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 alkynes. 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.

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1. Introduction

The construction of peptide-based fibrous biomaterials is receiving increased attention [12]. 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 sidechain 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, gabcdf (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-
the organic layer was separated and dried over MgSO4, and the solvent stirred at RT for 12 h. The solvent was removed. Imidazole-1-sulfonyl azide hydrochloride (1.06 g) was added and the mixture stirred at RT for 12 h. Potassium carbonate (2.03 g) and copper sulfate (20 mg) were added. Further peptide sequences and HPLC and mass spectrometry data are given in Table S1 and Fig. S7, Supporting Information.

2.2. Synthesis of Lysine ε-azide

Fmoc-Lysine ε-azide was prepared using the method of Goddard-Borger and Stick [27]. Fmoc-Lysine-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, and the residue partitioned between water (200 ml) and chloroform (150 ml) containing isopropanol (50 ml). The organic layer was separated and dried over MgSO4, and the solvent removed in vacuo. The crude product was purified by 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 Tsuda et al. [28].

2.3. Synthesis of rhodamine-alkyne

Rhodamine-alkyne was synthesized using the method of Punna and coworkers [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 CH2Cl2 (2 × 5 ml) and diethyl ether (2 × 5 ml) to afford a red-colored solid (41 mg, 82%). Spectral analysis matched those reported by Lee et al. [30]. The mass was confirmed by ESI-MS: calculated mass for C32H37N4O3S [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-colored solid (46 mg, 73%). Spectral analysis matched those reported by Wang et al. [32]. Mass was confirmed by ESI-MS: calculated mass for C32H37N4O3S [M + H]+ 556.58 Da; observed, 556.13 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 cm2 dmol−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, 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 CuSO4.5H2O 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...
the 100 µM SAF-p1z2 and SAF-p2a derivatized fibers (assembled overnight) in 10 mM MOPS buffer, pH 7.4, for a further 3 h. Excess rhodamine-alkyne was removed by washing the fibers 2 x MOPS buffer, excess biotin-alkyne was removed by washing the grid 2 x deionized water. Decoration of the fibers was either observed directly with the fluorescence microscope, or by adding 2.5 µM 5 streptavidin-gold (KPL) to the fibers, then visualized by TEM. (n.b. These gold nanoparticles synthesized by a proprietary method to minimize background binding.) Control samples for fluorescence microscopy were incubated with rhodamine B instead of rhodamine-alkyne; biotin-alkyne was omitted from TEM controls.

2.9. Thiol–ene click reactions

A 100 µM SAF-p2a and SAF-p1z2 derivatized fiber preparation in 10 mM MOPS buffer, pH 7.4, was mixed with the thiol-containing (BCP1) peptide, and 0.1% photoinitiator ((1-[4-(2-hydroxyethoxy)-phenyl]-2-hydroxy-2-methyl-1-propane-1-one)), then transferred to a quartz cuvette and exposed to UV light (UVP UVGL-58, 6W) at 365 nm, at a distance of 5 cm for 10 min. The samples were then placed on an ice-pack with a 1 mm spacer (between the sample and ice-pack) to prevent overheating. The samples were then transferred to a 0.5 ml tube, and incubated with 25 nM 5 nm streptavidin-gold, in the dark, for 1 h, before being analyzed by TEM.

2.10. Transmission electron microscopy (TEM) image analysis

TEM grids were prepared by spotting the relevant sample (5 µl) onto a carbon coated copper grid. The grid was washed with distilled H2O (2 x 5 µl) to minimise non-specific gold binding, excess solution was wicked with a filter paper. Fibers that required staining were spotted onto the carbon grid, stained with 1% uranyl acetate (5 µl), then washed with distilled H2O (2 x 5 µl). Fibers decorated with biotin-alkyne were visualized using a JEOL JEM 1200 EX MKII. Images were recorded digitally with a Mega View II digital camera, using Soft Imaging Systems GmbH analysis 3.0 image analysis software. 210 images of single fibers from n = 3 experiments were analyzed for each x:y:z ratio of single functionalized fibers; 150 images of single fibers from n - 3 experiments were analyzed for each x:y:z ratio of dual functionalized fibers. Images were analyzed at 100,000 magnification, only images containing individual fibers were used for analysis. Each unit area was defined by drawing a box of 70 x 300 nm, 2 boxes were placed manually (non-overlapping) on each fiber and the number of gold particles averaged.

2.11. Fluorescence microscopy and image analysis

Fibers were visualized using a Leica DM IRB inverted epifluorescence microscope equipped with a TRITC filter and a cooled CCD camera and recorded using Velocity software. Images were recorded at 1000x magnification using a 50 s exposure time with a gain of 240 and offset of zero. A TRITC filter was used to capture rhodamine fluorescence. Images at 1000x magnification were analyzed by measuring pixel intensity units for individual fibers in Image J. 210 single fibers from n - 3 experiments were analyzed for each x:y:z and x:y:z ratio of single and dual functionalized fibers.

2.12. Analysis of conjugated fibers by HPLC

HPLC analysis was used to test the fibers after the click reactions and to demonstrate unbiased uptake of modified SAF peptides. Fibers were washed 2 x 10 mM MOPS buffer, pH 7.4, then resuspended in water-acetonitrile buffer (80:20) and analyzed by HPLC (Kromatex HQ-SIL, C18 analytical column, 5 µm, 100 Å, 4.6 mm x 150 mm ID). Unless stated otherwise, samples were eluted using a linear gradient of 20–80% acetonitrile over 28 min; the solvent system was water-acetonitrile with 0.1% TFA.

3. Results and discussion

3.1. Effect of peptide modifications on the stability of the SAF fibers

To start, an azido group was introduced at the N-terminal of Fmoc-L-lysine prior to standard solid phase peptide synthesis (SPPS) [18]. In this way, two variants of SAF-p1 were synthesized each containing a single azide moiety, either at the central, or the C-terminal f position of the heptad repeat; these were named SAF-p1z2 and SAF-p1z4, respectively, Table 1. The f positions were chosen as the furthest away from the coiled-coil interface. In separate preparations, the variants were mixed 1:1 with the complementary SAF-p2a peptide, and fiber formation was assessed by circular dichroism (CD) spectroscopy and negative-stain transmission electron microscopy (TEM), Fig. S1C. These SAF-p1z2 led to aberrant amyloid-like β-structured assemblies, Fig. S1B. We have no firm explanation for this, but suggest that the azido-lysine (z) somehow disrupts the regular hydrophobic (H)-polar (P) pattern of the coiled-coil heptad repeat of SAF-p1z2 in the middle of the peptide—i.e., from ...HPPHP... to ...HPPHPzP... which interferes with correct fiber assembly. With the azido group at the C-terminus in SAF-p1z4, however, the effect of this change is less—the sequence ends HPPHPzP. Indeed, fibers made from SAF-p2 plus SAF-p1z4 had closely similar physical properties and microscopic characteristics to the parent SAFs [13,15], confirming that fibers tolerate the full inclusion of the modified peptide. Thus, and in contrast to any of our previous reports that introduce modified SAF peptides into the fabric of the fibers [14,19,20], we have achieved complete replacement of SAF-p1 with the azide-functionalized peptide SAF-p1z4.

3.2. Optimising copper(I)-catalyzed click reactions with SAF peptides and fibers

Conditions for the copper(I)-catalyzed azide-alkyne click reaction compatible with maintaining intact fibers were optimized using unmodified fibers. This was necessary to test if SAFs are non-covalent assemblies that do not tolerate all conditions; specifically, maintaining fully aqueous solutions buffered to ~ pH 7 critical. The fibers were least affected by a 1:1 mixture of copper sulfate and ascorbic acid each at 1 mM, Fig. S2, Supporting Information. Increasing the concentrations of these reagents to 2 mM, or the addition of tri(s)[1-benzyl-1H-1,2,3-triazol-4-yl]methyl]amine (TBTA) to retain copper in its copper(I) state [21], adversely affected the assembled fibers. In addition, we explored the timing of adding the reagents to assembling or assembled fibers. Recently, we have shown that SAF peptide assembly occurs by an initial nucleation event, of around 2 h, followed by fiber growth [22]. Adding copper (I) to the peptide mixtures before nucleation prevented fiber assembly.

We tested these SAF-compatible click reaction conditions first on free SAF-p1z4 with a 2-fold excess of biotin-alkyne and the copper(I) source, following the reaction by HPLC and mass spectrometry. 59% of the peptide was labeled with biotin-alkyne in 3 h, and longer incubations did not increase yields, Fig. 2. The resulting and purified SAF-p1-biotin conjugate did not assemble efficiently into fibers when mixed with SAF-p2a. This might be expected on the basis of our previous reports of massively sub-stoichiometric incorporation of heavily modified, or special, SAF peptides into fibers [14,19,20].

3.3. Post-assembly functionalization of SAFs

Next, we tested post-assembly conjugation to fibers containing the azido peptide. First, fibers with 1:1 mixtures of SAF-p1z4: SAF-p2a matured overnight at 20 °C were mixed with biotin-alkyne in the presence of the copper(I) source. The products were separated under denaturing conditions by HPLC. The yield of conjugated peptide reduced from 59% to 32%. This is expected, however, because the SAFs are solid cylinders and most of the peptides—we estimate ~ 60%—are buried within the fibers, and not available for labeling [23]. Nonetheless, the composition and the absolute labeling of the fibers could be varied further by mixing SAF-p1 + SAF-p1z4 with SAF-p2a in the ratio x:y:z:1; where, x and z are the fractions of SAF-p1 and SAF-p1z4, respectively, and x + z = 1. Fibers were prepared with x:y ratios of 0:0, 0.2:0.8, 0.5:0.5 and 1:0 labelled with biotin-alkyne, and the components separated by HPLC. The integrated peaks confirmed that the amount of incorporated functionalized peptide correlated with z, Fig. 3.
3.4. In situ functionalization of SAFs using copper (I)-catalyzed click

To demonstrate functionalization in situ, 5 nm streptavidin-gold nanoparticles were added to biotin-alkyne conjugated fibers prior to visualization by TEM (without additional stain). This revealed a high level of coverage with nanoparticles, Fig. 4e, as compared to control fibers without the azide, Fig. 4a. In further experiments with different x:z ratios, the degree of decoration was gauged by counting the number of gold nanoparticles on the fibers. This correlated, approximately linearly, with the amount of SAF-p1z4 in the initial mixture, Fig. 4. Substituting the biotin-alkyne with rhodamine-alkyne gave fibers with rhodamine dye attached, for direct visualization in solution by fluorescence microscopy, Fig. 6c2.

3.5. Functionalization of SAFs using thiol-ene click

To add a second function to the SAFs, we turned to thiol-ene click chemistry, a photoinitiated reaction between an alkene and a thiol [17,24]. Fmoc-c-allylglycine was used to introduce an alkene into SAF-p1. Again, two variants, SAF-p1y2 and SAF-p1y4, were synthesized each with a single allyl functional group at the second, or the fourth position, respectively Table 1. In contrast to the azide modifications, both were tolerated in fibers. Presumably, the smaller allylglycine side chain has less influence than azido-lysine on the folding and assembly into fibers of SAF-p1. However, dual functionalized, SAF-p1y2z4, did not assemble with SAF-p2a, highlighting again the limits to which the relatively short SAF peptides can be modified and still be competent for assembly. The following experiments used the variant with allylglycine at the second heptad, SAF-p1y2.

The thiol-ene reaction was optimized using SAF-p1y2, varying its ratio to a water-soluble thiol-containing peptide with biotin at the N-terminus, BCP1, Table S1, Supporting Information, the intensity and exposure to the UV source (6W, λmax 365 nm), and with or without the photoinitiator, 1-[4-(2-hydroxyethoxy)-phenyl]-2-hydroxy-2-methyl-1-propane-1-one [25]. Progress of the thiol-ene reaction was followed by HPLC and mass spectrometry. To reduce photodegradation of the peptides, exposure times were limited to 10 min. Under these conditions, 11% yields were obtained with a 2:1 ratio of thiol/alkene and 0.1% photoinitiator, Fig. 5.
Fig. 3. Post-assembly functionalization of SAF fibers as analyzed by HPLC. A – D, dilution ratios \(x:z\) as shown for the ratio of SAF-p1:SAF-p1z4 in the starting mixture. Key: 1, internal control peptide, 120 \(\mu\)M; 2, SAF-p2a + SAF-p1z4; 3, SAF-p1z4-biotin-alkyne conjugate; and 4, SAF-p1. Fibers were prepared by mixing SAF-p1, SAF-p1z4 and SAF-p2a in a ratio of \(x:z:1\); where \(x\) – fraction of SAF-p1, \(z\) – fraction of SAF-p1z4 and \(x + z = 1\). The sequence of the internal control peptide is given in Fig. 2. AUC ratios (\(n = 3\) experiments) for the peaks \((x:z)\) are as follows: A, 0:190 ± 5; B, 41 ± 4:160 ± 6; C, 141 ± 4:80 ± 5; D, 232 ± 6.0. Percentage SAF-p1z4 conjugated: A, 32 ± 2; B, 29 ± 3; C, 33 ± 2; D, 0.
These conditions were used to conjugate the biotin- and thiol-containing peptide to fibers comprising SAF-p1 and SAF-p1y2 in varying ratios \((x:y)\) and SAF-p2a, followed by decoration with 5 nm streptavidin-nanogold for visualization by TEM, Fig. 5a\textemdash c. In this case, because of the low conjugation yield, HPLC analysis was only suitable for fibers with 1:1 SAF-p1y2: SAF-p2a, which revealed 14% conjugation in fibers. Although this yield is slightly higher than that obtained for free peptide, this may reflect the reaction kinetics in two different systems neither of which goes to completion in the given time.

### 3.6. Dual functionalization of SAFs

Our ultimate aim is to add, control and tune different functions to the SAFs. Towards this, the two reactive peptides could be used provided that they incorporate stoichiometrically when mixed, and can be labeled orthogonally and to known extents, leading to reproducible decoration. To begin assessing this, we performed the following experiment: SAF-p1y2 and SAF-p1z4 were mixed with SAF-p2 in ratio 0.5:0.5:1 to form fibers, which were sampled over time, spun down, denatured and subjected to HPLC analysis. The ratio of the peptides incorporated into matured fibers remained constant over time at ~ 0.5:0.5:1, showing that the modified peptides incorporated into bulk fibers stoichiometrically, and with similar rates of uptake, Fig. S3, Supporting Information.

This type of analysis of the three-component mixtures has a caveat, however: it is a bulk measurement, and it is possible that the azido- and allyl-modified peptides form two distinct subsets of fibers. To test this, we prepared SAFs with different proportions of SAF-p1y2 (y) and SAF-p1z4 (z) in ratios with SAF-p2a of \(y:z:1\); various proportions of \(y:z\) (1:0, 0.8:0.2, 0.5:0.5, 0.2:0.8, and 0:1)
were explored. In this way, we could also evaluate the possibility of modulating function on the surface of the fibers. The resulting fibers were then functionalized in a step-wise manner: first, with the biotin- and thiol-containing peptide illuminated at 365 nm to effect the thiol-ene click reaction, followed by addition of the copper(I) source and rhodamine-alkyne for the second click reaction. This order circumvents possible thiol-yne cross-reactions [26]. The resulting samples were split for visualization by fluorescence analysis.

![HPLC and mass spectrometry analysis for the thiol-ene click reaction. A, HPLC trace of 100 μM SAF-p1y2 peptide incubated with 200 μM BCP1 peptide in the presence of 0.1% of photoinitiator, and exposed to light at 365 nm. B, MALDI-TOF analysis of peak 3, expected [M + Na]^+. C, HPLC trace of relative ratios of SAF-p1y2 and SAF-p1y2-BCP1 conjugate in allyl-derivatized fibers post-assembly. Fibers were assembled in a 1:1 ratio of SAF-p1y2 with SAF-p2a, then functionalised using thiol-ene click.](image)

Fig. 5. HPLC and mass spectrometry analysis for the thiol-ene click reaction. A, HPLC trace of 100 μM SAF-p1y2 peptide incubated with 200 μM BCP1 peptide in the presence of 0.1% of photoinitiator, and exposed to light at 365 nm. B, MALDI-TOF analysis of peak 3, expected [M + Na]^+. C, HPLC trace of relative ratios of SAF-p1y2 and SAF-p1y2-BCP1 conjugate in allyl-derivatized fibers post-assembly. Fibers were assembled in a 1:1 ratio of SAF-p1y2 with SAF-p2a, then functionalised using thiol-ene click. Key: 1, internal control peptide, 100 μM; 2, SAF-p1y2-BCP1; 3, SAF-p1y2 and 4, SAF-p2a. AUC analysis (n = 3 experiments) showed that 13.8% of SAF-p1y2 peptide was conjugated with BCP1. The sequence of the internal control peptide is given in Fig. 2.
microscopy, and by TEM using streptavidin-nanogold as the decoration and contrast agent. Several points are clear from the resulting representative images from these experiments, Fig. 6 and Fig. S4: Firstly, all fibers were decorated, which discounts the possibility of subsets of azide- or allyl-only fibers. Secondly, for each preparation the majority (\(\geq 95\%\)) of the fibers were decorated to similar extents, demonstrating reproducible decoration. Finally, the extent of decoration with fluorophore or nanogold was proportional to the amount of allyl or azido peptide used in the initial mixture. These experiments demonstrate dual incorporation of the different chemical handles into the SAFs at prescribed ratios; and the dual functionalization of the resulting three-component systems.

4. Conclusion

In summary, we have shown modification of SAFs using two orthogonal routes under conditions that are compatible with maintaining fiber integrity at physiological pH. The fibers incorporate either or both of two click-compatible peptides at specified ratios. This allows the preparation of increasingly complex and, we believe, unprecedented, two- or three-component self-assembling peptide fiber systems. We demonstrate that these labeled fibers can be decorated, and then visualized by adding functionalized gold particles and imaging dried-down, but otherwise unstained, fibers by TEM; or, directly, by adding a fluorophore and imaging in aqueous solution by fluorescence microscopy. These realizations have come about in part through the thorough understanding that we have of the model SAF system, in terms of sequence-to-structure relationships, assembly kinetics and the details of the final structure. Our future effort will focus on applying the methods that we have developed to similar systems where real-life applications are increasingly possible.

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Appendix. Supplementary data

Supplementary data associated with this article can be found in online version at doi:10.1016/j.biomaterials.2010.12.002.
Appendix

Figures with essential color discrimination. Figs. 1 and 6 in this article are difficult to interpret in black and white. The full color images can be found in the online version, at doi:10.1016/j.biomaterials.2010.12.002.

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