The non-covalent decoration of self-assembling protein fibers

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Abstract

The design of self-assembling fibers presents challenges in basic science, and has potential for developing materials for applications in areas such as tissue engineering. A contemporary issue in the field is the construction of multi-component, functionalized systems. Previously, we have developed peptide-based fibers, the SAF system, that comprises two complementary peptides, which affords considerable control over assembly and morphology. Here we present a straightforward route to functionalizing the SAFs with charged peptide tags, which offers advantages such as further control, reversibility, and future prospects for developing recombinant tags. We demonstrate the concept by appending fluorescent labels and biotin (and thence gold nanoparticles) to the peptides, and visualising the resulting decorated SAFs by light and electron microscopy. The peptide tags bind in the nm–μm range, and show specificity compared with control peptides, and for the SAFs over similar α-helix-based peptide fibers.

In particular, non-covalent decoration is attractive because of the potential for control and reversibility, and the possibilities of building up multi-component systems [15]. In this vein, Yu’s group has functionalized collagen-based scaffolds with Collagen Mimetic Peptides (CMPs) [16,17]; and has employed anionic CMPs to attach cell-growth factors to collagen scaffolds [18]. Coulombic interactions have also been used to organize gold nanoparticles and coiled-coils into reversible systems [19]. Here we describe the facile, non-covalent decoration of α-helical fibers, called Self-Assembling peptide Fibers (SAFs) [20–22], using straightforward charged peptides, called “SAF-tags”.

The SAF system comprises two complementary leucine-zipper peptides, SAF-p1 and SAF-p2a, Fig. 1. We have designed and characterized several iterations of SAFs [20,21]. The 2nd-generation SAFs, which comprise peptides SAF-p1 and SAF-p2a, are our model system and used herein. The SAF peptides associate in water to give sticky ended, heterodimers, which self-assemble to give unbranched fibers tens of microns long and 50–70 nm thick [21]. We have built upon this framework incorporating auxiliary, or special peptides to introduce branches [23] and kinks [24] and to recruit nanogold particles and active proteins [25]. However, these special peptides incorporate only sparingly in the SAF scaffold, which we attribute to the highly ordered, near-crystalline structure of the SAFs, evident from X-ray diffraction and high-resolution transmission electron microscopy (TEM) [22]. In TEM, the SAFs show striations at a spacing of 4.2 nm, consistent with end-to-end and side-by-side alignment of peptides; leading to bands of

1. Introduction

The construction of building blocks for the reliable self-assembly of biomaterials presents challenges in basic science, and opportunities in bionanotechnology and synthetic biology [1,2]. Benefits of this research include improved understanding of biomolecular assembly, and the development of new biomaterials for applications in tissue engineering [3]. With this potential in mind, many groups have proffered polymer-, biomolecule- and hybrid-based building blocks and demonstrated fibrous assemblies [4–7]. Though less-well developed, linear, proteinogenic peptides also offer versatile and synthetically accessible building blocks, with the added benefits of biocompatibility and the potential for rational design and recombinant production [3,8]. Indeed, over the past decade, work on peptide self-assembly has mushroomed, and systems utilizing α-helical [8,9], β-sheet [10–12], and collagen [8] building blocks have been realized to produce both stiff fibers or entangled networks, and hence hydrogels [2,13,14]. Many of the resulting materials, however, are bare scaffolds, and a key challenge is to produce decorated materials by supplementing them with functional auxilliary peptides.

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surface-exposed charged residues [22]. The assemblies carry an overall charge of +1 per sticky ended building block [21]. It is binding to such charge patterns that we seek to explore and exploit using the SAF-tags, Fig. 1. In addition, we have described a 3rd-generation SAF design [21], and also a single-peptide α-helical fiber-forming system, MagicWand [26], which serve as useful controls in the studies reported here.

2. Materials and methods

2.1. Peptide synthesis and purification

SAF and SAF-tag peptides were synthesized using Liberty CEM Microwave synthesizers and standard Fmoc chemistry with a para-hydroxy benzene linker on a Tentagel support, or a Rink-amide resin, respectively. For the latter, the resin was divided into three, and either capped by N-terminal acetylation or labeled with rhodamine or biotin. Rhodamine labeling was carried out using rhodamine-B isothiocynate (Sigma) using N,N-diisopropyl-ethylamine (DIPEA) (10 equivalents) and dye (10 equivalents) in N-methylpyrrolidone (NMP) at room temperature for 3 h. For biotin labeling, the peptides were first extended by adding serine and glycine to the N-termini using standard Fmoc chemistry. Biotin (Sigma) was added to the resin in the presence of four equivalents N,N,N,N’-tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) and DIPEA (10 equivalents) in a 1:1 mix of dimethylsulfoxide (DMSO) and dimethylformamide (DMF) at room temperature for 2 h. Peptides were purified using reversed-phase HPLC on a Kromatek HiQ-Sil C18 analytical column (5 μm, 100 Å, 4.6 mm × 150 mm ID) and peptide identity was confirmed by MALDI-TOF mass spectrophotometry using a-cyano-4-hydroxycinnamic acid (CHCA) matrix. Peptide sequences and calculated and observed molecular weights are given in Tables S1 and S2 of the Supporting information. Peptide concentrations were determined in 10 mM 3-(N-morpholino)propanesulfonic acid (MOPS) buffer at pH 7.2 using a molar extinction coefficients of 1420 mol cm⁻¹ cm⁻¹ at 274 nm for the capped and biotin-labeled peptides, and 97,231 mol cm⁻¹ cm⁻¹ at 555 nm for the rhodamine-labeled peptides. SAF peptides stocks were made up in deionised water, and tag peptide stocks in 10 mM MOPS buffer, pH 7.2.

2.2. Circular dichroism spectroscopy

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 in 10 mM MOPS buffer at pH 7.2, to which a solution of tag peptide was added to varying final concentrations. CD spectra were recorded in 1 mm quartz cuvettes at 20 °C using with a scan rate of 50 nm/min, 1 nm interval, 1 nm bandwidth and a response time of 1 s. The spectra were converted from ellipticities (deg) to molar ellipticities (deg cm² dmol⁻¹) by normalizing for concentration of peptide bonds and cell pathlength.

2.3. Transmission electron microscopy

Standard (bare) fibers were made by mixing SAF peptides, SAF-p1 and SAF-2a, to a final concentration of 100 μM in each peptide in 10 mM MOPS buffer at pH 7.2,
and matured for either 6 h or overnight at 20 °C; n.b., previously we have described and used overnight incubations; however, recently we have demonstrated that 6 h is ample for complete maturation of fibers [28]. Matured SAFs were mixed with biotin-labeled SAF-tag peptides and allowed to equilibrate for 30 min. Streptavidin-5 nm gold (KPL) was added to each sample to give a 1 in 100 dilution, mixed with biotin-labeled SAF-tag peptides and allowed to equilibrate for 30 min. The samples were incubated for 20 min before 5 μl were spotted onto 400 mesh carbon coated copper grids (Agar). The excess solution was wicked off with filter paper, and the grid washed two times with 5 μl water. Samples were air-dried and visualized using a Joel JSM 1200EX transmission electron microscope operating at an accelerating voltage of 120 keV and fitted with a Soft Imaging System Megaview II digital camera. Images were recorded using Volocity software. Fibers were prepared as described above, and matured for either 6 h or overnight at 20 °C; these gold nanoparticles. The samples were incubated for 20 min before 5 μl were spotted onto 400 mesh carbon coated copper grids (Agar). The excess solution was wicked off with filter paper, and the grid washed two times with 5 μl water. Samples were air-dried and visualized using a Joel JSM 1200EX transmission electron microscope operating at an accelerating voltage of 120 keV and fitted with a Soft Imaging System Megaview II digital camera. Images were recorded at magnifications of 60,000, 100,000 and 500,000. Control experiments contained only matured SAFs and the streptavidin–nanogold particles, and otherwise were prepared identically.

TEM images were captured and used in analysis as follows: 60,000× magnification, 8-bit color, 2153.36 × 1615.02 nm regions each containing a single SAF fiber were cropped. 100 such images from each experiment at different concentrations were analyzed using Image J software. Data are presented as a ratio of gold nanoparticles (GNPs) on the fiber to the number in the background. This ratio was determined using the formula \( \frac{F_1}{A} \), where \( F_1 \) is the total number of GNPs in the defined field of view; \( A \) is area of fiber, \( A_2 \) — area of the whole view. This normalized for slightly different fiber widths observed in any SAF preparation. Error bars are standard deviations for \( n = 3 \) experiments.

2.4. Widefield fluorescence microscopy

Binding interactions of rhodamine-labeled peptides were visualized using a Leica DM IRB inverted epifluorescence microscope with a cooled CCD camera and recorded using Velocity software. Fibers were prepared as described above, rhodamine-labeled peptides were added, and the mixtures observed after the stated incubation periods. All images were recorded for a 50 ms exposure time with a gain of 240 and offset of zero. Images were exported in TIFF and analyzed using Image J software. Differential interference contrast (DIC) was also used to acquire bright-field images of the fibers.

Images at 1000× magnification were used for image analysis. For each analysis, 150 images were used over a range of SAF-tag concentrations. Image analysis was carried out using Image J software. Single fibers were selected for generating an intensity profile. This gave the average intensity across each fiber, which was taken as the intensity of rhodamine bound to the fiber. Binding intensities were compared to control samples, which were prepared as standard fibers incubated with rhodamine-B only; this accounted for background binding of rhodamine to fibers. Error bars represent standard deviations from \( n = 3 \) experiments.

3. Results and discussion

3.1. Design of the SAF-tags

SAF-tags were designed with sequences of the type R-GG-(xy)-GyG, where \( x \) and \( y \) are charged or control amino acids; tyrosine supplies a chromophore; and \( R \) was made either biotin or rhodamine-B, to introduce probes for TEM and light microscopy, respectively. The core \( xy \) sequences were set as DEDEDE (DE3), KRKRKR (KR3), or AQAQAQ (AQ3), to give negatively and positively charged, or neutral peptides, respectively (Supporting information).

![Fig. 3. Recruitment of gold nanoparticles to SAFs using the SAF-tags and visualized by TEM. A–C, electron micrographs following the binding and subsequent non-covalent labeling with 5 nm streptavidin–gold (5 μm) for the various negatively charged, biotin-labeled SAF-tags that do bind SAFs: A, 10 μm DE3; B, 200 μm GE4; C, 200 μm GE6. D–F, electron micrographs from similar control and other experiments: D, 200 μm EEGEE; E, 200 μm KE6; and F, an untreated SAF control. In each case binding was quantified by calculating the ratio of gold nanoparticles on the fibers and in background: A, 32 ± 2.4; B, 44 ± 2.8; C, 46 ± 2.9; D, 5 ± 0.3; E, 5 ± 0.3; and, F, 5 ± 0.4. All images are unstained and contrast comes from the fibers and gold nanoparticles only. Scale bars, 200 nm.](image3)

![Fig. 4. Analysis of the binding of gold nanoparticles to SAFs via the biotin–DE3 peptide. A, SAF control alone; B, plus 1 μm B-DE3; C, plus 5 μm B-DE3; D, SAFs plus 10 μm B-DE3. Error bars represent standard deviation for \( n = 3 \) experiments. See Methods for details of these experiments and the data analysis.](image4)
Tables S1 and S2). Flexible glycine-based linkers were placed at the termini with future use of the tags in recombinant systems in mind.

First, to test the effect of SAF-tags on the stability of the SAF peptides, tag peptides (at 1–100 μM) were added to the individual SAF-p1 and SAF-p2a, and mixtures of these matured to fibers for 6 h as described above. At the upper 1:1 ratio, DE3 interacted with the individual SAF-p2a peptide to produce a fine visible aggregate, Fig. 2. This was not observed with any other SAF-tag:SAF peptide combinations under any conditions studied. We attribute this to an interaction of oppositely charged peptides—five of the eight N-terminal residues of SAF-p2a are positively charged, and it is likely that this segment of SAF-p2a interacts with the highly negatively charged DE3 tag. None of the peptides grossly affected the fiber secondary structure or stability, Fig. 2.

3.2. Visualization of SAF-tag binding to SAFs: electron microscopy

To explore further the binding of the SAF-tags to fibers, biotinylated SAF-tags (at 0.01–100 μM concentrations) were incubated with fibers, and binding followed by the addition of 5 nm streptavidin—gold nanoparticles. TEM revealed that only the negatively charged tags recruited gold particles to fibers, Fig. 3; the control of streptavidin—gold alone, and the biotin—KR3 and biotin—AQ3 peptides showed little or no decoration. The biotin—DE3 tag recruited gold particles when used at concentrations down to 1 μM, and also exhibited concentration-dependent binding up to 10 μM, Fig. 4. Concentrations above 10 μM did not increase the level of decoration, and in some cases led to a loss of fiber integrity, which may have resulted from the aforementioned competition for SAF-p2a by biotin—DE3.

As an interesting aside, the dose-dependence of and the plateau in binding around the μM range might be rationalized as follows: Considering the SAFs as solid cylinders and from their dimensions and those of the SAF peptides [22], it is possible to deduce that ~10–15% of these peptides are at the surface of the fibers; in addition, matured fibers account for ~70 μM of the peptide in solution [28]; together, these effectively reduce the concentration of SAF peptides incorporated in fibers and available for binding to Fig. 5.

Recruitment for fluorescently labeled SAF-tags to SAFs imaged directly by light microscopy. A, 1 μM rhodamine—DE3; B, 1 μM rhodamine—AQ3; C, 1 μM rhodamine—KR3; and, D, 1 μM rhodamine (control). Scale bars, 10 μm.

Fig. 6. Binding of the GE-based SAF-tag peptides to SAFs. A, 1.5 μM rhodamine—GE3; B, 1 μM rhodamine—GE4. Scale bar, 10 μm.
Fig. 7. TEM images for SAF-tags binding to 3rd-generation SAFs and MW1 fibers. A, 10 μM biotin–DE3 with 3rd-generation SAFs; and, B, 10 μM biotin–DE3 with MW1 fibers. In both cases, the tags were visualized by treatment with 5 nm streptavidin–gold nanoparticles. The ratios of GNPs bound to the fibers and in the background were $24 \pm 3.2$ for A, and $4 \pm 1.2$ for B; these measurements were taken over 21 fiber samples from $n = 3$ experiments. Scale bar, 100 nm. In both cases fibers were stained with 0.05% uranyl acetate to improve the contrast, which contrast with the images shown for 2nd-generation SAFs, which did not require any enhancement.

Fig. 8. Resilience of the binding of the DE3 SAF-tag to SAFs. A, after incubation with 1 μM rhodamine–DE3; B, similar samples to A after washing with 10 mM MOPS containing 30 μM SAF peptides; C, SAFs incubated with 1 μM rhodamine–DE3 for 10 min, followed by incubation with 1 μM unlabeled DE3 peptide for one week; D, SAFs treated with a pre-mixed solution of 1 μM rhodamine–DE3 and 1 μM HEWL, and incubated for 24 h; E, plot showing the change in binding intensity over time for SAFs mixed with rhodamine–DE3 (solid circles), and rhodamine–DE3 in competition with unlabeled DE3 (solid squares); F, similar plot for rhodamine–DE3 pre-mixed with HEWL (open squares). Scale bar, 10 μm. Error bars represent standard deviations from three experiments.
~7–11 μM. This means that at the μM concentrations of SAF-tags used in the present study it is possible, at least in principle, to saturate the available binding sites. Of course, there are caveats in making this argument, and even interpreting the data semi-quantitatively: Firstly, the SAF-tags will be in equilibrium between SAF-bound and unbound forms. We do not know where this equilibrium sits, and this would be difficult to determine, but from the experiments reported here the dissociation constant is likely in μM or sub-μM. Secondly, it is not possible to take the gold nanoparticles above ~5 μM—this is because they aggregate above this concentration leading to unacceptable backgrounds [25]—which limits the sensitivity of the experiments. Nonetheless, it is interesting that we do observe a dose-dependent binding of the DE3 SAF-tag to SAFs in the expected μM range based on the likely available binding sites.

To test alternative negatively charged peptides, the R-GG-(xy)-GYG peptides were reconfigured by varying the core xy sequence to give different numbers and spacings of glutamic acid residues; for example, the inserts GGEEGG, EEGGEE, GEGEGE (GE3) and GECEGECE (GE4) were all tested (Supporting information Table S2). The biotin-GGEEGG and biotin-EEGGEE sequences showed little or no binding, but clear recruitment was observed with the biotin-GE3 and biotin-GE4 peptides, Fig. 3. For these revised peptides, the best surface coverage was achieved with 200 μM of either biotin-GE3 or biotin-GE4, without affecting fiber stability or integrity. Thus, at least 3 alternately spaced negatively charged side chains appear to be important for tag binding to the SAFs. A peptide with equal positive and negative charged residues—biotin-KE3—was also tested, but did not show any binding to the fibers, indicating that positively charged groups do not contribute significantly to SAF decoration.

3.3. Visualization of SAF-tag binding to SAFs: light microscopy in solution

For the development and potential applications, it is crucial to establish stable decoration in aqueous solution. Therefore, we turned to rhodamine-labeled SAF-tags and widefield fluorescence microscopy. This showed differences in the uptake of positively and negatively charged peptides to the fibers, Fig. 5. Again, the rhodamine-KR3 peptide did not recruit to the SAFs; any binding of this peptide was indistinguishable from the background rhodamine-B fluorescence. The rhodamine-DE3 peptide, however, recruited at lower concentrations than observed indirectly in the gold nanoparticles-TEM experiments, with the lowest visible binding at 10 nM tag peptide. Interestingly, the rhodamine-AG3 peptide also decorated fibers; though binding was ~10-fold less tight, and less resilient to washing than that for rhodamine-DE3. To test the origin of binding by AG3, we made a rhodamine-labeled AG3 peptide. This did not bind fibers (n.b. The corollary, GQ3 control peptide synthesized poorly, aggregated, and could not be characterized). On this basis, we posit that amide side chains can also interact with the SAFs—possibly via the glutamine residues spaced every seven residues on the outer surfaces of the fibers—which is consistent with our recent findings that amide hydrogen-bonding interactions foster fibril–fibril interactions in SAF-based hydrogels [27].

Regarding the reconfigured rhodamine-GE3 and rhodamine-GE4 tags with reduced number of charge, these showed reduced binding to the fibers; whereas the rhodamine-DE3 tag bound at sub-μM concentrations, greater than μM concentrations of rhodamine-GE3 and -GE4 tags were required to achieve similar binding intensities, Fig. 6; again, these tags were more resilient to washing than AQ3.

3.4. Specificity and longevity of binding

Specific binding of the tags for the SAFs is clearly an important criterion; hence any binding of the biotin–DE3 tag, the best binder of the SAFs studied above, was tested against 3rd-generation SAFs and MagicWand (MW1) [21,26]. Both systems have an overall charge of ~1 per building block (a heterodimer in the case of 3rd-generation SAFs, and a monomer for MW1) and clear, persistent ultrastructure indicative of high levels of order. However, the sequence features of the designs, and how these may present in the ultrastructure and order are very different: both the 2nd and the 3rd-generation SAFs have a cluster of positive charge on the outer surfaces of the coated-coil [22], whereas the MW1 has a single positive charge [26]. Interestingly, the biotin–DE3 tag recruited to the 3rd-generation SAFs, but not to MW1 fibers, Fig. 7. This indicates strongly that it is not just positive charge that is important, but as we posit, its organization and density on the surface of the fibers, which contributes to binding of the tags to the SAF systems.

Further on this point, negatively charged peptides tags could bind non-specifically to basic globular proteins in some applications. Clearly, we cannot test against all possibilities here, and such background binding would have to be tested on a case-by-case basis. As an example, we probed the persistence of binding of the DE3 tag to SAFs in the presence of the potential competitor protein hen egg white lysozyme (HEWL), which has a charge of +8.6 at pH 7.0. 1 μM each of HEWL and rhodamine–DE3 tag were pre-mixed and added to SAF fibers, and binding observed by widefield fluorescence microscopy. Comparison of binding intensities for the HEWL-free and HEWL-containing samples showed that binding of the rhodamine–DE3 tag was affected, but not abolished in the presence of protein; the initial binding was ~97% of the HEWL-free signal, with ~60% still bound after 24 h, Fig. 8.

With unlabelled DE3 as the competitor, a rapid drop in binding intensity was observed over the first 48 h, this gradually stabilized to ~70% binding intensity by the end of one week, Fig. 8. These data illustrate that the binding of the tags is dynamic, and unlabeled tags can compete for available binding sites on the fiber.

4. Conclusion

In summary, we present a facile method for the non-covalent decoration of self-assembling peptide fibers (SAFs) using straightforward charged peptides as tags. These SAF-tags bind fibers at sub-μM to μM concentrations, and binding can be varied by using tags with different patterns and degrees of charge. We demonstrate specific and persistent binding of the SAF-tags over time, resilience to washing and peptide and protein competitors. The non-covalent interaction can be reversed under mild conditions, presenting the potential for design of dynamic systems. Importantly, this non-covalent decoration is mild and has little effect on fiber stability. The tags and our observations reported here add versatility to the SAF systems, and allow us to take the first steps towards the design of functional multi-component peptide systems.

Acknowledgements

We thank the Woolfson group for helpful discussions, and the BBSRC for funding (grant BB/E022359/1).

Appendix. Supporting information

Supporting information giving the full list of peptide sequence is available on-line. Supporting information associated with this article can be found in the on-line version, at doi:10.1016/j.biomaterials.2010.06.041.
Appendix

Figures with essential color discrimination. Figs. 1, 2, 5, 6 and 8 in this article are difficult to interpret in black and white. The full color images can be found in the on-line version, at doi:10.1016/j.biomaterials.2010.06.041.

References