

## Polar Assembly in a Designed Protein Fiber\*\*

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Self-assembling systems are found extensively in biology.<sup>[1]</sup> An improved understanding of these systems would fuel efforts to engineer novel, bioinspired assemblies through de novo design. Interest in the area is intense because an ability to engineer or design water-soluble self-assembling systems offers routes to novel biomaterials with potential applications in nanobiotechnology.<sup>[2–6]</sup>

Self-assembling systems based on peptides, proteins, DNA, and RNA are all being explored.<sup>[2–6]</sup> Furthermore, “living templates”, namely fungal hyphae, are being used to direct the hierarchical assembly of nanoparticles.<sup>[7]</sup> Our focus has been on peptide-based assemblies.<sup>[8–10]</sup> Specifically, we are interested in making self-assembling fibers and networks from straightforward, synthetically accessible peptide building blocks.

Considerable work has been done to mimic fibrous protein assemblies by using predominantly  $\beta$ -structured peptides that form amyloid-like fibrils.<sup>[2,11,12]</sup> Relatively less has been done with  $\alpha$ -helix-based assemblies.<sup>[3,8,13]</sup> We have focused on fibrous assemblies based on the  $\alpha$ -helical coiled coil, which is a widespread and well-understood protein–protein interaction motif.<sup>[14–16]</sup>

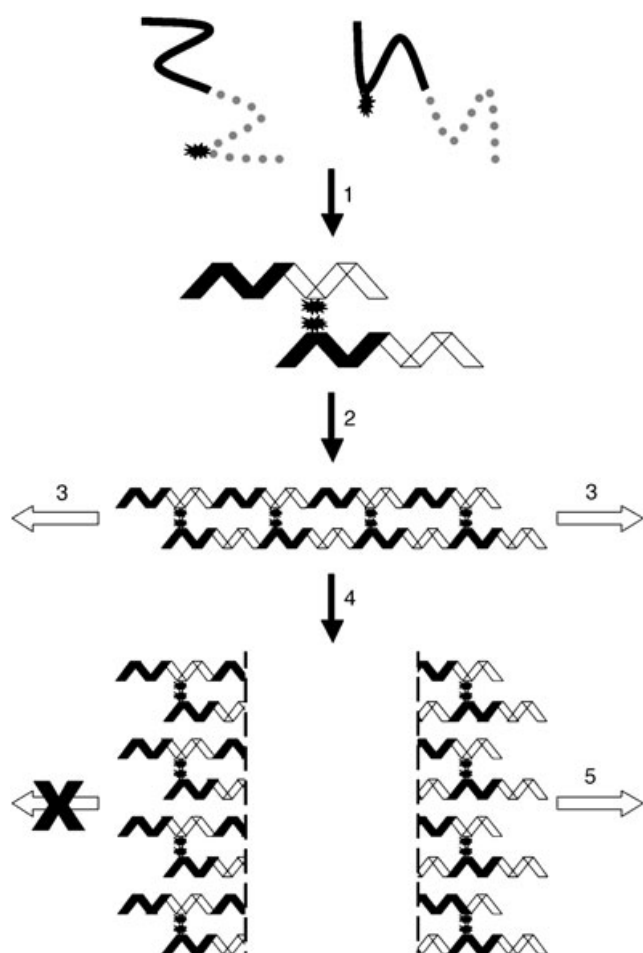
Specifically, we have combined rules for coiled-coil assembly to design a self-assembling-fiber (SAF) system comprising two complementary peptides, SAF-p1 and SAF-p2.<sup>[8]</sup> Unlike natural and previously designed coiled coils, which form blunt-ended structures,<sup>[14,17]</sup> the SAF peptides were engineered to make offset dimers with complementary sticky ends to promote longitudinal assembly into fibers (Figure 1). Helical assembly was confirmed by using circular dichroism spectroscopy and X-ray fiber diffraction,<sup>[8]</sup> and

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**Figure 1.** A model for SAF fibrillogenesis. Each SAF peptide is represented by a polar strand with a basic (solid lines) N-terminal half, an acidic (broken/hollow lines) C-terminal half, and a single asparagine residue (starred). These features direct the assembly of a sticky-ended heterodimer (step 1). These heterodimers then assemble longitudinally to give double-stranded coiled-coil protofibrils (step 2). We assume that, at this stage, extension occurs by the addition of peptide building blocks to either end of the protofibril (step 3). At some point the protofibrils have enough affinity for one another to assemble laterally and thicken (step 4). These thickened fibers are polar; that is, when one type of the SAF peptide is added to matured fibers it is selectively recruited to just one end of the fibers (step 5).

more recently by FTIR spectroscopy (unpublished data). Fiber formation was confirmed by electron microscopy (EM), which revealed that the fibers were linear, extended many microns, and were  $\approx 45$  nm thick;<sup>[8]</sup> approximately 20 times thicker than expected for coiled-coil dimers.<sup>[18]</sup> One explanation for thickening is that two-chain assemblies form nascent structures (protofibrils) that assemble further laterally to form the matured, thick fibers (Figure 1). Herein we describe experiments initially conceived to follow lateral assembly directly in water. We also show that this relatively straightforward system displays the unexpected and potentially exploitable feature of polar assembly.

To follow the process of lateral assembly directly in solution we performed the following experiment: A cysteine residue was introduced into SAF-p2a—a variant of SAF-p2—

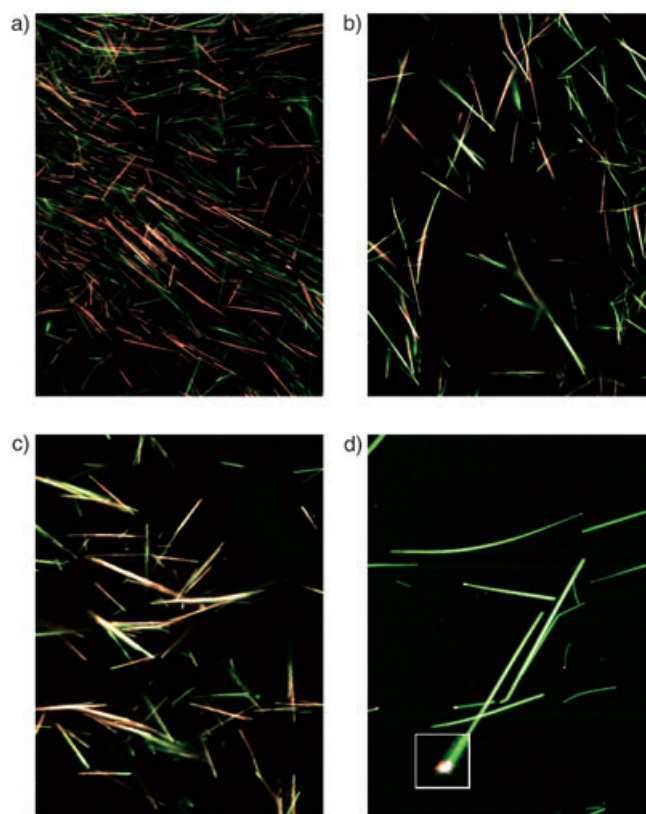
to make SAF-p2aCys (Table 1). This peptide was used to make two fluorescently labeled peptides: one containing fluorescein and the other rhodamine. Each labeled peptide was incorporated separately and at low levels (0.05 % total

**Table 1:** SAF peptide sequences.

Peptide	Sequence <sup>[a]</sup>
SAF-p1	K IAALKQK IASLKQE IDALEYE NDALEQ
SAF-p2	K IRALKAK NAHLKQE IAALKEQE IAALKEQ
SAF-p2a	K IRR <b>RLKQK</b> NAR <b>LKQE</b> IAAL <b>EYE</b> IAALKEQ
SAF-p2a-Cys	K IRR <b>LKCK</b> NARLKQE IAAL <b>EYE</b> IAALKEQ
SAF-p2a-Lys	K IRR <b>LKCK</b> NARLKQE IAAL <b>EYE</b> IAALKEQ

[a] Successive mutations are highlighted in bold, italic font.

peptide) into otherwise standard SAF-p1:SAF-p2a fiber preparations. When visualized by confocal fluorescence microscopy, the resulting fibers appeared green and red, respectively, and otherwise normal. By mixing these fibers and recording images over time we observed the formation of matured fibers through the emergence of thickened yellow structures (Figure 2a–c). The yellowing did not result from subunit exchange between the solution and fibers, however,



**Figure 2.** False-color images of merged red- and green-filtered confocal microscope data: a) recorded immediately after mixing two separately prepared samples of rhodamine- and fluorescein-labeled fibers; b and c) for samples prepared as described in A, but left to mix for 24 and 48 h, respectively; d) for a matured fluorescein-labeled sample, which was treated subsequently with rhodamine-labeled SAF-p2a. The inset shows an approximately fourfold further magnification of one fiber tip. The short axis of each of these panels is approximately 100  $\mu$ m.

because some of the fibers showed distinct red and green halves and yellow central regions, thus indicating that red and green fibers must have merged in a manner consistent with our model for fibrillogenesis (Figure 1).

We conducted the following additional experiment to probe how individual (or at least small numbers of) peptides were recruited to matured fibers: Fibers containing the fluorescein-labeled peptide were assembled as described above and matured for 16 h, after which time fiber formation had essentially ceased, and the concentration of free monomers had fallen to the critical concentration for fibrillogenesis ( $\approx 30 \mu\text{M}$ ).<sup>[19]</sup> At this point, rhodamine-labeled SAF-p2a was added. Intriguingly, as shown in Figure 2d, the rhodamine-labeled monomers appeared to be selectively recruited to just one end of the matured fibers.

To examine this finding further, and to test the possibility of making nano-to-micron-scale barcodes,<sup>[20]</sup> but from self-assembling biomolecules, we conducted a further experiment: First, unlabeled fibers were grown for 24 h. Rhodamine-labeled SAF-p2a was then added, and the sample was left to mature for 24 h, followed by the addition of fluorescein-labeled SAF-p2a and further maturation for 24 h. The result was fibers with one end labeled red followed by green (Figure 3). Although there was some background staining of the unlabeled fibers, it was clear that only one end of each fiber was labeled and, moreover, that labeling was in the correct (red–green) order as prescribed in the experiment.

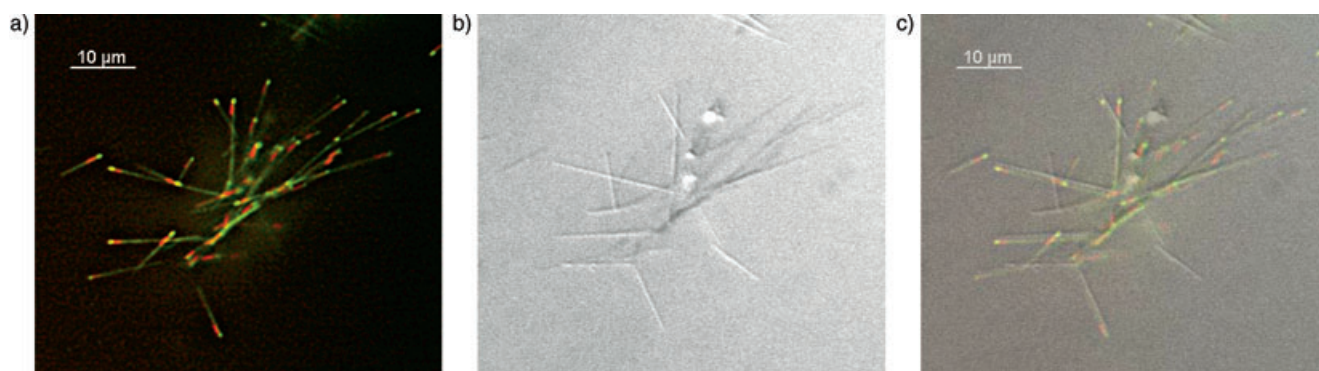
What gives rise to polar assembly in our system? The SAF peptides themselves are polar: They each have a positively charged N-terminal half and a negatively charged C-terminal half (Figure 1).<sup>[8]</sup> As a result, the designed heterodimeric building block for the fibers, in which the C-terminal half of SAF-p1 binds to the N-terminal half of SAF-p2a and vice versa, will be polar. Thus, the longitudinally extended coiled-coil protofibrils will be polar. On this basis, there may be preferred protofibril–protofibril arrangements that could lead to polar matured fibers. In turn, the different ends of these matured fibers could be distinguished by new incoming peptides (Figure 1), as observed. Indeed, a theory for this type of polar assembly has been advanced,<sup>[21]</sup> although examples of this type of assembly for soft materials appear to be restricted to natural protein fibers, such as microtubules, intermediate

filaments, and collagens,<sup>[1,22,23]</sup> and, most recently, the fibril form of the yeast prion protein Ure2p.<sup>[24]</sup> This unexpected mimicry in our relatively simple, designed binary peptide system is intriguing and may shed light on both natural and synthetic self-assembly processes in general. Furthermore, and in view of current attention being given to the generation of bioinspired materials for applications in nanobiotechnology,<sup>[2,3,11,25–28]</sup> our findings offer promise for nano-to-micron-scale engineering of self-assembling systems from the bottom up in water; for example, the directed incorporation of functionalized peptides—that is, either regularly spaced along the fibers, or specifically located at one end of the fibers—can be envisaged based on the observations presented herein.

## Experimental Section

**Peptide synthesis:** Peptides were made chemically on a pioneer peptide synthesis system (PE Applied Biosystems, CA, USA) by using standard Fmoc-based solid-phase protocols (Fmoc = 9-fluorenylmethoxycarbonyl). Two approaches were taken to introduce the fluorescent labels: For our initial experiments (Figure 2), labeled peptides were prepared by conjugating SAF-p2a-Cys (Table 1) with either fluorescein-5-maleimide or tetramethylrhodamine-5-maleimide (Molecular Probes) by the protocols of the supplier. The labeled peptides were purified on PD 10 columns (Amersham Biosciences, Buckinghamshire, UK) by using the protocol of the supplier. For the later experiments (Figure 3), succinimidyl esters of 5- (and 6-)carboxyfluorescein and 5- (and 6-)carboxytetramethylrhodamine were used to label the lysine residue introduced into SAF-p2a-Lys (Table 1), which was specifically deprotected and subjected to reaction during peptide synthesis by analogous procedures to those published elsewhere.<sup>[10]</sup> Peptides were purified by semipreparative reversed-phase HPLC and, when necessary for dye–peptide conjugates, beforehand by using PD-10 columns. The final constructs were identified by MALDI-TOF mass spectrometry (Micromass Ltd, Manchester, UK): *m/z*: calcd for SAF-p1: 3174, found: 3175 [*M* + *H*]<sup>+</sup>; calcd for SAF-p2: 3128, found: 3129 [*M* + *H*]<sup>+</sup>; calcd for SAF-p2a: 3325, found: 3326 [*M* + *H*]<sup>+</sup>; calcd for SAF-p2a-Cys: 3300, found: 3301 [*M* + *H*]<sup>+</sup>. Mass increases of 359, 413, 427, and 481 Da were detected for carboxyfluorescein-, carboxytetramethylrhodamine-, fluorescein-5-maleimide-, and tetramethylrhodamine-5-maleimide-labeled SAFs, respectively.

**Fluorescence microscopy:** For the “kinetic-mixing” experiments (Figure 2a–c), the unlabeled peptides SAF-p1 (100  $\mu\text{M}$ ) and SAF-p2a (100  $\mu\text{M}$ ) were mixed with the labeled peptide (0.1  $\mu\text{M}$ ) in MOPS (3-



**Figure 3.** a) Merged false-color image showing unlabeled fibers treated first with rhodamine-labeled peptide, and then with fluorescein-labeled peptide. b) The corresponding DIC image highlighting the full lengths of the fibers. c) Merge of A and B showing that the fluorescent labels are located predominantly at the fiber tips and in the correct order.

(*N*-morpholino)propanesulfonic acid; 10 mM, pH 7). The sample was allowed to mature for 16 h at 20 °C prior to each experiment. The matured solutions were then mixed and either visualized immediately or incubated for a further 24 or 48 h before examination. In the first “polar-assembly” experiment (Figure 2d), fluorescein-labeled fibers were prepared as described above. After the 16-hour incubation the rhodamine-labeled peptide (0.1  $\mu$ M) was added, and the solution was left for 24 h before examination. For these experiments, samples were visualized by using an x60 oil-immersion lens on a BioRad MRC 600 confocal microscope with a krypton/argon mixed-gas laser, dual-excitation filter, and K1 (520 nm, fluorescein) and K2 (585 nm, rhodamine) filter-block set. Data were collected by using COMOS (BioRad). Images were contrast stretched and false colored red or green before overlaying the two halves.

For the experiments presented in Figure 3, unlabeled fibers were formed first by using our standard protocol (100- $\mu$ L samples, 100  $\mu$ M in each of SAF-p1 and SAF-p2a, MOPS (10 mM, pH 7), incubation at 20 °C for 24 h); the sample was then made up to 1  $\mu$ M in rhodamine-labeled SAF-p2a and incubated for a further 24 h; finally, the sample was made up to 1  $\mu$ M in fluorescein-labeled SAF-p2a and incubated for a further 24 h. The experiment was repeated with the introduction of the labels in the opposite order (data not shown). Fluorescence and differential interference contrast (DIC) images were recorded on a Carl Zeiss Vision widefield microscope fitted with x10 and x40 LD lenses, a krypton/argon mixed-gas laser, a dichromic mirror, and two light filters (520 nm (filter set 10) for fluorescein and 585 nm (set 15) for rhodamine). Data were collected by using an ORCA ER camera. Images were false colored red or green before they were overlayed by using the Carl Zeiss Vision imaging software.

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