide spacers are partially connected by coiled-coil forming peptides, and partially by an enzyme that dimerizes. Each protein building block has an additional function: the coiled coils are chelated with osmium moieties via histidine residues, rendering the hydrogels conductive, and in the dimeric form the enzyme uses electrons for the catalytic reduction of dioxygen to water. Thus, when the hydrogels are formed on electrodes this combination of functions allows the bioelectrocatalytic reduction of dioxygen to water, with possible application in fuel cells. (Figure 21).<sup>138</sup>

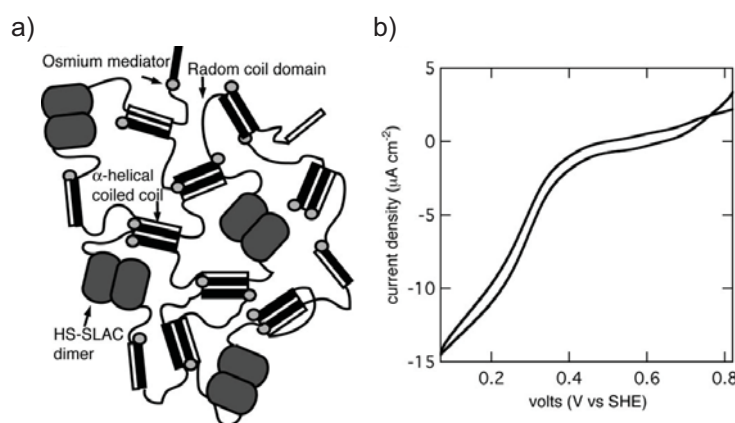


Figure 21. a) Diagram of a supramolecular hydrogel that relies on coiled-coil folding and enzyme dimerization to gelate. b) Bioelectrocatalysis of such a mixed hydrogel.<sup>138</sup>

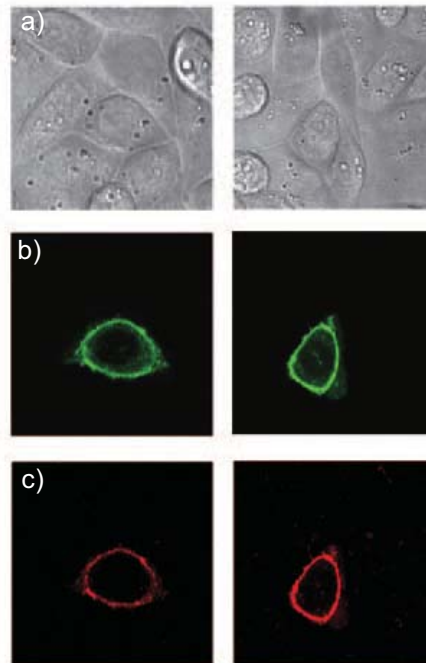
### Coiled-coil Systems

The goal of synthetic-biology is to create functional systems, which implies the interaction of multiple self-assembled components. The aim is therefore to incorporate units or assemblies, such as those discussed in the previous sections, with other self-assembled structures, such that functional systems emerge from the combination of the properties of the components, and the effects that they exert on one another. All of the systems developed to date have been rather basic, using coiled-coil units rather than assemblies. Additionally, each system aims to modify or model natural processes – there has not yet been a synthetic-biology system with an original purpose.

Biologists have made use of coiled-coil synthetic biology for some time. One technique used to visualize protein complexes in living cells is biomolecular fluorescence complementation. The concept is that moieties with highly specific associations are fused to protein fragments and the interaction of these moieties *in vivo* leads to the protein fragments forming a functional and fluorescent complex. Coiled-coil peptides which bind in a stable and specific manner such as the coiled-coil region from the GCN4 transcription factor and designed peptides have been used for such applications.<sup>139, 140</sup> Alternatively the interactions of native coiled-coil containing proteins can be visualized by fusing them with fragments of small fluorescent proteins.<sup>141</sup> As a specific tag-probe example, a hetero dimeric coiled-coil pair was utilized to label proteins in living cells. One of the peptides

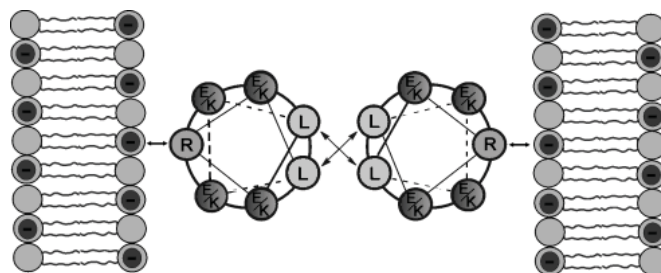


was recombinantly attached to the surface exposed terminus of a transmembrane receptor protein. The corresponding peptide was synthesized with a fluorescent label, and added to the culture medium. Within one minute the fluorescently-labeled peptide had coated the cell surface as hetero coiled coils were formed. The formation of the coiled coil did not affect the receptor function, hence they were an efficient small tag-probe pair (Figure 23).<sup>142</sup>



*Figure 23.* a,b) Cells were expressed bearing a coiled-coil tagged surface protein. Upon addition of the complementary peptide (three heptads, left; four heptads, right) the specific molecular recognition localized the peptide to the surface (c). The labeling was more effective with four heptad repeats on the probe peptide.<sup>142</sup>

Slightly more synthetic examples follow in which coiled-coil units and lipid assemblies are combined. In one case different coiled-coil forming peptides were added to solutions of liposomes. The positively charged peptides adsorbed to the surfaces of the liposomes and caused aggregation of the vesicles (Figure 24). Although the lipid packing was disturbed there was no liposome fusion or leakage. This model system could be used to study the interrelated effects of lipid membranes and coiled-coil peptides on one another.<sup>143</sup>



*Figure 24.* Coiled-coil folding and the interaction of the cationic coiled coil with negatively charged lipid membranes results in vesicle aggregation.<sup>[143]</sup>



As explained in the ‘native coiled coils’ section, enveloped viruses enter cells by way of a pH triggered conformational change involving a coiled-coil complex. Peptides that form an extremely stable complex with the viral envelope proteins may be an effective way to reduce viruses from infecting cells. Such inhibitors could be screened for with an efficient sensor platform. To that end a coiled-coil trimer based on a native viral protein was anchored to supported lipid bilayers and peptide binding to the coiled coil was monitored. The concept was demonstrated with two known inhibitor peptides and binding was monitored with AFM and ellipsometry, so there is some way to go to make this a practical system.<sup>144</sup>

A synthetic-biology system that is intended not to prevent, but to mimic viral membrane fusion has been developed. A peptide that forms an  $\alpha$ -helical trimer at low pH was anchored in liposome bilayers via the C-terminus and displayed a tryptophan residue at the N-terminus. At low pH when the peptides have a helical configuration there is fusion of the liposomes, albeit very slowly and with contents leakage, which is proposed to occur due to tryptophan insertion in a nearby liposome, analogous to the fusion sequence in viral fusion proteins.

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## CONCLUSIONS

By combining the basic units of coiled coils – amino acids, in different sequences an amazing variety of coiled-coil unit, assembly, and system properties are possible. Changing just two amino acids in a sequence can alter factors such as the coiled-coil binding strength or hierarchical aggregation size by many orders of magnitude. This extreme variability has lead, over billions of years, to coiled coils performing a vast range of functions in every living cell. These functions include, but are certainly not limited to: controlling the binding of cellular components, structural edifices of varying dimensions, levers, force transducers, hinges, and clamps.

These many functions are fertile ground for creating synthetic-biology systems, with the important benefit that the rules for mapping amino acid sequences to coiled-coil assembly are relatively well understood. There is a pyramid of examples in which coiled coils are used as building blocks in synthetic biology. There have been many investigations of coiled coils as units, in which their binding specificity and stability are probed. Based on and building upon this knowledge the self-assembly of these units into higher order structures has been probed, both assemblies composed wholly of coiled coils, and those composed of coiled-coil hybrids. Also their dependence on environmental conditions has been explored. At the tip of the pyramid, an area which has yet to be explored to any great extent is combining coiled coils or coiled-coil hybrids with other self-assembled structures in order to compose functional systems.

Although intricate and with a wealth of function, self-assembly as observed in nature is not always the best solution to a particular challenge. By reverse engineering nature we discover tools with which we can construct structures with a wider scope than biology. We can construct coiled-coil hybrids that are unavailable to nature, and investigate self-assembly via pathways that are not possible naturally. We can use the assembly of coiled coils in nature, as developed slowly over billions of years, as a jumping board to new areas of synthetic biology.

## SCOPE OF THIS THESIS

Each of the myriad coiled coils in nature operate in conjunction with other self-assembled units, be they motor domains, DNA-binding sequences, or lipid membranes. However, there have only been a limited number of coiled-coil assemblies or systems that mimic native coiled coils by incorporating orthogonal self-assembly. The aim of this thesis is therefore to explore this section of synthetic-biology space.

In order to achieve this, a range of hybrid molecules are synthesized which combine coiled-coil peptides with a hydrophobic component. In this way the highly specific coiled-coil self-assembly is juxtaposed with the non-specific, but structure-inducing aggregation of the hydrophobic section.

The same coiled-coil unit, made up of peptides 'E' and 'K', is used throughout the exploration, and is introduced in Chapter 2. Experimental characterization is compared and contrasted with a molecular dynamics simulation of the peptide binding. The results from the molecular dynamics simulation support the experimental results, briefly that the peptides E and K form a parallel coiled-coil dimer in aqueous solution. The added benefit of the molecular dynamics simulation is that each binding component is estimated, not just the cumulative average. In this way the E/K binding is understood in more depth, which helps to interpret results from subsequent chapters in which E and K are part of hybrid molecules.

In Chapter 3 the question is posed as to whether coiled-coil forming peptides are still able to fold if one is conjugated to an aggregating hydrophobic block. That is, if the self-assembly process can be balanced such that the intrinsic properties of both blocks persist when they are combined, and if so how they influence one another. To investigate this question the first hybrid is constructed by coupling peptide E with a short polystyrene chain. The self-assembly in solution is investigated of PS<sub>9</sub>-E on its own and together with peptide K and a hydrophilic hybrid K-PEG<sub>77</sub>. It is found that coiled-coil folding between E and K still occurs to a large extent when the peptides are conjugated with PS and/or PEG, resulting in linear noncovalent di- and triblock copolymers. Additionally, the intrinsic aggregation of polystyrene in aqueous solutions is not overpowered, and results in micelles. The morphology of the micelles is changed by reversibly unfolding the coiled-coil block.

The obvious question raised by the results of Chapter 3 is what is the limit of the hydrophobic block size? To approximate the hydrophobic block sizes that the peptides E and K are able to induce into well ordered aggregates, poly( $\gamma$ -benzyl L-glutamate), or PBLG, was polymerized from peptide E. The synthesis and self-assembly of PBLG-E hybrids with polypeptide block lengths ranging from 36 monomers to 250 monomers are discussed in Chapter 4. By making use of the binding of E and K-PEG even the longest of these hybrids underwent ordered assembly into vesicles in aqueous solution. This system allows one to independently vary both hydrophobic and hydrophilic sections of the amphiphiles, and in this way disk-like micelles, and vesicles of different sizes, membrane

thicknesses and surface chemistries were accessed. The synthesis of polypeptide-*b*-peptides is novel, and as well as the self-assembly flexibility of this new class of peptide, this route has the advantage that the block copolymers are very easy to purify.

The thesis continues with methodology in Chapters 5 and 6, in which two novel techniques to produce polymersomes are presented. Every method to produce polymersomes results in different polymersome characteristics, and has different requirements regarding equipment, time, energy input etc., some of which are not compatible with particular research situations or block copolymers. Therefore a wide range of preparative techniques are beneficial. In Chapter 5 a detergent removal technique that has been used for three decades to form liposomes is adapted to block copolymers. This method will be particularly useful for incorporating biological material into the vesicles as it is a benign process. Chapter 6 adapts another vesicle formation method that has long been applied to liposomes to make it suitable for polymersomes, the ‘water addition – solvent evaporation’ procedure. The main advantages of this technique are that well defined polymersomes with low polydispersity indices are produced with a high capacity for tuning the polymersome sizes, and that it is very practical in terms of time and equipment, requiring only five minutes and a rotary evaporator.

Chapter 7 returns once again to the main thrust of the thesis: extending the orthogonal self-assembly of coiled-coil hybrids. In this Chapter the aspect under investigation is whether E/K coiled-coil folding can guide not just the self-assembly of the hydrophobic blocks to which they are covalently bound, but also separate supramolecular assemblies. E and K are conjugated to lipids, by which means they are anchored into the lipid bilayers of liposomes. The effect of E/K binding on the liposomes is studied and it is found that the system represents a minimal model for SNARE protein mediated membrane fusion. The coiled-coil hybrids mimic the structural components of SNARE proteins, and importantly they also induce liposomes fusion in a way that mimics native membrane fusion.

Chapter 8 presents an overview of the different approaches available to synthesize polymer-peptide block copolymers. There is a focus on contemporary activities, and the advantages and limitations of each approach are touched upon.

Finally, in Chapter 9 the results are summarized and discussed, and possibilities for future research are presented.

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