Antibiotic Spider Silk: Site-Specific Functionalisation of Recombinant spider silk using 'click'- chemistry

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The use of functionalised recombinant spider silk as a sustainable advanced biomaterial is currently an area of intense interest owing to spider silk's intrinsic strength, toughness, biocompatibility and biodegradability. This paper demonstrates, for the first time, the site-specific chemical conjugation of different organic ligands that confer either antibiotic or fluorescent properties to spider silk. This has been achieved by the incorporation of the non-natural methionine analogue L-azidohomoalanine (L-Aha) using an *E. coli* methionine auxotroph and subsequent copper catalysed azide-alkyne cycloaddition (CuAAC) or 'click chemistry' functionalisation of 4RepCT^{3Aha}. The 4RepCT^{3Aha} protein can be modified either prior to, or post fibre formation increasing the versatility of this approach as demonstrated here by the formation of silk fibres bearing a defined ratio of two different fluorophores uniformly distributed along the fibres. Silk decorated with the fluoroquinone family broad spectrum antibiotic levofloxacin via a labile linker is shown to have significant antibiotic activity over a period of at least 5 days. The inherent low immunogenicity and pyrogenicity of spider silk should allow a diverse range of functionalised silks to be produced using these approaches that are tailored to applications including wound dressings and as tissue regeneration scaffolds.

Strength, biodegradability and biocompatibility make dragline silk an attractive candidate for use in biomedical applications. Unfortunately, the availability of natural spider silk is limited as the territorial and cannibalistic nature of spiders prevents large scale farming.[1] Several recombinant silk production methods have emerged in response to this problem, utilising various organisms, including *E. coli*.[2–7] Johansson and coworkers produced 4RepCT,a self-assembling recombinant dragline silk protein, derived from the nursery-web *Euprosthenops australis*.[5-6] This protein is a miniaturised version of the silk monomers (spidroins) found in nature and comprises of a repetitive domain and a non-repetitive C-terminal domain essential to fibre formation (CT) (Figure 1).[8,9]

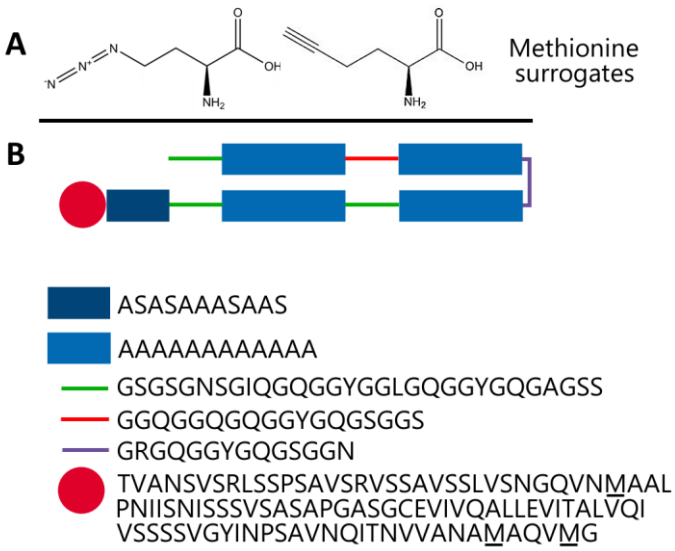

Figure 1: *L*-azidohomoalanine (L-Aha) and a schematic representation of 4RepCT^{3Aha}. **(a)** The bioorthoganol methionine surrogate L-Aha. **(b)** Composition of 4RepCT^{3Aha}; the blue boxes represent poly-alanine blocks and the dark blue box represents a serine and alanine rich tract, Red, green and purple lines represent glycine rich tracts. The non-repetitive C-terminal domain is represented by a red circle. Non-natural amino acid substitution sites are shown in the primary sequence of the C-terminal domain as underlined methionines. Figure adapted from.[5]

Thioredoxin-4RepCT can be cast into cell supporting films or, when digested with thrombin to generate the miniature spidroin 4RepCT, self-assembled into immune-tolerated fibres demonstrating the potential to be used as a biomaterial in man.[5,10–14] Currently, the

applications of 4RepCT are limited due to a lack of tuneable functionality. However function can be attained and tailored to specific applications by two means; genetic fusion of functional peptide sequences to silk genes or chemical conjugation of functional molecules onto amino acid side chains. The first approach has the advantage that post-translational manipulation of the silk is minimised, but the disadvantages that genetic manipulation is challenging due to the high GC content of the gene and is limited to the inclusion of a single ligand binding site per 25 kDa 4RepCT silk protein. If a large 'adapter' protein such as an antibody (150 kDa) is used to non-covalently recruit a second ligand as has been reported, the loading of the fibre will be reduced further. [15] Still, silk fusion proteins have been produced via this route that demonstrate improved cellular adhesion and varied antimicrobial potencies.[16–20] The chemical modification of silk proteins should allow the covalent attachment of multiple copies of a wide variety of organic and organometallic ligands through either robust or environmentally sensitive linkers as required. Making the modification site specific is more challenging and requires the residues to be modified to be accessible and chemically bio-orthogonal to the rest of the silk protein. Cysteine residues are commonly used to conjugate maleimide-tagged molecules to proteins via a Michael addition reaction.[21] However cysteine maleimide conjugates can undergo exchange reactions, resulting in the deconjugation of the functional molecule thus raising concerns over long term stability of these conjugates *in vivo*.[22] More recently azide functional groups have been conjugated to the N terminal of a dragline silk protein using EDC/NHS coupling, yielding glycopolymer-conjugated films with enhanced cell adhesion and DNA-silk chimeras with controllable micro-architectures.[23,24]

In this study we have incorporated 3 L-Aha residues into 4RepCT, yielding 4RepCT^{3Aha}. [25–28] The azide side chains of L-Aha allow highly specific and efficient site-specific conjugation to a large and varied repertoire of functional molecules via Staudinger ligation with phosphine reagents, Copper (I)-catalysed azide-alkyne cycloaddition (CuAAC) or Strain promoted azide-

alkyne cycloaddition (SPAAC) in so called 'click' reactions.[25,29,30] This paper demonstrates the CuAAC mediated conjugation of 4RepCT^{3Aha} with two different fluorophores and the antibiotic levofloxacin, showcasing the potential of covalently functionalised recombinant spider silk proteins as biomaterials with enhanced properties.

Results

Conjugation of fluorophores to soluble 4RepCT^{3Aha} and 4RepCT^{3Aha} fibres

The presence of the azides was verified by the successful conjugation of alkyne fluorophores to pre-assembled fibres and soluble protein (Figure 2). Labelled soluble 4RepCT^{3Aha} formed fibres upon incubation with thrombin (Figure 2 B) and, as with pre-assembled fibres, showed uniform intense fluorescence (Figure 2 C, D). Control fibres that did not contain L-Aha rapidly lost fluorescence upon washing; returning to the appearance of un-labelled fibres (Figure 2 E, F).

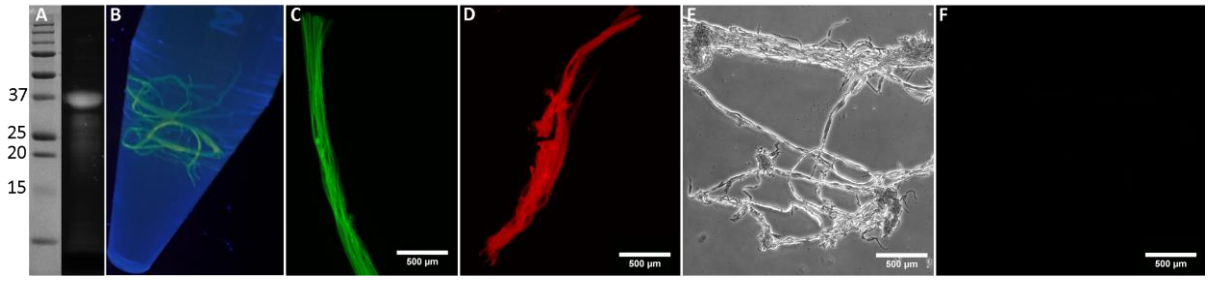

Figure 2: Successful conjugation of 4RepCT^{3Aha} with alkyne fluorophores. (A) A fluorescent band at 37 kDa corresponding to FAM labelled soluble 4RepCT^{3Aha} and (B) a fluorescent fibre produced from incubation with thrombin. (C) A pre-assembled fibre labelled with FAM and (D) a pre-assembled fibre labelled with Alexa Fluor 594®. (E) Control 4RepCT fibre (no L-Aha) and (F) control fibre showing no fluorescence.

Dual labelled macroscopic fibres.

FAM- and Alexa Fluor 594® -labelled 4RepCT^{3Aha} were combined in a 1:1 ratio and fibre formation initiated by the addition of thrombin. The resulting fibre displayed both green (FAM)

and red (Alexa Fluor 594®) fluorescence but appeared orange in colour to the naked eye. Composite images showed intense uniform yellow fluorescence along the length of the fibre, similar to mono-labelled fibres (Figure 3).

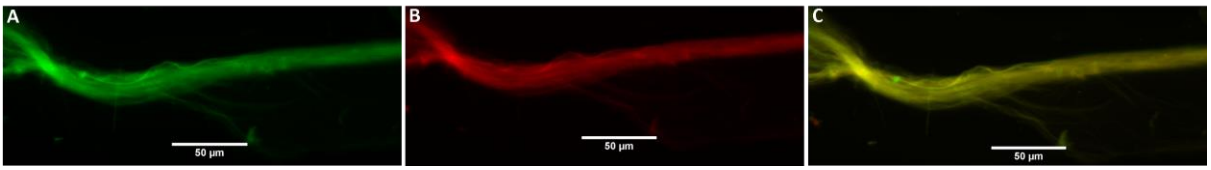

Figure 3: Fluorescence light microscope images of dual fluorophore labelled 4RepCT^{3Aha} fibres. (A) When excited by 467-498 nm light, (B) excited by 542-582 nm light and (C) a composite image of both fluorescent signals.

Levofloxacin labelled 4RepCT^{3Aha} exhibits antimicrobial activity

Pre-assembled 4RepCT^{3Aha} fibres were decorated with the antibiotic levofloxacin via an acid-labile linker (Figure 4).[31] Antimicrobial activity was tested against *E. coli* NCTC 12242 in a zone of inhibition assay and by monitoring cell density of liquid cultures. After a 28 hour incubation period of liquid cultures, the levofloxacin functionalised fibre had significantly reduced bacterial cell density to ~50% of the other fibre types and Luria-Bertani medium (LB) alone (p= <0.01). The levofloxacin functionalised and control fibre types both gave zones of inhibition after 24 hours incubation (3.5 cm and 2.0 cm respectively), as expected, the non-functionalised fibre did not produce a zone of inhibition. Upon further incubation (120 hours), colonies were growing in the previously clear zone of the levofloxacin control fibre, as the adsorbed levofloxacin was exhausted, but not in the zone generated by the levofloxacin 'click' functionalised fibre where the antibiotic was still being released.

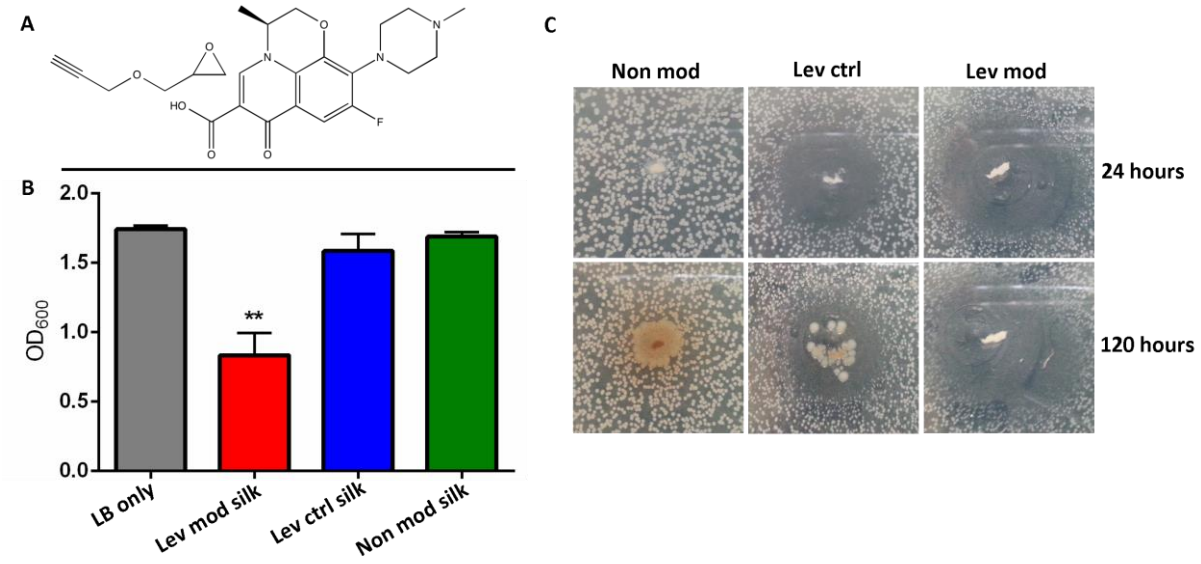

Figure 4: Levofloxacin functionalised 4RepCT^{3Aha} fibre. (A) Glycidyl propargyl ether linker (left) and levofloxacin (right) form labile ester bond between the epoxide and carboxylate group. (B) Optical densities of *E. coli* NCTC 12242 after 28 hours incubation with LB media alone (LB only), non-functionalised (non mod silk), levofloxacin control (lev ctrl silk) and levofloxacin-functionalised (lev mod silk) fibres. Data is mean of 3 replicates, error bars show standard error of the mean; two tailed t-test was used to determine significance (** p= <0.01). (C) Muller-Hinton agar plates showing zones of inhibition of *E. coli* NCTC 12242. Incubation times displayed on the right and fibre types displayed above.

Discussion

Amidst efforts to increase silk production in order to compete with other industrially produced materials, a considerable area of research is dedicated to the modification of these proteins to create novel silk materials and/or diversify the applications of silk. Here we report the incorporation of L-Aha into the methionine sites of a previously characterised recombinant silk protein 4RepCT, to give 4RepCT^{3Aha} and allowing for downstream site-specific chemical modification of the protein using click chemistry. Unlike previous attempts to incorporate azide groups into silk proteins that involve multi-step processes that are non-specific or only allow the introduction of only a single azide group at the N-terminal, our method achieves this in just one step and allows complete control over the number of azides per protein subunit, based on

the number of methionine codons it's gene contains.[23,24] Cu (I) is known to be cytotoxic, is used to catalyse the 'click' reaction. Therefore to conserve biocompatibility, copper must not be present in the final functionalised silk product. By washing functionalised fibres with buffers containing EDTA and by utilising THPTA which binds and stabilises the Cu (I) ion in the click reaction, the traces of copper (<0.1 % by weight) that were detectable by Energy-dispersive X-ray spectroscopy (EDX) in the fibres initially after the CuAAC reaction, have been removed (supplementary information). The potential for copper to be bound by the 4RepCT^{3Aha} protein is low due to the presence of only 2 glutamic acid residues and the complete lack of histidine residues. These residues are present in the thioredoxin solubilising fusion partner, however this does not cause issues since the thioredoxin is removed in order to trigger fibre formation, clearing all copper from the silk structure. Alternatively, copper free 'click' reactions such as the Staudinger ligation or SPAAC could be employed if the ligand itself had affinity for copper.

During production of unmodified 4RepCT^{3Aha} fibres we observed that, under conditions identical to those previously described, they appear within the same incubation time and share very similar appearance lengths and tensile strengths (17.5 MPa for the unmodified silk cf 17.9 MPa for the silk modified with FAM from an average of 3 measurements for each of the two silk types).[5,6,11] This suggests that critical interactions between C-terminal domains upon dimerization are unaffected by the incorporation of L-Aha and moreover the methionine residues it replaces are not crucial to the self-assembly processes.

In addition, we have engineered a multiple proteolytic cleavage site allowing macroscopic fibres to be formed upon incubation with thrombin, Lys-C or Enterokinase. Consequently this expands the variety of functional peptides with which 4RepCT^{3Aha} could be modified without fear of enzymatic digestion of the ligand during fibre formation. This provides a significant benefit for further functional applications over single enzyme methods.

Unlike previous studies from the Kaplan group that soaked silk worm silk in antibiotic solutions in order to load them with the drug through non-covalent adsorption or from the Tamura group who have genetically introduced fluorescent proteins into the silk sequence to give fluorescent fibres, we have generated fluorescent and antimicrobial silk through the successful conjugation of 4RepCT^{3Aha} with fluorophores and levofloxacin via a linker.[32,33] Considerable antimicrobial activity was observed when 4RepCT^{3Aha} was functionalised with levofloxacin, demonstrating that bioactive molecules can be temporarily conjugated to fibres. Additionally, levofloxacin functionalised fibres displayed a sustained release of antibiotic over at least 5 days, preventing the re-colonisation of the initial zone of inhibition. This observation indicates that the inhibition zone was not simply caused by 'burst release' of adsorbed levofloxacin (also displayed by the levofloxacin soaked control fibre), but the slower, successful breakdown of the covalent glycerol-ester linker over time. The glycerol-ester linker has been reported by Quan *et al.* to release a drug from a synthetic polymer such that that 34.3% of the drug was released at pH 1.2, 21.5% at pH 5.4 and 19.3% at pH 7.4 after 7 days[34] which is consistent with our observations. This valuable characteristic could be employed in wound treatment, where an initial pulse of antibiotic followed by sustained release over a period of days from a dressing would provide enduring protection. Functionalisation with fluorophores served as a highly sensitive detection method for the presence of L-Aha residues. Retention of fluorescence due to non-specific interactions between the fluorophore and silk fibre was not apparent as evidenced by the rapid loss of fluorophore from the control silks during washing. Moreover labelling soluble 4RepCT^{3Aha} with bulky fluorophores did not prevent fibre formation, meaning further processing steps to obtain fibres, such as artificial spinning, are not required. Furthermore differentially labelled soluble Thioredoxin-4RepCT^{3Aha} could be mixed in a desired ratio and then proteolytically cleaved to form fibres without detriment to the self-assembly process. This observation demonstrated the potential to generate multifunctional

fibres which would have considerable impact in applications such as wound dressings; where synergistic combinations of antibacterial and antifungal agents could be incorporated into a single dressing, or in regenerative medicine, where delivery of multiple different growth factors is often desired. The small number of previous attempts to create multi-functionalised silk materials have focused on genetically engineering functional peptides or domains into silk proteins. Examples of this include the creation of a potential gene delivery system by the addition of a poly L-lysine tract and RGD motifs to a silk-like protein and another 4RepCT construct where functional molecules can be presented via IgG molecules bound to engineered binding domain.[15,35] However the poly L-lysine and RGD functionalised silk can only be used for one application – gene delivery. Conversely, the other study harnessed the potential of IgG to bind an almost unlimited repertoire of ligands to create a highly versatile and multi-purpose silk material. Our protocol shares the same outcome but does not suffer the disadvantages of this method such as; the need to raise anti-ligand antibodies, the complication of releasing bound drug molecules and significant alteration of the silk protein primary sequence, bringing biocompatibility into question.

In conclusion, our approach allows the rapid generation of biocompatible, mono or multi-functionalised silk structures for use in a wide range of applications, being particularly useful in the fields of tissue engineering and biomedicine as biomaterials for cell matrices and scaffolds, and as advanced wound dressings. This approach to functionalisation can be extended to any other silks or protein-based materials that can be expressed using a methionine auxotroph.

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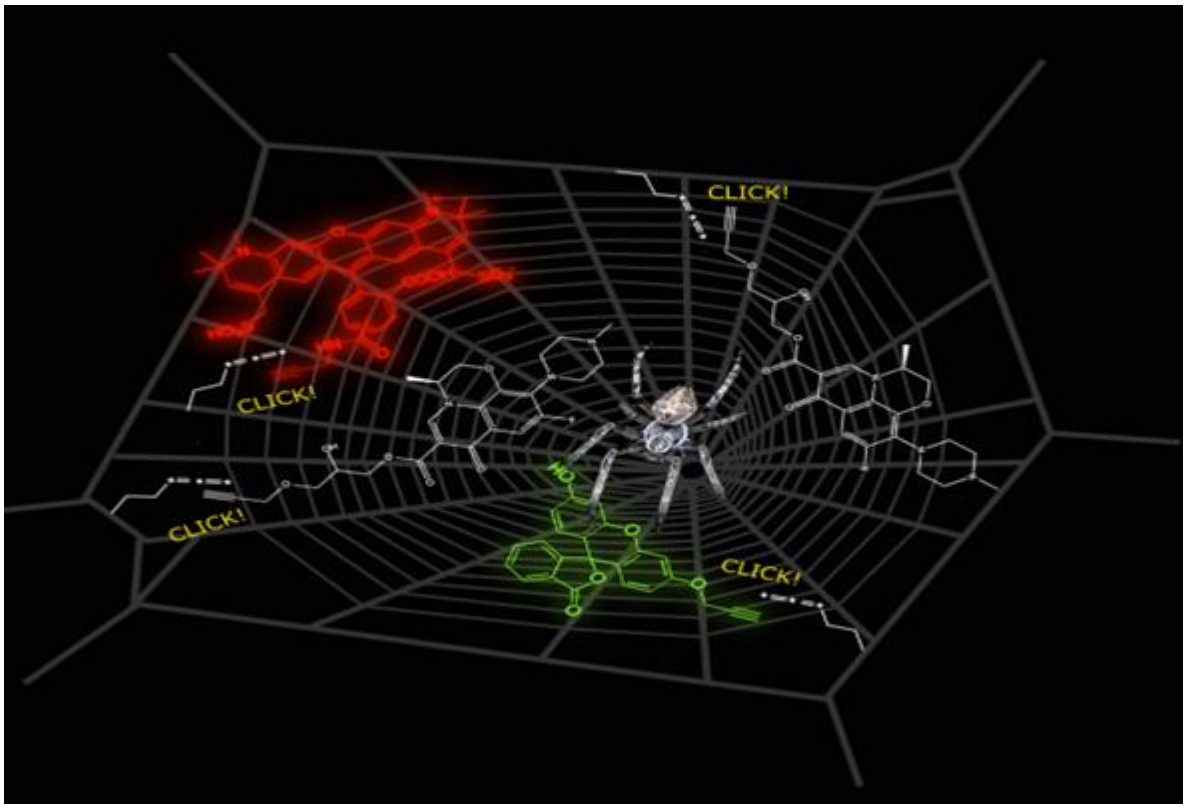

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In a new, versatile approach to functionalising recombinant spider silk, L-azidohomoalanine has been introduced site-specifically in the minispidroin protein 4RepCT through expression in an *E. coli* methionine auxotroph. Both fluorophores and the antibiotic levofloxavcin have been attached to this bio-orthogonal amino acid using copper-catalysed click chemistry, either before or after the silk fibres have self-assembled. The levofloxacin decorated silk fibres retain anti-bacterial activity for at least 5 days.