Self-Assembling Peptide Fibers

Introducing Branches into a Self-Assembling Peptide Fiber**

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There is a growing interest in employing peptides as building blocks in the self-assembly of supramolecular structures.[1] Such assemblies have potential as novel scaffolds for functionalizing surfaces and in tissue engineering.[2] Elsewhere,[3] we described a self-assembling fiber (SAF) system comprising two designed peptides (**1** and **2**, also known as SAF-p1 and SAF-2a, Figure 1). These sequences are based on established

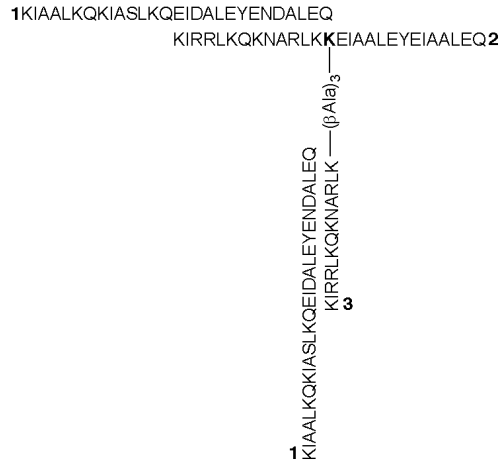

Figure 1. SAF and T-SAF peptide building blocks of linear and branched fibers. Lysine 14 of **2** is highlighted in bold.

design principles for leucine-zipper motifs.[4] However, unlike all other leucine zippers, which are blunt ended, the SAF peptides are designed to assemble with sticky ends that facilitate fibrillogenesis (Figure 1, see also Supporting Information). Like other systems,[1a,c,f] the SAF peptides are linear and form exclusively linear and nonbranching fibers when mixed; note, **2** is a slight redesign of the previously described SAF-p2,[3] this new design combines with **1** to give more-stable and better-ordered linear fibers (see Supporting Information). Natural protein fibers, such as those formed by actin, collagen, and fibrin form branches. We set out to design special units to complement the standard SAF building blocks and, so, engineer branched fibers de novo. Herein we report T-shaped peptides, T-SAFs, that coassemble with the standard SAF peptides to give branched self-assembling fibers. In T-SAFs the "bar" of the "T" is the complete SAF peptide **2** and the "stem" (peptide **3**) is the *N*-terminal half of **2**; the stem and the bar are joined through a linker, typically of three β-alanine (βALA) units, between the *C*-terminus of peptide **3** and the ε-amino group of the central lysine (lysine 14) of peptide **2** (Figure 1). In principle, when combined with peptides **1** and **2**, T-SAFs should promote assembly of orthogonally conjoined fibers.

We applied different approaches[5] to synthesize T-SAFs (supporting information), however, only two gave the desired product in reasonable yields: a) punctuated solid phase synthesis, in which the bar peptide was synthesized, followed by synthesis of the stem from the side chain of selectively deprotected lysine 14; b) chemoselective ligation by using deprotected bar and stem peptides, in which lysine 14 was activated as bromoacetyl to accept a *C*-terminal cysteine engineered into **3**. In both methods it was essential to extend the side chain of lysine 14 of the bar peptide with β-alanine or ε-aminohexanoic acid units before coupling it to the stem peptide.

We tested coassembly of T-SAF with **1** and **2** under our standard conditions (100 μM each of **1** and **2,** 10 mM 3-(N-morpholino)propanesulfonic acid (MOPS) pH 7, 20 °C, overnight). For **1** and **2** alone, transmission and scanning electron microscopy (TEM and SEM) revealed linear structures ≈50 nm thick that extend for many microns without deviation, junctions or branches (Supporting Information). However, with 0.01 to 100 μM T-SAF branches were visible

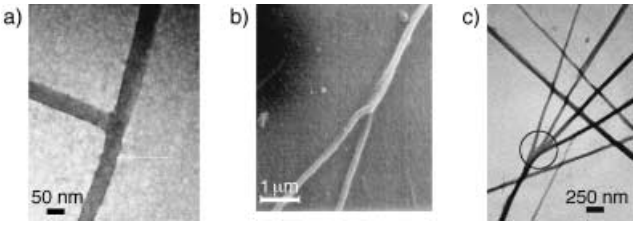

Figure 2. TEM (a and c) and SEM (b) images of fibers formed in the presence of T-SAF peptide. Peptides **1**, **2** and T-SAF were at 100 μM.

(Figure 2, see also Supporting Information). The highest density of the branched fibers was observed at equimolar ratios of T-SAF and SAF peptides — 100 μM in each of peptides **1**, **2** and the T-SAF — in which ≈30% of the fibers had branches. Otherwise, the appearance of these fibers was similar to that of the straight fibers.

Most of the branched fibers had just one branch; multiply branched fibers were also present (circled in Figure 2c), but these were less frequent. This is perhaps surprising given that, apart from the additions to lysine 14, the bar of the T-SAF was identical to **2**. Thus, incorporation of T-SAFs into fibers might have been expected to occur at a similar rate to peptide **2**, which would have resulted in multiply branched fibers and possibly cross-linked networks. However, the samples did not gel as might have been expected in this case. Thus, for the current design incorporation of T-SAFs and subsequent branching is not quantitative and has to be assessed empirically as a function of T-SAF concentration.

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We used circular dichroism (CD) spectroscopy to investigate further the effect of T-SAF on the coassembly of peptides **1** and **2**. CD spectroscopy allows the rate and extent of fiber assembly to be gauged in solution.[3] In isolation, peptides **1** and **2** were unfolded, but when mixed they coassembled to form α-helical fibers with a distinctive CD spectrum (Figure 3a). CD spectra recorded at intervals after

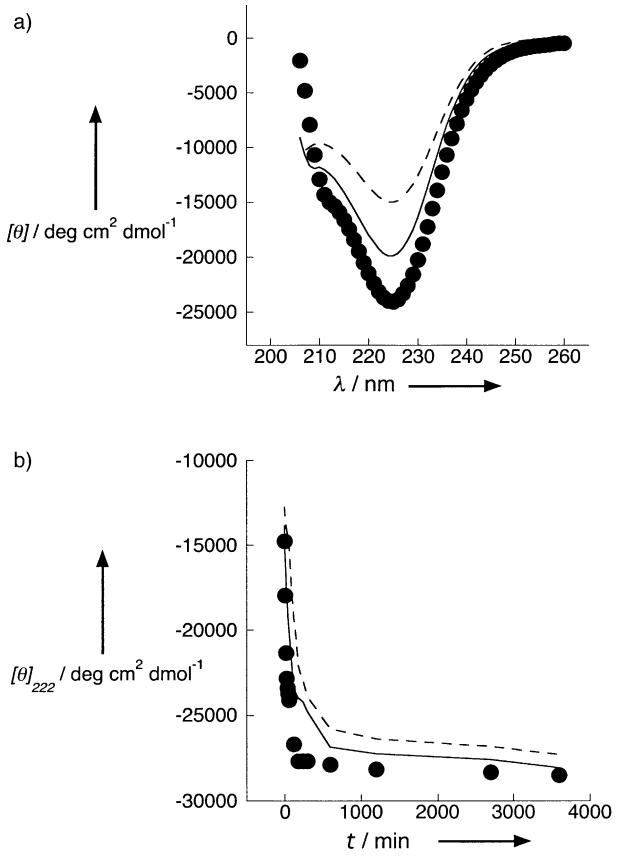

Figure 3. CD measurements following the assembly of peptides **1** and **2** in the absence and presence of T-SAF. a) CD spectra for a mixture of peptides **1** and **2** (black discs); representative spectra for mixtures with T-SAF at 0.1 μM (solid line) and 10 μM T-SAF (broken line). b) Build up of the CD signal at 222 nm for the various mixtures. Key: as in (a). Conditions: 100 μM in peptides 1 and 2, 10 mM MOPS, pH 7, 20 °C.

mixing showed the buildup of the α-helical structure, which was complete within several hours at 20 °C, at which point the fibers are "mature". In contrast, 1:1 mixtures of peptide **1** and T-SAF failed to yield CD spectra or electron micrographs indicative of fiber formation. Thus, T-SAF did not substitute completely for peptide **2** in fiber assembly. Figure 3a shows representative CD spectra recorded 1 hour after mixing peptides **1** and **2** in the presence of 0.01 to 100 μM T-SAF. Essentially, higher concentrations of T-SAF reduced α-helix formation in this time. T-SAF at a concentration of 1 μM provided an interesting cutoff: at this concentration and above the helical content was ≈50% of that for a pure peptide **1** + **2** mixture (Figure 3a). Furthermore, TEM images recorded for these samples showed no visible linear or branched fibers, and further incubations of 2 hours were required to observe these. At the lower T-SAF concentrations, the higher helical signals were achieved (Figure 3a), and fibers were observed by TEM. At all concentrations of T-SAF, the final helical signal did plateau. However, the time taken to achieve this increased with increasing concentration of T-SAF; for instance, with 100 μM of each of peptides **1** and **2** and T-SAF maturation took 2–3 days. Interestingly, the kinetics of fiber assembly in the presence of T-SAF also grouped with T-SAF concentration above and below 1 μM (Figure 3b). TEM images recorded for all of the matured samples revealed both linear and branched fibers.

A model consistent with the CD data, and the low density of branching observed by EM, is that **2** and T-SAF compete for **1** during fiber assembly with **2** being the preferred partner. What causes this preference? First, the mature fibers are multistranded and many peptide units thick.[3] Thus, appendages to the peptide building blocks, such as those in T-SAF, probably interrupt thickening and are not fully tolerated in fiber assembly. Second, fibers comprising purely **1** and **2** show considerable internal order; the leucine zippers are hexagonally packed and aligned along the long fiber axis.[7] This order is also likely to limit the inclusion of peptides other than **1** and **2** into the fibers, as these would effectively be imperfections. HPLC analysis of matured fibers confirmed that T-SAF incorporated into fibers at the expense of **2**. The incorporation increased and saturated with increasing T-SAF: at 1 μM T-SAF virtually all the peptide was incorporated (≈0.7% of the total peptide in the fibers), but only ≈ten times more incorporation was achieved at 100 μM T-SAF (Table S1, Supporting Information).

Further evidence for direct incorporation of T-SAF came from the following control. We made a T-terminator peptide (see Supporting Information) in which the stem was the *C*-terminal half of peptide **2** rather than its *N*-terminal half. This construction should not propagate orthogonal fibers as shown in Figure 1. Indeed, this was observed: in combination with peptides **1** and **2**, the T-terminator yielded short (< 1 μm), thin (< 20 nm) and unbranched structures at up to 1 μM, and completely inhibited fiber assembly above this concentration.

Peptides that form amyloid and amyloidlike structures also branch.[1g,6] In this case, the peptides form protofibrils that wrap around each other in a ropelike fashion to form the matured fibers, and some unfinished ends of the protofibrils produce branches. The assembly of our structures is different: first, the SAFs appear to be rigid rods that are unlikely to wrap around one another; second, we introduce the notion of rationally designed branching into a synthetic self-assembling fiber. We have demonstrated this through the coassembly of novel T-shaped peptides with linear building blocks. The bottom-up assembly of peptide-based nanostructures of this type holds promise for the development of novel scaffolds for applications in nanobiotechnology.[2]

Experimental Section

Peptide synthesis: Peptides were synthesized by using standard Fmoc protocols, purified by reversed-phase HPLC, and analyzed by MALDI-Tof mass spectrometry (supporting information).

Fiber assembly: All samples of fibers were prepared with peptide **1** and peptide **2** (each at 100 μM) with the designated amounts of T-SAF peptides, and incubated for 1–120 h at 20 °C in filtered 10 mM MOPS, pH 7.

CD spectroscopy: All peptide samples were prepared as above. CD Measurements were made in 1 mm quartz cuvettes. Data points for CD spectra were recorded at 1 nm intervals by using a 1 nm bandwidth and 4–16 s response times. After baseline correction, ellipticities in mdeg were converted to molar ellipticities ($[\theta]$, deg cm^2 dmol res^{-1}) by normalizing for the concentration of peptide bonds. The concentration of peptide bonds was taken as 54 μM ($2 \times (28-1)) \times$ 100 μM) for all spectra. This neglected any contribution to the CD from the T-SAFs. Time course measurements were made at 222 nm recording spectra every 10 min during first 60 min period and every 60 min after. The signals were converted to molar ellipticities ($[\theta]_{222}$) neglecting the concentrations of the T-SAF peptides.

HPLC analysis of incorporation of T-SAF peptides into fibers: 300 μL of fiber-forming mixtures (100 μM in peptides **1** and **2** SAF, 10 mM MOPS, pH 7) were incubated at room temperature for $\approx$30 h with different concentrations of T-SAF (100 and 1 μM). After incubation each sample was centrifuged to pellet the fibers. The pellets obtained from three or six samples containing the same starting concentration of T-SAF were combined, washed with the buffer and dissolved in 20 μL of HPLC buffer A for analytical reversed-phase HPLC. Peptide fractions were identified by MALDI-TOF, and collected, lyophilized and dissolved in 200 μL of water. The concentrations of peptides in these fractions were calculated based on the absorbance at 280 nm. This procedure was repeated three times for each measurement. The results are summarized in the Supporting Information.

EM visualization and analysis: Fiber suspensions were dried onto carbon-coated copper specimen grids and stained with uranyl acetate (all at 20 °C) for electron microscopy as described elsewhere.[3] The numbers of branched fibers present were measured over 200–250 fibers. Standard deviations on the measurements were 0.01 (for SAF/T-SAF, 100: $\leq$0.1 μM) and 0.15 (for SAF/T-SAF, 100: $\geq$1 μM). Additional TEM and SEM images are found in the Supporting Information.

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