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Bioorthogonal dual functionalization of self-assembling peptide fibers

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ABSTRACT

The ability to modify peptide- and protein-based biomaterials selectively under mild conditions and in aqueous buffers is essential to the development of certain areas of bionanotechnology, tissue engineering and synthetic biology. Here we show that Self-Assembling peptide Fibers (SAFs) can incorporate multiple modified peptides non-covalently, stoichiometrically and without disrupting their structure or stability. The modified peptides contain groups suitable for post-assembly click reactions in water, namely azides and alkenes. Labeling of these groups is achieved using the orthogonal Cu(I)-catalyzed azide-alkyne and photoinitiated thiol-ene reactions, respectively. Functionalization is demonstrated through the conjugation of biotin followed by streptavidin-nanogold particles, or rhodamine, and visualized by electron and light microscopy, respectively. This has been shown for fibers harboring either or both of the modified peptides. Furthermore, the amounts of each modified peptide in the fibers can be varied with concomitant changes in decoration. This approach allows the design and assembly of fibers with multiple functional components, paving the way for the development of multi-component functionalized systems.

1. Introduction

The construction of peptide-based fibrous biomaterials is receiving increased attention [1,2]. Such materials have potential for example as scaffolds in tissue engineering. At present, however, many of the materials are bare, and there is a pressing need to selectively modify peptides within them to produce functional systems [3–5]. Issues here include, avoiding non-specific side chain reactions, and introducing multiple functions through orthogonal modification. One potential route to this is *via* so-called "click" reactions [6–8], which combine high selectivity and high yields under ambient conditions in aqueous buffers. Click chemistry has found applications in chemical biology, and is starting to be used in biomaterials science [9–11]. Here we combine click chemistry and rational design to add multiple functional groups to a coiled coil-based Self-Assembling peptide Fiber (SAF) system.

The SAFs comprise two 28-residue α-helical coiled coil peptides—SAF-p1 and SAF-p2a—of *de novo* design [12]. These have heptad repeats, *gabcdef* (Table 1), typical of coiled coils in which residues at *g*, *a*, *d* and *e* specify a dimeric helix–helix interface; while, those at *b*, *c* and *f* are solvent exposed and can be used to add or tailor function. When mixed, the peptides self-assemble in aqueous buffers around neutral pH to form stiff fibers 40–80 nm thick, and tens of microns in length [12,13].

Previously, we have functionalized the fibers by introducing biotin and peptide antigens as branches into linear SAF peptides [14]. However, these branched peptides incorporate poorly and subsequent decoration is inefficient. We can rationalize this as the fibers have extremely high structural order [15], and tolerate modified peptides only sparingly (to ~ 0.5% total peptide). To realize applications of the SAFs, better routes to functionalization are required that allow: (1) increased and known levels of incorporation of functional groups; (2) the addition of multiple components; and, ideally, (3) spatial and temporal control over these inclusions. Towards these goals, notably objectives 1 and 2, here we describe two routes to functionalize assembled fibers. These use copper(I)-catalyzed azide-alkyne [16], and thiol-ene click reactions [17], Fig. 1.

2. Materials and methods

2.1. Peptide synthesis and purification

Peptides were synthesized on a CEM Liberty microwave synthesizer using standard Fmoc Chemistry, HBTU activation and TentaGel Fmoc-Gln(Trt)PHB or Rink-

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Table 1
Sequences of the SAF peptides used in this study. z and y denote side chains modified with azido and allyl moieties.

Name	Sequence and heptad register[a]
	gabcdef gabcdef gabcdef gabcdef
SAF-p1	H-KIAALKQ KIASLKQ EIDALEY ENDALEQ-OH
SAF-p2a	H-KIRRLKQ KNARLKQ EIAALEY EIAALEQ-OH
SAF-p1z2	H-KIAALKQ KIASLKz EIDALEY ENDALEQ-OH
SAF-p1z4	H-KIAALKQ KIASLKQ EIDALEY ENDALEz-OH
SAF-p1y2	H-KIAALKQ KIASLKy EIDALEY ENDALEQ-OH
SAF-p1y4	H-KIAALKQ KIASLKQ EIDALEY ENDALEy-OH

[a] Further peptide sequences and HPLC and mass spectrometry data are given in Table S1 and Fig. S7, Supporting Information.

amide Chemmatrix resin. For synthesis of *C*-terminal modified peptides SAF-p1z4 and SAF-p1y4, the corresponding Fmoc-amino acids, Fmoc-Lysine-ϵ-azide and Fmoc-L-allylglycine, respectively, were manually loaded onto Tentagel R PHB resin using 3 equivalents amino acid, 2.9 equivalents HBTU, 3 equivalents HOBt, 4.5 equivalents DIPEA and 0.1 equivalent DMAP. The resin was then washed with excess DMF, and loaded onto the synthesizer for further automated synthesis. Peptide purification was performed by reverse-phase HPLC on a Kromatek C18 column (semi micro, 5 μm, 100 Å, 10 mm ID × 150 mm L). Peptide identities were confirmed by MALDI-TOF mass spectrometry using α-cyano-hydroxycinnamic acid (CHCA) as the matrix. Calculated and measured molecular weights are shown in Table S1. Purity was confirmed using a Kromatek HiQ-SiL C18 analytical column (5 μm, 100 Å, 4.6 mm ID × 150 mm L). Peptide stocks were stored as freeze-dried powder and reconstituted in deionized water just before use. Peptide concentrations were determined using the molar extinction coefficient for tyrosine of 1405 $M^{-1}cm^{-1}$ at 274 nm.

2.2. Synthesis of Lysine ϵ-azide

Fmoc-Lysine ϵ-azide was prepared using the method of Goddard-Borger and Stick [27]. Fmoc-Lys-OH.HCl (4.2 mmol, 1.79 g) was dissolved in methanol (100 ml). Potassium carbonate (2.03 g) and copper sulfate (20 mg) were added. Imidazole-1-sulfonyl azide hydrochloride (1.06 g) was added and the mixture stirred at RT for 12 h. The solvent was removed *in vacuo*, and the residue partitioned between water (200 ml) and chloroform (150 ml) containing isopropanol (50 ml). The organic layer was separated and dried over $MgSO_4$, and the solvent removed *in vacuo*. The crude product was purified using a short plug silica column. The crude material was loaded and washed with a 5% solution of acetone in dichloromethane (500 ml) and eluted with a 20% solution of acetone in dichloromethane (500 ml) to yield the desired compound in 55% yield. Spectral analysis matched those reported by Isaad et al. [28].

2.3. Synthesis of rhodamine-alkyne

Rhodamine-alkyne was synthesized using the method of Punna and co-workers [29]. Propargylamine (15 μL, 0.23 mmol) was added to rhodamine isothiocyanate (50 mg, 0.1 mmol) in THF (15 ml), and the mixture stirred at RT overnight, then concentrated *in vacuo*, washed with CH_2Cl_2 (2 × 5 ml) and diethyl ether (2 × 5 ml) to afford a red-coloured solid (41 mg, 82%). Spectral analysis matched those reported by Seo et al. [30]. The mass was confirmed by ESI-MS: calculated mass for $C_{32}H_{37}N_4O_3S$ $[M + H]^+$ 556.58 Da; observed, 556.90 Da.

2.4. Synthesis of biotin-alkyne

Biotin-alkyne was prepared using the method of Link and Tirell [31]. N-(Biotinyloxy)succinimide (64 mg, 0.19 mmol) was dissolved in excess neat propargylamine. After 20 min, the solution was added dropwise to diethyl ether. A white precipitate formed and was collected by centrifugation. The precipitate was washed in diethyl ether (2 × 5 ml), and the solvent removed *in vacuo* yielding a white-coloured solid (46 mg, 73%). Spectral analysis matched those reported by Wang et al. [32]. Mass was confirmed by ESI-MS: calculated mass for $C_{13}H_{19}N_3O_2S$ $[M + H]^+$ 282.12 Da; observed, 282.13 Da.

2.5. Circular dichroism

CD measurements were made using a JASCO J-815 spectropolarimeter fitted with a Peltier temperature controller. SAF peptide samples were made up as 100 μM solutions each in 10 mM MOPS buffer at pH 7.4. CD spectra were measured in a 1 mm quartz cuvette at 20 °C using a scan rate of 50 nm/min, 1 nm interval, 1 nm bandwidth and a response time of 2 s. After baseline correction, ellipticities in deg were converted to molar ellipticities (deg cm^2 $dmol\text{-}res^{-1}$) by normalizing for the concentration of peptide bonds and path length. Thermal melts were acquired at 222 nm in a 2 mm quartz cuvette using a scan rate of 50 nm/min, 1 nm interval, 1 nm bandwidth and a response time of 2 s, over 20–80 °C. First and second derivatives of these curves were used to determine the TM.

2.6. SAF assembly

Standard fibers were assembled overnight by mixing 100 μM SAF-p2a and SAF-p1 each in 10 mM MOPS buffer, pH 7.4 at 20 °C, in 0.5 ml tubes. Fibers containing SAF-p1z4 and SAF-p1y2 were assembled with SAF-p2a using an *x*:*y*:*z* ratio, where *x*, *y* and *z* are fractions of SAF-p1, SAF-p1y2 and SAF-p1z4 respectively, and $x + y + z = 1$ (relative to SAF-p2a = 1).

2.7. Testing the stability of fibers in copper sulfate and ascorbic acid

The described quantities of $CuSO_4.5H_2O$ and ascorbic acid were added to SAFs, and the tubes were transferred to a rotator and agitated at 20 °C for the stated times. The effect on the stability of the fibers was observed by CD spectroscopy, TEM and widefield light microscopy after 3 h, 24 h and 72 h.

2.8. Copper(I)-catalyzed click reactions

1 mM copper sulfate and 1 mM ascorbic acid were pre-mixed with 2-fold excess of alkyne (either 200 μM biotin-alkyne or rhodamine-alkyne), then incubated with

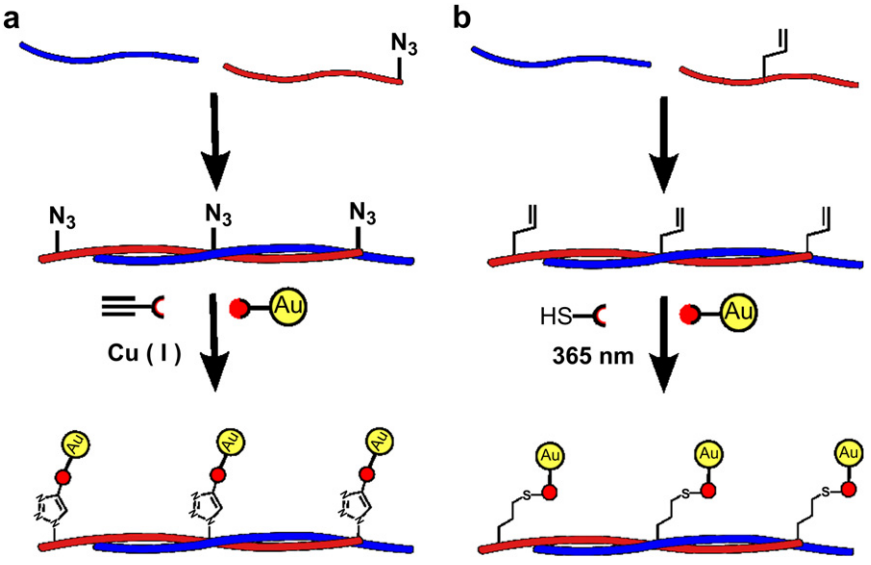

Fig. 1. Cartoon of fiber decoration *via* (*a*) copper(I)-catalyzed azide-alkyne click reactions; and (*b*) thiol-ene click reactions. Fibers are represented as two-stranded (fibril) structures, but in practice they are thickened with many fibrils packed laterally.