

Functionalisation of spider silk protein 4RepCT using un-natural amino acid mutagenesis and click chemistry

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Abstract

Interest in spider silk as a biomaterial has recently increased owing to its strength, biocompatibility, biodegradability, lack of immunogenicity and pyrogenicity. Unfortunately large scale production from spiders is highly impractical, as their cannibalistic nature prevents them from being farmed. As a consequence of this, scalable production of recombinant spider silk sequences in *E.coli* has emerged. There is a demand for functionalised silk materials tailored for specific novel applications. Previously this has been achieved through genetic fusion, which can prove difficult when using highly GC rich silk sequences, or classical chemical conjugation methods that are non-residue or non-site specific. The aim of the research described in this thesis was to produce a chemically modifiable self-assembling spider silk protein through the incorporation of a non-canonical amino acid into a miniature spidroin construct (4RepCT).

To achieve this, a methionine auxotrophic strain of *E.coli* has been used to incorporate a methionine analogue (L-azidohomoalanine (L-Aha)) in place of all three methionine residues in the mature 4RepCT sequence to give 4RepCT^{3Aha}. I have demonstrated that this did not adversely affect the normal self-assembly process of the protein into fibres. Subsequently the silk protein was functionalised using either copper-catalysed azide-alkyne cycloaddition (CuAAC) or strain-promoted azide-alkyne cycloaddition (SPAAC) in so called click chemistry reactions for a number of purposes. Firstly, the presence of

L-Aha residues was verified by the successful conjugation of alkyne modified fluorophores to both soluble spidroin protein and pre-made fibres. Following this proof of principle 4RepCT^{3Aha} fibres were then functionalised with the broad spectrum antibiotic levofloxacin endowing the fibres with antimicrobial activity which has been demonstrated against *E.coli* NCTC 12242.

Another potential area of application for spider silk is as a cell scaffold for use in wound healing and tissue engineering.

4RepCT^{3Aha} has been modified through conjugation of alkyne modified cyclic peptides bearing an RGD motif to improve its capacity to support mesenchymal stem cells. This was demonstrated to be more effective in supporting growth of the cells compared to routinely used tissue culture plastics. The successful modifications of 4RepCT^{3Aha} reported in this thesis highlight the versatility of the residue-specific click chemistry modification approach, generating new bespoke silk-based materials relevant in a wide range of applications.

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Abbreviations

Three and one letter codes for the twenty proteinogenic amino

acids

L-Alanine	Ala	A
L-Arginine	Arg	R
L-Asparagine	Asn	N
L-Aspartic acid	Asp	D
L-Cysteine	Cys	C
L-Glutamic acid	Glu	E
L-Glutamine	Gln	Q
L-Glycine	Gly	G
L-Histidine	His	H
L-Isoleucine	Ile	I
L-Leucine	Leu	L
L-Lysine	Lys	K
L-Methionine	Met	M
L-Phenylalanine	Phe	F
L-Proline	Pro	P
L-Serine	Ser	S
L-Threonine	Thr	T
L-Tryptophan	Trp	W
L-Tyrosine	Tyr	Y
L-Valine	Val	V

Unnatural amino acid three letter codes

L-azidohomalanine	Aha
L-homopropargylglycine	Hpg
L-homoallylglycine	Hag

Bases

Adenine	A
Cytosine	C
Guanine	G
Thymine	T

Silk worm species

Bombyx mori *B. mori*

Spider species

<i>Araneus diadematus</i>	<i>A. diadematus</i>
<i>Euprostenops australis</i>	<i>E. australis</i>
<i>Nephila clavipes</i>	<i>N. clavipes</i>
<i>Tegenaria domestica</i>	<i>T. domestica</i>

Yeast

Pichia pastoris *P. pastoris*

Bacteria

<i>Bacillus subtilis</i>	<i>B. subtilis</i>
<i>Escherichia coli</i>	<i>E. coli</i>

Staphylococcus aureus *S. aureus*

Spider silk proteins

<i>Araneus diadematus</i> fibroin	ADF/eADF
Major ampullate spidroin 1	MaSp1
Major ampullate spidroin 2	MaSp2

Growth factors

Basic fibroblast growth factor	bFGF
Bone morphogenic protein	BMP
Epidermal growth factor	EDF
Fibroblast growth factor	FGF
Insulin-like growth factor	IGF
Platelet – derived growth factor	PDGF
Transforming growth factor beta	TGF- β

Enzymes

Aminoacyl tRNA synthetases	aaRSs
Matrix metalloprotease 2	MMP-2
Matrix metalloprotease 9	MMP-9
Tissue type plasminogen activator	TPA

Other biological molecules

Deoxyribonucleic acid	DNA
Green fluorescent protein	GFP
Human neutrophil defensins	HNP
Messenger ribonucleic acid	mRNA
Lipopolysaccharide	LPS
Transfer ribonucleic acid	tRNA

Cell types

Baby hamster kidney cells	BHK
Commercially immortalised mesenchymal stem cells	CiMSCs
Human mesenchymal stem cells	h/MSC
Immortalised mammary alveolar cells with large T-antigen	MAC-T
Mesenchymal stem cells transfected with GFP	MSC GFP
Neuronal stem cells	NSC
Schwan cells	SC
<i>Spodoptera frugiperda</i> insect cells	Sf9

Analytical techniques

Atomic force microscopy	AFM
Energy-dispersive X-ray spectroscopy	EDX
Enzyme-linked immunosorbent assay	ELISA
Electrospray ionisation time of flight	ESI-TOF
High performance liquid chromatography	HPLC
Matrix assisted laser desorption ionisation	MALDI
Nuclear magnetic resonance	NMR
Scanning electron microscopy	SEM
Sodium dodecyl sulfate polyacrylamide gel electrophoresis	SDS PAGE
Transmission electron microscopy	TEM

Synthetic polymers

Polyethylene glycol	PEG
Polylactic acid	PLA
Poly lactic-co-glycolic acid	PLGA
Polyoxazoline	Pox

Click chemistry and associated reagents

Bicyclononyne	BCN
Copper catalysed azide alkyne cycloaddition	CuAAC
Dibenzocyclooctynol	DIBO
Difluorinated cyclooctyne	DIFO
(3'-hydroxy-6'-(2-propyn-1-yloxy)-3H-spiro [2-benzofuran-1,9'-xanthen]-3-one))	FAM
Strain promoted azide alkyne cycloaddition	SPAAC
(3[tris(3-hydroxypropyltriazolylmethyl)amine])	THPTA

Other

Basement membrane extract	BME
C terminal	CT
Dulbecco's modified eagle medium	DMEM
1-ethyl-3-(3-dimethylaminopropyl)carbodiimide	EDC
Ethylenediaminetetraacetic acid	EDTA
Extra cellular matrix	ECM
Fluorescein isothiocyanate	FITC
Food and drug administration	FDA
Hexafluoroisopropanol	HFIP
Hospital acquired infection	HAI
Isopropyl β -D-1-thiogalactopyranoside	IPTG
Luria-Bertani	LB
Methanol	MeOH
N-hydroxysuccinimide	NHS
Nickel immobilized metal affinity chromatography	NiIMAC
Non-tissue culture plastic	NTCP
Numerical aperture	NA
Optical density	OD
Phosphate buffered saline	PBS
Tissue culture plastic	TCP
Total internal reflection	TIRF

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1. General Introduction

1.1 A brief history of silk

Silk is a natural proteinaceous material that is produced and typically spun into fibres by many arthropods including mites, scorpions and flies¹. However the most prolific of silk-producing organisms are spiders and the Lepidoptera larvae or silk worms (*Bombyx mori*)²⁻⁴.

Domestication and high density farming of *B. mori* permitted the production of large quantities of silk, leading to the establishment of the silk textile trade^{5,6}. Conversely, spiders cannot be farmed like silk worms since most, but not all, are highly territorial and sometimes cannibalistic⁷. This behaviour dictates that a spider 'farm' would require more space per spider than is required for silk worms, raising practical difficulties and cost. Furthermore, silk worms generally have a greater yields than spiders and the harvesting process is much simpler since the worms do not need to be immobilised and their silk 'reeled' from them⁸. Consequently spider silk has rarely been used in the textile industry, but has instead been used for alternative applications such as the fabrication of fishing nets and wound dressing as far back as ancient Greek times^{6,9-11}. These applications reflect the impressive strength and toughness of spider silk as well as the potential for aiding in healing. This combination of physical and biochemical attributes are un-matched by *B. mori* silk and many other natural materials. It was this fact that continued man's use of spider silk.

1.2 Types of spider silk

Spiders produce silk in glands located within the abdomen. The orb weaving spiders (*Nephila clavipes* and *Araneus diadematus*) have seven glands and can therefore produce seven different types of silk (Figure 1.1)¹². Each of the different types of silk are used for specific tasks including prey capture, structural supports, attachment (glue) of structures to surfaces and protection of offspring. The strongest of these silk types, dragline silk, originates from the major ampullate gland and is used to form the non-sticky 'frame' structures within the orb web and as a life line when evading predators¹³. Dragline silks have very impressive physical characteristics and are often heralded as the strongest natural material known to man, only recently been outcompeted by limpet teeth¹⁴. On average a dragline has a tensile strength of around 0.8-1.6 GPa outperforming steel on a weight for weight basis, and a toughness of 95-520, outperforming Kevlar®-man's toughest material^{15,16}. For these reasons dragline silk has been the focus of many groups within the field of silk research. Efforts have been spent on finding out how exactly the spider is able to produce dragline silk at such a prolific amount with relative ease, how the anatomy of the spider plays a role in silk production and the protein structure and biochemistry of the dragline fibres.

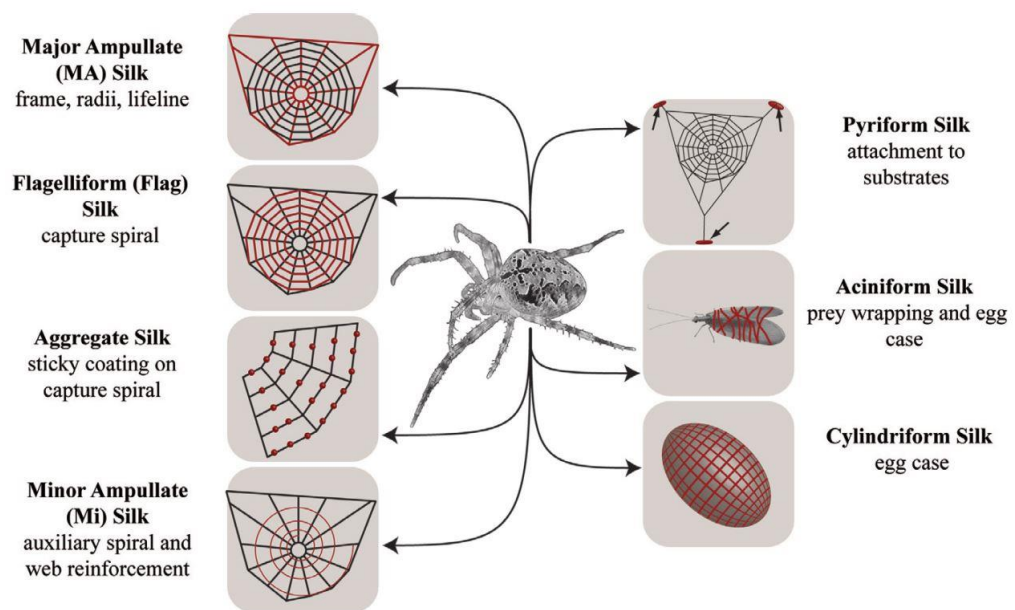


Figure 1.1: A representation of the seven different types of silk orb weavers can produce.

Each silk type comes from its own gland and they each have different physical properties making them appropriate for specific applications¹⁷. Differences in physical properties are thought to arise from differences within the primary sequence of the silk proteins. However very few studies focus on silk types other than dragline silk (major ampullate); therefore not enough evidence exists to substantiate this hypothesis and so this remains an area of uncertainty.

1.3 How the spider produces dragline silk

As previously mentioned, silk proteins are produced within glands located in the abdomen of the spider. The major ampullate gland can be divided into three distinct areas; the tail, the sac and the duct (Figure 1.2)¹⁸. Silk protein is secreted into the lumen of the gland as 'protein dope' which is a highly concentrated protein solution. This process occurs in the tail and the sac area of the gland, however the relative rates of production between the two areas suggest the tail is the main source of protein dope; with a rate four times greater than the sac¹⁹.

Therefore it appears that the main function of the sac is to store protein dope, given its size, large volume and the fact that protein dope is found in high concentration there (30-50% w/w)^{20,21}.

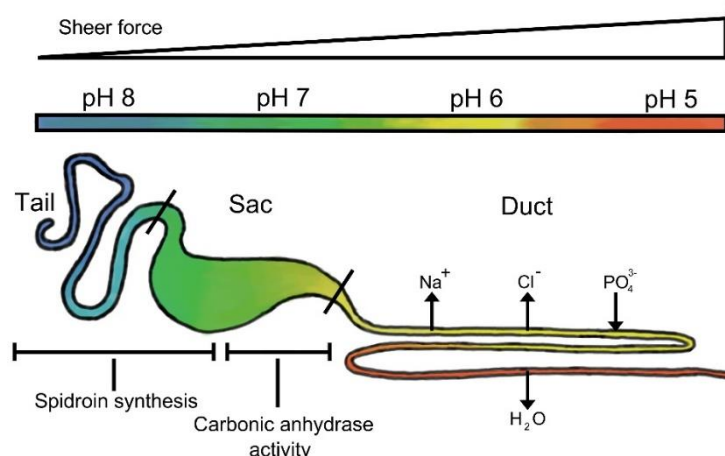


Figure 1.2: A schematic of the major ampullate gland.

Spidroins are secreted into the lumen of the tail and sac area. Ion concentration, pH and water content are changed with progression down the duct as well as increasing shear force.

The duct connects the sac to the spigot and spinnerets (where the solid silk fibre emerges from) and progressively narrows with its three 'limbs' folded into an S-shape¹⁸. It is within this structure, specifically the third limb, that the aqueous protein solution is converted into a water insoluble fibre²². This process is not fully understood, particularly how the process is controlled in order to prevent premature fibre formation or protein aggregation. However it is known that shear force (arising from the flow of silk dope against the duct wall that push the silk dope in opposite directions Figure 1.3) generated by narrowing of the duct, changes in pH, changes in ion concentration and more recently carbonic anhydrase activity which maintains the pH gradient and

increases the intraluminal partial pressure of CO₂ all contribute to the controlled formation of solid, water insoluble fibres^{16,22–25}.

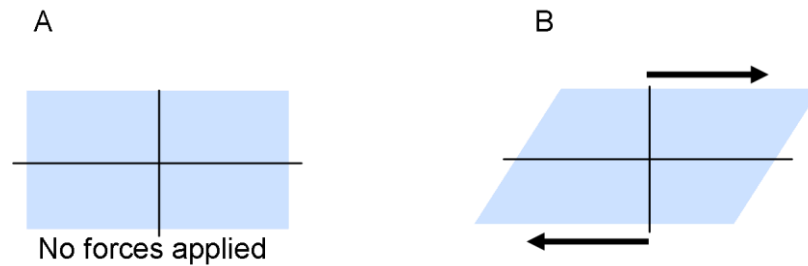


Figure 1.3 A schematic explaining shear force

The force at the top pushes in the opposite direction to the force at the bottom causing deformation through shear force.

1.4 Dragline silk proteins

Dragline fibres are not composed of just one protein, but instead are made up of two polypeptide building blocks known as spidroins; major ampullate spidroin 1 (MaSp1) and major ampullate spidroin 2 (MaSp2) or the MaSp-like polypeptides from *Araneus diadematus* fibroin (ADF-3) and ADF-4^{26–28}. Spidroins are large proteins ranging from 3000-4000 amino acids with reported masses of 200-720 kDa^{26,29,30}. In addition, dragline spidroins have an unusually high alanine and glycine content, with over 50% of the amino acid composition attributed to these residues. MaSp1 and 2 have similar primary sequences and both can be divided into clear domains; a repetitive domain comprised of poly-alanine blocks interspersed with glycine rich tracts, a non-repetitive N terminal domain and a non-repetitive C terminal domain (Figure 1.4)

^{26,27,31}. The differences in primary structure between MaSp1 and 2 are found in the glycine rich regions of the repetitive domain, with specific motifs belonging to each MaSp. For MaSp1 the glycine motifs are GA (glycine alanine) and GGX (where X can be Y, L or Q) whereas in MaSp2 the motifs feature P residues giving GPGXX (where X can be G, Y or Q). It is this motif that contributes to the higher proline content observed for MaSp2 spidroins^{32,33}

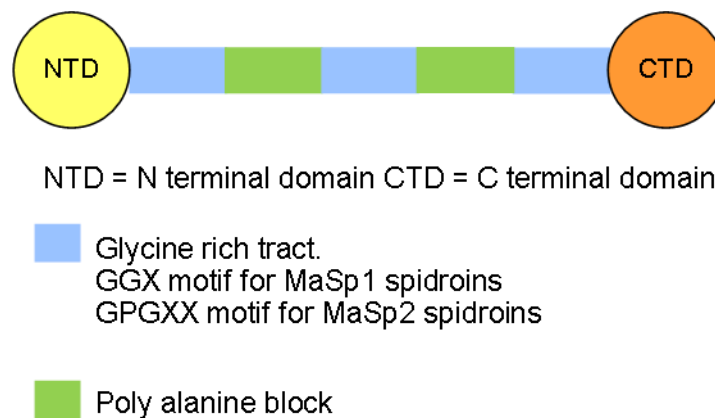


Figure 1.4 : Schematic representation of domains of MaSp1 and 2

The non-repetitive N and C terminal domains are shown in circles. The repetitive domain is shown between the terminal domains, differences between MaSp1 and MaSp2 glycine rich tracts are shown in the legend.

Owing to the similarities in primary structure of the repetitive domain, both spidroins have similar secondary structures. Upon polymerisation into a solid fibre the poly alanine blocks form crystalline anti-parallel β sheets whereas the glycine rich tracts form more amorphous coil structures^{34–37}.

The non-repetitive and highly conserved C terminal domain consists of five α helices and contains a single cysteine residue (Figure 1.5)^{38,39}.

This residue is responsible for the permanent parallel dimerization of C

terminal domains via a disulphide bond, 'clamping' two spidroins together.

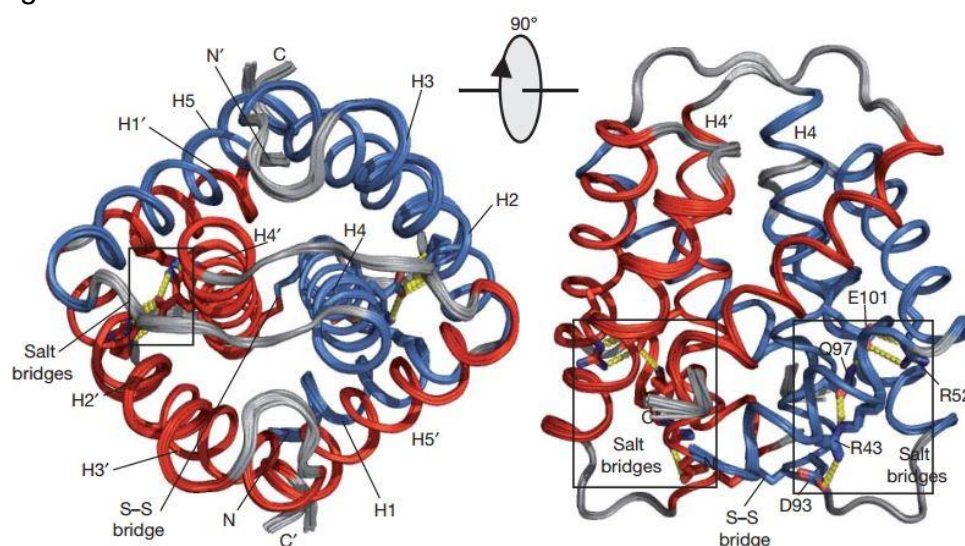


Figure 1.5: The non-repetitive C terminal domain

Two C terminal domain fragments (one red, one blue) of 123 residues each (with mutations V34A, V40A, K67N and H68Q) dimerize at pH 6.0 forming a parallel five helix bundle (protein database number 2KHM). Salt bridges in helices 1 and 2 at one side of helix 4 (shown in black boxes). Disulphide bridges are formed between helix 4 of each C terminal domain ³⁹.

Upon dimerization hydrophobic residues are buried at the interface between the folded C terminal domains whereas the more polar or hydrophilic residues are located on the solvent exposed surface of the dimer, presumably aiding with solubility. Interestingly it is the correct dimerization of C terminal domains which is critical to fibre formation more so than the formation of the disulphide bridge. Exemplified by mutagenesis studies where the single cysteine was mutated to serine or arginine^{40,41}. Here the mutation to the cysteine isostere, serine, did not affect fibre formation. However mutation to arginine which greatly altered the domain surface properties arrested fibre formation.

Indicating that perturbations in the highly conserved hydrophobic loop

(where the single cysteine is situated) affect C terminal dimerization and therefore fibre formation⁴⁰. The C terminal domain has also been implicated in the recruitment of accessory proteins to coordinate folding and keeping the protein soluble before extrusion^{38,42}.

The N terminal domain, like the C terminal domain, consists of five α helices and dimerises with another N terminal domain (Figure 1.6)^{43–46}.

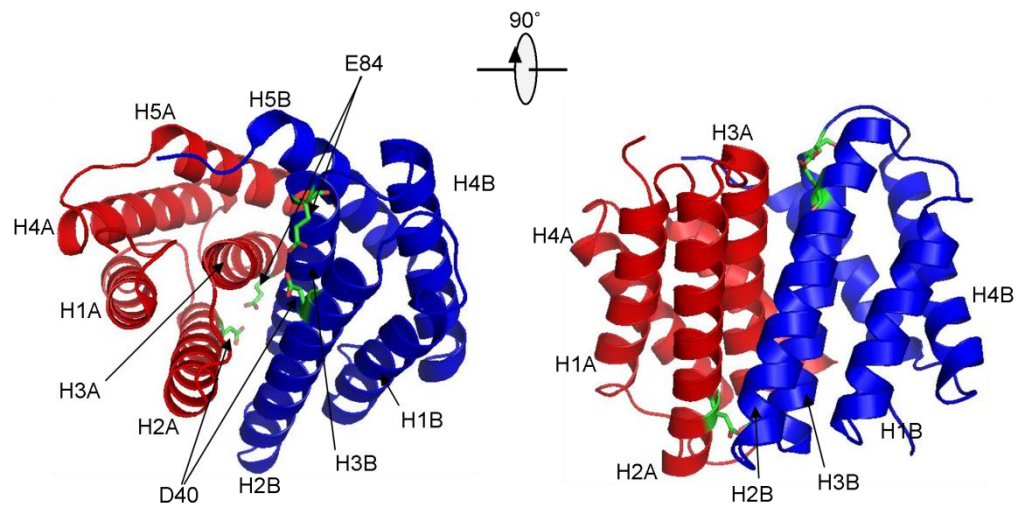


Figure 1.6: The non-repetitive N terminal domain

Two N terminal domain fragments (one red, one blue) of 137 residues each dimerize at pH 6.3 forming an antiparallel five helix bundle (protein database number 3LR2). Key acidic residues D40 and E84 are shown as green sticks.

However it is not critical for the formation of fibres. Instead the N terminal domain seems to be a pH sensitive switch that is primarily responsible for the storage and prevention of aggregation of the spidroins in the sac⁴⁶. Only when the spidroin has migrated further down the gland into the S bend of the duct and the pH has become more acidic (<6.4) does the N terminal promote fibre formation via dimerization with another N terminal domain. Unlike the C terminal

domain, dimerization is not locked into place with a covalent bond and forms a tight antiparallel dimer. However N terminal dimerization does depend on key acidic residues aspartate 40 and glutamate 84. Mutation of these residues to their corresponding amides (D40N and E84Q) prevented the successful dimerization of the domains⁴³.

1.5 Spidroin distribution and dragline fibre structure

The distribution and abundance of spidroins in a dragline fibre is not even. MaSp1 is more abundant and evenly spread whereas MaSp2 tends to be localised in certain areas of the fibre^{26,27,47}. How MaSp1 and 2 become separated from a seemingly evenly distributed mixture in the dope is still unknown. The macroscopic arrangement of dragline silk has seen several contradictory theories arising from different experimental techniques^{48–51}. The general arrangement of a dragline fibre is a core composed of spidroins that is covered by a thinner layer of protein or 'skin'. However it is the number of layers that has led to conflicting results. Early light microscopy observations described the core-skin arrangement, which was then later modified by atomic force microscopy (AFM) analysis; suggesting that the core had two fibrillar layers which is surrounded by a thin skin^{49,51}. These findings were corroborated with findings taken from ¹³C nuclear magnetic resonance (NMR), scanning electron microscopy (SEM) and confocal microscopy^{48,50}. Interestingly the same skin-core arrangement was not observed in one study which used transmission electron microscopy (TEM) and light microscopy⁵². However further TEM studies on dragline

silk bound with specific antibodies for MaSp1 and 2 led to the proposal of a three layered skin-core model. Here the spidroins are located in the core, surrounded by a skin of unknown protein which in turn is surrounded by a layer of glycoproteins; identified by concavilin A conjugated gold particles^{48,53}.

1.6 Recombinant dragline silk production

As mentioned previously, obtaining dragline silk from ‘farmed’ spiders is fraught with problems meaning that alternative means of production have been used. Consequently silk research has turned to the recombinant production of silk proteins in a variety of host systems including yeast, insect cells, mammalian cells, tobacco plants, potato plants, transgenic goats and bacteria in order to solve this issue (summarised in Table 1.1)^{54–60}. Each host has distinct advantages and disadvantages relating to protein expression in general (summarised in Table 1.2). However only partial spidroin sequences have been successfully expressed because entire MaSp gene sequences have not yet been cloned. Furthermore, many problems have arisen with the expression of the various spidroin sequences. The genes encoding dragline spidroins have a high guanine (G) cytosine (C) content; reflected by the unusually high alanine and glycine content in the spidroins themselves⁵⁸. This results in mRNA transcripts that possess extensive secondary structures which have been postulated to lead to translational pauses or the complete dissociation of the ribosomal unit causing truncation of the protein⁵⁸. Even if the problematic secondary

structure of the mRNA is overcome, truncations can also occur through the exhaustion of alanine and glycine tRNA pools (spiders have larger tRNA pools to facilitate sustained expression)⁶¹. Low protein solubility is a major issue and requires the use of strong denaturing agents such as 8 M urea and 6 M guanidine hydrochloride to denature and solubilise proteins^{58,62,63}.

Naturally the problems described here are intensified when attempting to express long spidroin sequences. Therefore attention has been focused on the recombinant production of 'miniature spidroins' which have, in general, smaller repetitive domains and either none, one or two non-repetitive domains^{64–66}. These greatly smaller silk proteins represent the minimum sequence length that, in most cases, can still be generated into a fibre via mechanical spinning or extrusion into a coagulation bath or spontaneous self-assembly following cleavage of a solubility enhancer protein. The latter technique has demonstrated the production of long (up to 1 metre) dragline silk fibres under physiological conditions and gentle physical agitation⁶⁵. Using this technique to form fibres has some advantages over other methods that rely on spinning or extrusion. Since there is no need for spinning/extrusion apparatus, lengthy optimisation processes are avoided as is the use of organic solvents, acids or alcohol solutions to produce the fibre.

1.6.1 Production in yeast *Pichia pastoris* (*P. pastoris*)

The expression and purification of dragline spidroins derived from *N. clavipes* and *E. australis* have been carried out in *P. pastoris*. Using this host, spidroins of up to 3000 residues in length have been expressed with no evidence of truncations caused by translational pauses or terminations⁵⁷. Additionally the inserted spidroin genes demonstrated good stability, undergoing no alterations for at least 100 doublings. However levels of expression declined with increasing gene length, presumably because of the reasons discussed above. Despite this, impressive yields of shorter spidroin sequences (1g/L) were expressed and purified, however the use of guanidine hydrochloride was required to solubilise the protein. Moreover no attempts to make fibres or other silk architectures were made. Other dragline spidroins (of undefined size) expressed in yeast were also insoluble and necessitated the use of 10% (v/v) saturated lithium chloride 90% (v/v) concentrated formic acid solution to solubilise the protein; however the yield of spidroin attained in these studies was not stated^{67,68}. Even so, microporous 3D scaffold plugs were fabricated from the spidroin protein using salt leaching and a drying process.

In contrast to these studies, soluble spidroin protein has recently been expressed in yeast⁶⁹. Factors such as temperature, pH and expression time were reported to influence the amount of secreted soluble protein found in the extracellular fraction. Unlike the homogeneity of purified spidroins observed in Fahnstock and Bedzyk's 1997 study, full length

proteins and degraded proteins were obtained. Full length protein was able to form fibres following enzymatic removal of a solubility enhancer, whereas degraded protein was not. Analysis of these protein species revealed that the C terminal domain was post-translationally glycosylated and, in the case of degraded protein, enzymatically cleaved.

1.6.2 Production in Insect Cells

Miniature MaSp1 spidroins based on sequences from *Araneus diadematus* (*A. diadematus*) ADF-3, rADF-4 and ADF-4 have been expressed using insect cell line Sf9 (derived from the fall armyworm *Spodoptera frugiperda*)^{10,40,54}. Interestingly, each spidroin behaved differently when expressed, even though their primary sequences are similar. ADF-3 was consistently expressed as a soluble protein, although yields were not given, whereas rADF-4 and ADF-4 were found as insoluble aggregates and fibres in the cytosol of the cells. The contrast in behaviour was blamed on the difference in hydropathicity between ADF-3 and 4. The insoluble structures were purified and analysed; revealing fibre filaments with diameters of 200 nm – 1 µm and lengths of up to 100 µm. The small dimensions of the fibres were attributed to the size constraints of each insect cell, meaning that fibre size may not be able to be improved upon.

1.6.3 Production in mammalian cells

The expression of ADF-3 and MaSp1 and 2 fragments from *N. clavipes* in immortalised mammary alveolar cells with large-T antigen (MAC-T) and baby hamster kidney cells (BHK) was investigated. Both cell types were shown to successfully express and secrete the 60 kDa ADF-3 spidroin with yields of 25-50 mg/L. However larger spidroins (110 and 140 kDa) did not express as well as the 60 kDa ADF-3 monomer. More interestingly pellets of the 60 kDa ADF-3 were easily re-dissolved in phosphate buffered saline (PBS); suggesting greater solubility than previously expressed silk proteins. Furthermore macroscopic fibres of 8-23 μm in diameter were formed by spinning ADF-3 protein dope of concentration 10-28% w/w into a coagulation bath⁵⁵.

1.6.4 Production in plants

Potato (*Solanum tuberosum*) and tobacco (*Nicotiana tabacum*) plants have been used to express partial MaSp1 spidroins (up to 100 kDa) taken from *N. clavipes*^{59,70}. Here the spider silk gene was cloned into a cauliflower mosaic virus controlled expression vector, resulting in ubiquitous expression of the silk gene. Further to this, a signal peptide was fused to its C terminus allowing the expressed spidroin to linger in the endoplasmic reticulum of the cell. It was reported that the silk protein accumulated well in the transgenic plants; with the best producer plants accumulating silk protein >2% (w/w) of all soluble

protein. Ammonium sulphate was ultimately used to purify and enrich the silk protein, however spidroin pellets were not re-dissolved in aqueous buffer. Interestingly buffer containing guanidine hydrochloride was required even though the protein was obtained from the soluble fraction of the leaf extracts⁵⁹.

1.6.5 Production in *Escherichia coli* (*E. coli*)

Standard expression strains of *E. coli* have been previously shown to be capable of expressing spidroins up to 163 kDa in size⁵⁸. However as seen for other expression hosts large spidroins are not able to be consistently expressed to their full length. These observations were attributed to instability of spidroin genes and translational pauses or terminations caused by secondary structure elements of mRNA, as reported by others⁶². Despite this, in a more recent study a spidroin of comparable size to natural spidroins, 284.9 kDa, was expressed⁷¹. Importantly the *E. coli* were metabolically engineered to simultaneously over express glycine tRNAs enabling the bacterium to cope with the elevated demand for glycine residues. By doing this, high density fermentation of the transformed *E. coli* permitted silk protein yields of 0.5 – 2.7 g/L, however denaturants were once again required to solubilise the spidroin during cell lysis and after ammonium sulphate purification. Fibres were created by wet spinning spidroin (dissolved in hexafluoro-2-propanol (HFIP)) into a coagulation bath of 90% (v/v) of pure methanol 10% (v/v) water.

In light of the difficulties associated with the expression of large spidroins, the most recent studies have focused on the expression of miniaturised spidroins.

The Johansson group expressed a soluble miniature dragline spidroin (4RepCT) derived from the nursery web spider *Euprosthenops australis* (*E. australis*)⁶⁵. This 39 kDa (inclusive of his and thioredoxin tags) miniature spidroin was N terminally fused to a solubility enhancer, thioredoxin, and gave yields of 40 mg/L of *E. coli* grown in shake flasks. The spidroin was easily purified using Nickel immobilised affinity chromatography (NiIMAC) and was demonstrated to self-assemble into fibres of various lengths (up to 1 metre). They observed that in order to form fibres four poly alanine blocks and a C terminal domain (CT) are required. Constructs that only had the repetitive domain did not self-assemble into fibres upon enzymatic removal of the solubility enhancer; underpinning the importance of the C terminal domain in fibre formation^{38,39}. It was also demonstrated that other solid structures such as films, foams and meshes could be fabricated from 4RepCT⁷².

Other miniature dragline spidroins, eADF 3 and eADF4, have also been successfully expressed in *E. coli*^{39,73,74}. These proteins consist of 16 repeating poly alanine blocks and a C terminal domain. However unlike 4RepCT, eADF is not fused to a solubility enhancer and therefore requires the use of denaturing agents to solubilise the protein post expression. Macroscopic structures were generated by optimising the spinning apparatus, including microfluidic devices, to mimic the changes in ionic strength, pH and sheer force observed in the duct of

the Major Ampullate gland. Similarly to 4RepCT, other silk protein structures have been produced from eADF such as films. However microparticle and microsphere architectures of controllable size were also generated by exposing the protein to different salts and salt concentrations^{74–77}.

Organism	Spidroin type	Origin	Spidroin size	Amount expressed	Solubility	Comments	Silk structures	Reference
Yeast <i>P. pastoris</i>	Partial MaSp1 repeats	<i>N. clavipes</i>	<3000 residues	1g/L	Insoluble	~45% recovery of expressed protein	No attempts made	57
Yeast <i>P. pastoris</i>	Synthetic MaSp1	<i>N. clavipes</i>	Not stated	Not stated	Insoluble	Not much information given	3D porous disks	68
Yeast <i>P. pastoris</i>	Miniature MaSp1 repeat + C terminal	<i>E. australis</i>	32 kDa	Not stated	Soluble	Enzymatic degradation and post translational modifications evident	Fibres	69
Insect cell line Sf9	ADF-3 (MaSp2) ADF-4 (MaSp1)	<i>A. diadematus</i>	~60 kDa	Not stated	Soluble Insoluble	ADF-4 exclusively insoluble. Formed aggregates and small fibres in cytosol	Aggregates and fibres	54
Mammalian cells BHK & MAC-T	ADF-3 MaSp1 MaSp2	<i>A. diadematus</i> <i>N. clavipes</i> <i>N. clavipes</i>	60-140 kDa	25-50 mg/L (60 kDa protein)	Insoluble	60 kDa protein expressed well, larger proteins did not	ADF-3 Fibres	55
Tobacco & Potato plants	MaSp1 and 2	<i>N. clavipes</i>	60-100 kDa	0.01- 2 % of total soluble protein	Soluble	Protein pellets solubilised with guanidine hydrochloride	No attempts made	59, 70
Bacteria <i>E. coli</i>	MaSp1 repeats	<i>N. clavipes</i>	15-41 kDa	2-15 mg/l	Insoluble	Purification complicated by precipitation of protein	No attempts made	56
Bacteria <i>E. coli</i>	MaSp1 including C terminal MaSp1/2 repeat mix	<i>N. clavipes</i>	43 kDa 55 kDa	4 mg/ml Not stated	Insoluble	Use of denaturant required	Fibres	62, 63
Bacteria <i>E. coli</i>	ADF-3 and 4 repeats, C terminal domains and repeats + C terminal	<i>A. diadematus</i>	12-60 kDa	10-30 mg/L (140-360 mg/L in fermenter)	Insoluble	Use of denaturant required	No attempts made	73
Bacteria <i>E. coli</i>	MaSp1 and 2 repeats	<i>N. clavipes</i>	65-163 kDa	300 mg/L	Insoluble	Size heterogeneity of purified protein	Fibres	58
Bacteria <i>E. coli</i>	Synthetic MaSp1	<i>N. clavipes</i>	100.7-284.9 kDa	0.5-2.7 g/L	Insoluble	Use of <i>E. coli</i> with enhanced tRNA pools. Highly insoluble protein requiring 8M urea 2M thiourea	Fibres	71
Bacteria <i>E. coli</i>	MaSp1 repeat+ C terminal	<i>E. australis</i>	39 kDa	40 mg/L	Soluble	Stable and soluble protein. Easily purified	Fibres	65

Table 1.1: A summary of recombinant silk protein expression hosts.

Expression host	Advantages	Disadvantages
Bacteria	- Easy to handle and non pathogenic - Easy to manipulate genetically - Possibility to increase tRNA pools - Target proteins expressed from plasmids - Variety of ways to induce protein expression - Easily scalable; high density fermentation - No post translational modification	- Protein expression decreases with increasing spider length (but can be improved by increasing tRNA pools) - Target genes must be optimised for bacterial codon usage - Repetitive gene sequences may be unstable; product inhomogeneity. - Potential lipopolysaccharide contamination
Yeast	- Production of larger spider proteins with less chance of premature termination - Secretion of silk proteins - Easily scalable; fermentation	- Protein expression decreases with increasing spider length - Multiple gene insertions; product inhomogeneity - Secretion of proteases into media, reducing yield - Post translational modifications; unknown impact on fibre formation and biocompatibility
Plants	- Easy to handle and non pathogenic - Production of larger spider proteins - Easily scalable	- Much longer generation times - Genetic manipulation more complicated - Scale up requires more space for planting transgenic plants; space which could be used for food - Transgenic plants may impact other plant species through cross pollination - Potential mycotoxin contamination
Insect cells	- Production of larger spider proteins possible - Scale up by fermentation - Most closely resembles spiders - No translational pausing - Secretion of silk proteins	- Genetic manipulation more complicated - Longer generation times - Silk protein aggregation in cytoplasm of cells; protein insolubility
Mammalian cells	- Production of larger spider proteins possible - Secretion of silk proteins	- Reduced yield; tRNA pool exhaustion, translational pausing and termination - Inhomogeneous protein expression - Genetic manipulation more complicated - Susceptible to infection

Table 1.2: Advantages and disadvantages of specific expression hosts

1.7 Physical properties of naturally occurring and recombinant silk

1.7.1 Mechanical properties

Mechanical properties, such as tensile strength, of silk fibres are thought to be heavily influenced by the secondary structure and GGX, GPGX motifs in the repetitive domain of the spidroins⁴. Therefore explaining the differences in dragline tensile strengths not only between species but even between individual spiders⁷⁸. Currently it is thought that the crystalline β sheets formed in the alanine blocks allow spidroins to oligomerize and, because of the large number of hydrogen bonds, impart strength to the fibre. The less ordered glycine rich regions are theorised to confer elasticity via the formation of spring like structures and 3_1 helices^{32,79}. The combination of these two secondary structures contributes to the 'toughness' of dragline silks; a combination of tensile strength and extensibility that man-made materials cannot match. The high degree of toughness permits efficient energy dissipation a key function allowing the silk to perform its various tasks including prey capture⁸⁰⁻⁸². However, because recombinantly produced spidroins are, in general, much smaller than natural spidroins their mechanistic properties are diminished. Only one study thus far has produced a recombinant silk protein with comparable toughness to natural dragline silk⁸³. Table 1.3 summarises and compares the mechanistic properties of natural (native) and recombinant dragline silks.

Dragline	Tensile strength (GPa)	Extensibility (%)	Toughness (MJ/m ³)	Reference
Native dragline silks	0.8 – 1.6	15 – 50	96 – 520	4, 78, 84 - 87
Recombinant ADF spidroins	0.01- 0.308	6 - 110	0.3 - 189	83
Recombinant 4RepCT	0.08 – 0.2	Not stated	Not stated	41, 65
Other recombinant spidroins	0.5	Not stated	Not stated	71

Table 1.3: Mechanical properties of native dragline silk and recombinant dragline silk.

1.7.2 Supercontraction

Supercontraction is a phenomenon observed in silk materials and occurs when the silk fibre is immersed in water and alcohol solutions. Upon immersion, the initial length of the silk fibre can decrease by up to 50%; impacting on some of the aforementioned mechanical properties⁸. The purpose of supercontraction within the webs spiders weave is unclear. However it may be a molecular mechanism that prevents ‘sagging’ of webs that have become wet or have accumulated droplets of water during a rain shower. Since, within a web, fibres are normally attached to solid structures (branches for example) and therefore limited on how far they can contract. Consequently fibres that are trying to contract, but are constrained, would have increased tension thus removing any bowing caused. The structural changes within dragline spidroins during supercontraction have been investigated using several methods, including X-ray diffraction, Raman spectroscopy, NMR^{8,80,88,89}. Cumulatively the data indicates that upon wetting, the

glycine rich regions undergo hydrogen bonding disruption, triggering the crystalline β sheets to rotate.

1.8 Application of un- modified native and recombinant silk materials

Unmodified silk materials derived from native and recombinant silk protein have found several applications within life sciences such as; cell culture matrices, drug delivery vehicles and wound dressings. Recent studies demonstrated the ability to fabricate a range of silk material morphologies. Consequently this has expanded the potential applications in which both native and recombinant silk can be utilised.

1.8.1 Native silk

Dragline silk obtained from live spiders by means of 'reeling' have been demonstrated to be capable of supporting mammalian cells. Allmeling (2006) showed that dragline silk in combination with acellularised vein could support the growth of Schwann cells (SC)⁹⁰. Seeded SCs were observed to adhere and proliferate well, ultimately enveloping the silk fibre completely. Following this, the vein dragline silk construct was later shown to facilitate the regeneration of a 6 cm tibial nerve defect in sheep within 10 months and a 2 cm sciatic nerve defect in rats within 6 months^{91,92}. In another study a woven mesh of dragline silk supported the co-culture of fibroblasts and keratinocytes, emulating dermis and epidermis of the skin. Cells were observed to be confluent within one week and continued cell culture (35 days) developed a macroscopic cell

structure⁹³. These studies show promise for the use of silk structures in regenerative medicine and wound healing.

1.8.2 Recombinant silk proteins

Similarly recombinant spidroin proteins have also been investigated as potential cell culture and tissue scaffolds. In contrast to native spider silk, a range of silk structures or morphologies have been studied.

1.8.2.1 Recombinant silks in cell and tissue culture

Recombinant spidroin analogues 1F9 and rS1/9 were fabricated into highly porous 3D structures and both shown to support the growth of 3T3 fibroblasts under normal cell culture conditions^{67,68}. More interestingly, several different rS1/9 structures were further tested *in vivo*; subcutaneous implantation in BALB/c mice and inserted into bone defects in Wistar rats⁶⁷. The subcutaneous implants were analysed after 8 weeks and displayed adhesion to the surrounding tissues. In addition, histological analysis revealed in-growth of fibroblasts, extra cellular matrix (ECM) proteins, fat tissue, blood vessels (of various sizes) and nerve fibres. Biosorption of the silk scaffold was also noted and appeared to be cell (macrophage and multi nuclear giant cell) mediated. Bone implants performed similarly and after 4 weeks displayed in-growth of osteoblasts and osteoclasts. The influx of osteogenic cells resulted in the production of mature bone tissue within the pores of the scaffold. For both implant scenarios, it was reasoned that the overall positive charge and porous structure of the silk scaffolds

was responsible for greater cellular adhesion and slower cellular migration when compared to controls.

The miniature spidroins eADF and 4RepCT have been processed into a range of morphologies and have displayed differing performances as cell culture aids. In a 2012 study, eADF4 was processed into films, hydrogels and non-woven mesh scaffolds and were used in attempts to culture mouse 3T3 fibroblasts⁹⁴. It was observed that adhesion of fibroblasts to eADF4 hydrogels and films after four hours was very poor, <5% and ~25% respectively. Consequently the proliferation of cells over a 6 day period was greatly reduced in comparison to the control; with eADF4 films having a very marginal increase in proliferation at the 6 day time point over hydrogels. The inability of these structures to adhere cells was attributed to the lack of specific cellular adhesion domains typically observed in the ECM. Whereas high levels of hydration causing a thin layer of water to be present on the surface of hydrogel structures was thought to prevent adhesion, a phenomenon observed for other hydrogel materials⁹⁵. Interestingly the lack of cell adhesion has proved useful in the prevention of fibrotic capsule formation associated with manmade implants. eADF4 protein was used to coat silicon materials, providing a temporary shield between implant and host that reduced inflammation⁹⁶.

In contrast to films and hydrogels, fibroblasts adhered and proliferated well on eADF4 non-woven meshes. Here, just like the porous 3D scaffold discussed earlier, it was noted that cellular adhesion is mediated by the physical properties of the scaffold. The diameter of the

fibres within the non-woven mesh was shown to influence how well the fibroblasts adhered and consequently proliferated. The widest fibres (diameter of ~700 nm) yielded the greatest adhesion (~80%) after four hours. Additionally, because of the wider diameter, larger spaces between fibres contributed to the protrusion of cellular processes and better cytoskeletal arrangement.

Several morphologies of 4RepCT have been shown to support the growth of primary human fibroblasts⁷². In this study films, foams, fibres and fibre meshes (non-woven) were employed and shown to be, despite the lack of specific cellular binding domains, hospitable to the cells. 4RepCT films, fibres and meshes outperformed the positive control (tissue culture plastic), harbouring significantly more cells after 7 days continuous cell culture (high cell seeding density) and 9 days (low cell seeding density). Foams however displayed significantly more cells than the positive control from day 9 in the low cell seeding density condition, but was not significantly different to the control in the high density condition. Hybrid silk scaffolds comprising film and fibres were shown to harbour significantly more cells when compared to film and fibre alone as early as day 3 of continuous culture. In this case it is more than likely that the film and fibres work synergistically; the film prevents loss of cells because the hydrophobic plastic surface is better covered, and the fibres provide even more surface area for the cell to proliferate onto.

However the positive control was not outperformed when a different cell type (neural stem cells (NSC)) was cultured on 4RepCT films and

foams. In this study the positive control, poly-L-ornithine and fibronectin coated polystyrene well plates, was matched in terms of cell viability, self-renewal and differentiation potential⁹⁷. Demonstrating that 4RepCT is equally capable of supporting an arguably more sensitive cell type (in comparison to fibroblasts). Furthermore, 4RepCT was still able to produce this result without the specific cell binding domains and glycoprotein motifs which are present in fibronectin⁹⁸.

1.8.2.2 Recombinant silks for drug delivery

Drug delivery vehicles are another area of great interest because they increase target specificity, bioavailability and cellular uptake of therapeutics, whilst reducing toxicity (lower doses required)^{99,100}.

Morphologies of eADF including microparticles and microspheres have shown potential as drug delivery vehicles. Solid particles and microspheres were fabricated by the addition of potassium phosphate solution to solubilised eADF protein⁷⁴⁻⁷⁷. In addition the size of the particles and microspheres was shown to be influenced by the concentration of potassium phosphate used, eADF concentration and how intensely the two solutions were mixed.

Several drug molecules with different charges, *logP* values (a measure of hydrophobicity) and chemical nature (acid/base etc.) have been used to test the loading of and subsequent release from eADF microparticles⁷⁵. It was demonstrated that positively charged (at pH 7) drug molecules that are sufficiently hydrophobic gave the best loading efficiency (98.2%, ethacridine lactate 98.1% methyl violet (Figure 1.7))

whereas more hydrophilic uncharged compounds did not efficiently load (0.2%, acetaminophen 0.0% phenol red (Figure 1.7)) .

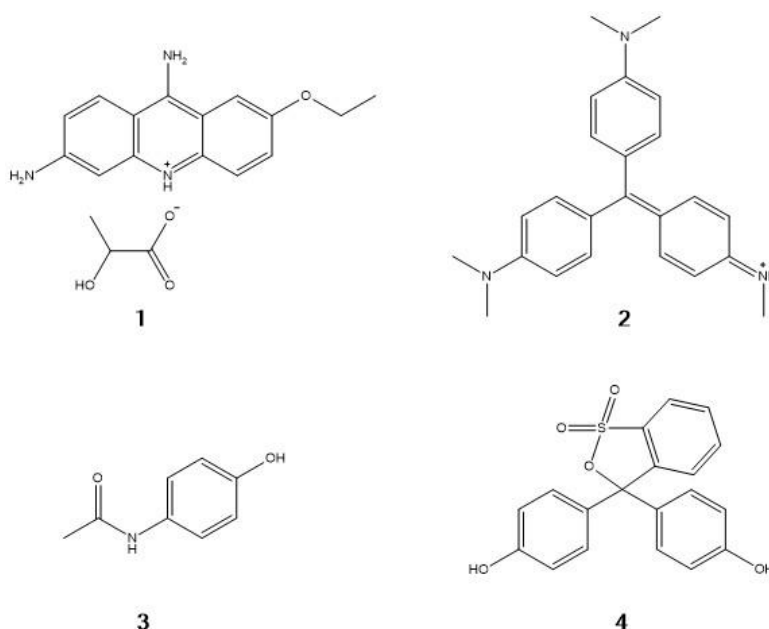


Figure 1.7: Compounds used to load eADF spider silk particles.

Ethacridine lactate (1) and methyl violet (2) are charged at pH 7, whereas acetaminophen (3) and phenol red (4) are uncharged.

This observation was attributed to the negative charge of eADF particles at pH7, consequently attracting positively charged compounds to the particle surface followed by diffusion into the hydrophobic core of the particle. Release of ethacridine lactate and methyl violet from eADF microparticles was studied *in vitro* and shown to occur over a period of 35 days. During this time frame 100% of methyl violet was released whereas ~80% of ethacridine lactate was released from the particles. However, the release of drugs from the microparticles is accelerated in the presence of enzymes such as trypsin and elastase which digested

the silk protein and in turn degraded the microparticles. Here, at the highest enzyme concentrations, maximum release rates were reached after just 3 days incubation leading to 60% release after just 7 days (12-15 days without enzymes). Whilst the ability of trypsin and elastase to degrade the microparticles is essential for their removal from a living host, the release of drugs from microparticles *in vivo* may be considerably accelerated.

1.9 Functionalisation of silk proteins and impact on application

There are two well-established methodologies for modifying or functionalising proteins; chemical conjugation of functional ligands to residue side chains or fusion of the protein with extrinsic functional domains.

1.9.1 Chemical functionalisation

Dragline spidroins, unlike typical globular proteins, are predominantly composed of unreactive alanine and glycine residues. Therefore leaving only a small number of reactive residues to be modified¹⁰¹. In solution, the largely unfolded state of glycine rich regions grants easier access to reactive residues, aiding chemical conjugation. Furthermore elevated thermal stability of spidroins (compared to proteins in general) permits increased reaction temperatures; potentially speeding up reaction times^{102,103}. The chemistry used to modify reactive residues is well defined, and a number of options exist depending on the target.

Carboxylate groups of glutamate residues are potential conjugation targets. These groups can be reacted with carbodiimides followed by *N*-hydroxysuccinimide (NHS), making them selectively reactive towards primary amine groups^{104,105}. Reaction of NHS with a primary amine, such as those on lysine side chains, forms a stable amide bond between protein and ligand. One study used this method to immobilise β -galactosidase enzyme on the surface of premade silk films¹⁰⁶. The enzyme remained hydrolytic activity after conjugation, demonstrated by the formation of blue precipitates upon incubation with 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal). Similar studies have only embedded enzymes within silk films, but have reported retention of enzymatic activity ten months later¹⁰⁷. These results describe the potential for silk materials to be used as biosensors; with application within medicine for the early detection of disease or in the detection of controlled substances for example^{108,109}.

The sulfhydryl groups of cysteine residues are another target for chemical modification. Ligands carrying a maleimide group can be reacted with sulfhydryl groups forming a thioether bond via a Michael addition reaction or bonds can be formed between sulfhydryls and metal particles^{104,105,110}. Maleimides have been used to conjugate cyclic arginine, glycine and aspartate (RGD) peptides, biotin and gold nanoparticles to premade silk films^{66,111}. RGD peptides are known to mediate cellular adhesion to the ECM via integrin proteins¹¹². These transmembrane proteins recognise and bind RGD motifs forming focal adhesion points through which the cytoskeleton connects to the ECM.

These connections not only anchor the cell to the matrix they also deliver signals to the cell. Consequently when silk films were conjugated with RGD peptides, the adhesion and subsequent proliferation of fibroblasts was greatly improved, compared to non-RGD films⁶⁶. The conjugation of biotin to silk films offers the opportunity for further functionalisation. Here the high affinity of streptavidin for biotin ($K_d = 10^{-15}$ M) could be exploited to 'bolt on' additional molecules and ligands as each streptavidin is a homotetramer with four biotin binding pockets. Lastly, gold nanoparticles present the potential of electrical conductivity. Maleimide bearing gold nanoparticles were conjugated to premade silk films and shown to be evenly distributed. Furthermore, once conjugated, the nanoparticles could be enlarged; suggesting that, if enlarged sufficiently, a continuous conducting layer of gold could be produced¹¹¹. Conducting silk materials could possibly be used as sensors as demonstrated in a separate study where electrically conducting silk fibres were used to sense methanol vapours¹¹³. However in this study the gold nanoparticles were not covalently linked to the silk protein. Instead strong interactions with amine groups were thought to bind the negatively charged gold nanoparticles.

1.9.2 Genetic modification

Genetic modification involves the fusion of functional peptides/ motifs/domains with the primary sequence of the silk protein. RGD motifs were once again employed to improve cellular adhesion and proliferation. Films made from RGD spider silk (ADF) fusion protein

performed similarly to chemically modified films, demonstrating both methods could be used⁶⁶. In another study, RGD containing silk films improved wound healing in rats by increasing hydroxyproline synthesis and upregulating expression of basic fibroblast growth factor (bFGF); factors thought to improve the healing process^{114–116}.

Bone regeneration is a key area of biomaterial research and attempts to facilitate this with silk scaffolds have been made. R5 peptide (derived from silafin) and dentin matrix protein were fused to dragline spidroin sequences, enabling the deposition of silica particles and hydroxyapatite mineralisation of the silk^{117,118}. Silica silk films supported the growth of human mesenchymal stem cells (hMSCs), reaching 85% confluency after 5 days incubation. Furthermore osteogenic gene markers were shown to be upregulated when grown on silica silk films when compared to control films; suggesting that these films enhance osteogenesis.

Attempts to produce silk materials that can prevent or fight infection have also been made by fusing silk protein with human antimicrobial peptides (human neutrophil defensins (HNP)^{119,120}. Paper discs were soaked in solutions of silk protein fused with HNPs and exhibited antimicrobial activity towards Gram positive and negative bacteria, however only small zones of inhibition were observed; presumably because the HNP is fused to the silk protein and cannot diffuse away to act upon the bacteria¹¹⁹.

Recombinant silk structures have previously been shown capable of drug delivery. However the fusion of poly L-lysine and RGD domains to the spidroin sequence generated a targeted gene delivery system. Here the positively charged poly L-lysine domain functions to bind negatively charged plasmid DNA and the RGD domain mediates cell entry^{121,122}.

Gene delivery efficiency was shown to be dependent on how many RGD motifs were introduced, silk structure, cell type and vector. The variability suggests that a lot of optimisation is required to attain satisfactory transfection efficiencies, however the potential is clear.

To summarise, functionalisation by chemical conjugation or genetic fusion can improve attributes such as cell binding or endow silk proteins with functions that they would ordinarily not possess. Consequently the application of silk materials is no longer limited to the obvious *i.e.* structural scaffolding, but can be pushed into new applications such as electronics or biosensing.

1.10 Biocompatibility

As outlined previously, spider silk (both recombinant and native) has shown clear potential in biomedicine, but developments will count for nothing if a host reacts negatively when exposed to silk materials.

Biocompatibility is a term often misused to simply mean a lack of immune response to an implanted material; such as titanium hip replacements, plates and screws for example¹²³. However materials that are truly biocompatible should also facilitate integration with

surrounding host tissues and ideally be biodegradable leaving no traces of itself behind. Dragline silks have been shown to have these qualities.

1.10.1 Native silk

Native dragline silk 'reeled' from live spiders has demonstrated good levels of biocompatibility (an observation first made by ancient Greeks who used spider silk as wound dressings¹¹). When applied to epicutaneous skin wounds, only a microscopic inflammation response over a two week incubation period was observed. Here the infiltration of leukocytes and tissue granulation is typical for a wound inflammation response, as opposed to inflammation caused by rejection of a foreign body¹²⁴. This observation was echoed by two other *in vivo* studies, where dragline silk based grafts provided a scaffold for neuronal regeneration^{91,92}. Unlike the skin wound study, the silk grafts were implanted for a much longer time period; 6 and 10 months respectively without signs of macroscopic inflammation. This indicated that dragline silk was well tolerated and did not antagonise the host immune system. Furthermore all of the studies did not detect silk structures in histological analysis, highlighting the ability of spider silk to be biodegraded within a host.

However not all spider silks appear to have the same level of biocompatibility. Egg sack silk was shown to deter cell growth and invoke a large and sustained immune response, displaying excessive leukocyte infiltration, fibrosis and granulation of the surrounding tissue^{125,126}. This difference in immune response could be explained by

differences in amino acid sequence meaning that, by chance, egg sack silk comprises peptide motifs that look similar or are the same as antigens already recognised by immune cells. Similarly patterns of post translational modifications such as glycosylations that the immune system is already programmed to recognise as foreign may also contribute to this outcome.

1.10.2 Recombinant silk

Recombinant dragline silk proteins like those discussed previously, present additional challenges regarding biocompatibility. The most common expression host, *E. coli*, whilst easily scalable and easy to manipulate is a Gram negative bacterium. Consequently proteins expressed by *E.coli* are contaminated with pyrogenic lipopolysaccharide (LPS) after lysis of the bacterial cells¹²⁷.

Furthermore the contaminating LPS persists even after affinity purification of target proteins; seemingly making the protein unsuitable for implantation. Alternative bacterial expression hosts such as the Gram positive *Bacillus subtilis* (*B. subtilis*) and *Staphylococcus aureus* (*S. aureus*) lack LPS, therefore removing contamination at its source^{128,129}. Unfortunately Gram positive bacteria are more difficult to manipulate genetically than *E. coli* and normally express proteases that ultimately reduce the yield of the target protein¹³⁰. Furthermore the pathogenic nature of *S. aureus* may cause more complications than LPS contamination alone.

Tobacco and potato plants offer an easily scalable route to recombinant silk production, however similar drawbacks relating to biocompatibility are found^{59,70}. Plants produce a wide variety of compounds such as alkaloids, xanthines, salicylates, terpenoids and steroids¹³¹. Some of these compounds have medicinal uses (aspirin for example), however many of the alkaloid species are poisonous (and have been used as such in the past). Both tobacco and potato plants belong to the nightshade family of plants (*Solanaceae*) and produce the glycoalkaloid toxin α -solanine, which has been shown to cause gastrointestinal disturbances through the inhibition of acetylcholinesterase¹³². Furthermore post translational modifications of the silk protein may cause issues with immune system recognition, leading to greater inflammatory responses – although this is yet to be examined.

Seemingly the solution to host related biocompatibility issues would be to express silk protein in human cell lines; removing the potential for contamination with pyrogens and toxic compounds. However the higher complexities of mammalian cells unfortunately cause more complications. Similar to plant hosts, a wide array of post translational modifications may significantly impact yield, protein functionality and biocompatibility¹³³. Tyrosine and serine phosphorylation has been observed in spidroins, however the impact of these modifications is unclear¹³⁴. Moreover mammalian cell lines are susceptible to infection from bacteria, yeasts, mycoplasma and viruses; resulting in a complete loss of yield if infection takes hold¹³⁵.

Despite these host related problems, several studies have used recombinant silk *in vivo* and did not observe severe immune responses^{67,68,96,116,136}. However, in the case of insoluble silk proteins, the use of strong denaturants, acids and extensive dialysis may have contributed to the reduction of toxins/pyrogens; making them amenable for implantation.

Fortunately a simpler method has been devised that greatly reduces LPS contamination from *E. coli*¹³⁷. The process consists of simple washing steps prior to cell lysis and protein purification. From washing alone the LPS content of purified protein was reduced to 0.1 µg/mg of pure protein from 1 µg/mg of pure protein. LPS contamination was further reduced by passing soluble pure protein through an EndoTrap® Blue column; yielding an LPS contamination level of ~320 pg/mg of pure protein. Interestingly LPS levels of only 40 pg/mg of protein were detected in fibres generated from LPS depleted soluble protein, suggesting LPS remains in the soluble fraction. Therefore this simple procedure can greatly reduce LPS contamination such that the silk material is acceptable for implantation into humans and animals. Moreover the use of *E.coli* (arguably the easiest to handle and scale up) as the expression host will no longer pose a risk to biocompatibility.

1.11 Aims and Objectives

Previous studies into functionalising recombinant silks have focused on classical chemical or genetic modification processes. However these approaches can lead to functionalisation that may be unstable or nonspecific or may perturb the natural function and biocompatibility of the silk.

Therefore the main objective of this project was to define a versatile method that could be utilised to modify 4RepCT; with a variety of ligands appropriate for numerous applications.

The work presented here aims to explore this objective in three steps:

Chapter 3 aims to incorporate a non-natural amino acid (L-azidohomoalanine) into a miniature silk spidroin (4RepCT) in order to allow functionalisation of the silk protein through 'click chemistry'.

Chapter 4 aims to examine whether bioactive ligands can be conjugated to the modified silk and furthermore whether these can be subsequently released into the environment whilst retaining their bioactivity.

Finally Chapter 5 aims to determine whether silk, permanently functionalised with bioactive ligands, can be used to enhance cell growth through the production of optimised silk scaffolds.

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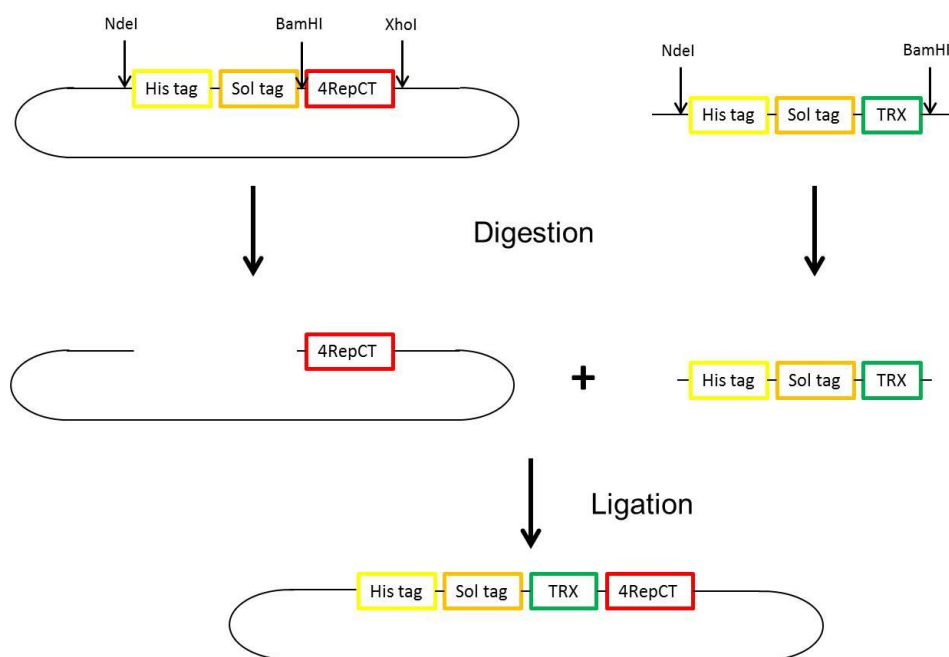
2. General Methods

Reagents were purchased from Sigma Aldrich unless stated otherwise.

Buffer recipes, solution components, SDS PAGE components and media recipes are included in the appendix section.

2.1 Construction of TRX4RepCT gene

A pJExpress 401 plasmid encoding his-tag, sol-tag (small solubilising tag originally included in DNA 2.0 4RepCT gene design), 4RepCT was obtained from DNA 2.0 (U.S.A). The insert encoding a his-tag, sol tag, thioredoxin (TRX) was obtained from Life technologies. Scheme 2.1 shows how the insert was cloned into the plasmid encoding the 4RepCT gene.



Scheme 2.1: Construction of TRX4RepCT gene in pJExpress 401 plasmid

First the plasmid and insert were digested with NdeI and BamHI restriction enzymes (New England Biolabs Inc) for 1 hour at 37°C to remove the his-tag and sol tag from the plasmid and to generate appropriate ends for the insert. After digestion the insert was ligated into the digested plasmid using a T4 DNA ligase rapid ligation kit (Roche) to give TRX4RepCT (sequence shown in Figure 2.1). The ligation reaction was incubated for 15 minutes at 20°C. XL1 Blue cells were transformed with the ligation reaction and plated onto Luria-Bertani (LB) agar plates containing 50 µg/ml of kanamycin. Colonies were selected and their plasmids isolated using a Wizard Plus SV Miniprep kit (Promega).

MAHHHHHKETAALKFERQHMDSSSGLVLPQGSMSDKIIHLTDDSFDT
DVLKADGAILVDFWAEWCGPCKMIAPILDEIADEYQGKLTVAKLNIQ
NPGTAPKYGIRGIPTLLLFKNGEVAATKVGALSKGQLKEFLDANLAGT
DDDDKLVPRGSGNSGIQGQGGYGGLGQGGYGQGAGSSAAAAAAAAA
AAAAGGQGGQGGYGQSGGSAAAAAAAAAAAAAAAAAAGRQGGY
GQSGGNAAAAAAAAAAAAAAAAAGQGGQGGYGRQSQGAGSAAAAA
AAAAAAAAAGSGQGGYGQGGYGQSSASASAAASAASTVANSV
SRLSSPSAVSRVSSAVSSLVSNQVNMAALPNIISNISSSVSASAPGA
SGCEVIVQALLEVITALVQIVSSSSVGYINPSAVNQITNVVANAMAQVM
G

Figure 2.1: Primary sequence of TRX4RepCT

The his-tag is highlighted in yellow, the sol tag is shown in orange text, TRX is shown in green text and 4RepCT is shown in red. The molecular weight of the protein is 39967 Da and theoretical isoelectric point (pI) is 5.87 as calculated by ExPASy ProtParam tool (<http://web.expasy.org/protparam/>)

2.2 Transformation of the methionine auxotroph *E. coli* DL41

An aliquot of calcium competent *E. coli* DL41 (obtained from Yale Coli Genetic Stock Center) cells were removed from -80°C storage, combined with 1-2 µl of pJExpress 401 TRX4RepCT plasmid DNA and incubated on ice for 30 minutes. After cold incubation, cells were transferred to a 42°C water bath and heat shocked for 45 seconds immediately followed by a 2 minute incubation on ice. Following incubation 800 µl of LB medium (containing no antibiotics) was added to the heat-shocked cells, and the tube transferred to a 37°C incubator for 1.5 hours. Subsequently 200 µl of the cell suspension was spread onto selective LB agar plates containing 50 µg/ml of kanamycin. Plates were inverted and incubated at 37°C for an overnight period.

2.3 Expression of 4RepCT in LB media

A transformed colony of DL41 *E. coli* was selected and used to inoculate a subculture of 5 ml LB media containing 50 µg/ml of kanamycin (LB^{KAN}). The subculture was incubated at 37°C with shaking (180 rpm) for an overnight period (~16 hours) and subsequently used to inoculate 1L of LB^{KAN}. The 1L culture was incubated at 37°C with shaking (180 rpm) until an optical density (OD₆₀₀) of 0.6 – 0.8 was achieved and chemically induced with IPTG to a final concentration of 1 mM. The induced culture was incubated for a further four hours at 37°C with shaking (180 rpm). After this final incubation period, bacteria were

harvested by centrifugation using a Beckman Avanti J-25 I equipped with a JLA – 10.500 rotor at 3500 x *g*, 4°C for a period of 12 minutes. Following centrifugation, the supernatant was discarded and the bacterial cell pellets were resuspended in a total of ~35 ml of binding buffer/ Buffer A (see Appendix 7.3.1). The resuspended bacteria were transferred to a 50 ml Falcon tube, flash frozen in liquid nitrogen and stored at -80°C.

2.4 Expression of 4RepCT in M9 minimal media

A transformed colony of DL41 *E. coli* was selected and used to inoculate a subculture of 5 ml LB^{KAN}. The subculture was incubated at 37°C with shaking (180 rpm) for an overnight period (~16 hours) and subsequently used to inoculate an intermediary culture of 50 ml LB^{KAN}. The intermediary culture was incubated at 37°C with shaking (180 rpm) for a further two hours and harvested by centrifugation using a Heraeus Primo R at 3500 x *g*, 4°C for 12 minutes. The supernatant was discarded and the bacterial cell pellet was resuspended in ~ 5 ml 1 x M9 salts solution (see Appendix 7.2). Subsequently, resuspended cells were used to inoculate 1L M9 minimal media (see Appendix 7.3.1) containing 50 µg/ml kanamycin (M9^{KAN}) and incubated at 37°C with shaking (180 rpm) until an OD₆₀₀ of 1.0 -1.3 was achieved. Once a satisfactory OD₆₀₀ was achieved the culture was chemically induced with IPTG to a final concentration of 1 mM and incubated at 37°C for a further four hours. After incubation the culture was harvested by

centrifugation using a Beckman Avanti J-25 I equipped with a JLA – 10.500 rotor at 3500 x *g*, 4°C for 12 minutes, the supernatant discarded and the bacterial cell pellets resuspended in a total of ~35 ml Buffer A (see Appendix 7.1). The resuspended bacteria were transferred to a 50 ml Falcon tube, flash frozen in liquid nitrogen and stored at -80°C.

2.5 Expression of 4RepCT^{3Aha} in M9 minimal media

As described in section 2.4 however the culture was not induced as soon as an OD₆₀₀ of 1.0-1.3 was achieved. Instead the culture was harvested by centrifugation using a Beckman Avanti J-25 I equipped with a JLA – 10.500 rotor at 3500 x *g*, 4°C for 12 minutes, the supernatant discarded and the pellets resuspended in 1 x M9 salts solution and transferred to 50 ml Falcon tubes. The resuspended pellets were subjected to 3 washing steps with 1 x M9 salts solution (see Appendix 7.2). A wash involved centrifugation using a Heraeus Primo R at 3500 x *g*, 4°C for 12 minutes, removal of the supernatant and resuspension of cell pellet with fresh 1 x M9 salts solution. After the washes were complete, the resuspended pellet was used to inoculate another 1L of M9^{KAN} media devoid of methionine. The culture was incubated at 25°C with shaking (180 rpm) for a period of 10 minutes after which 50 mg of the methionine analogue, L-azidohomoalanine (synthesised in house ^{1,2}), was added into the culture followed by IPTG to a final concentration of 0.5 mM. The culture was incubated at 25° C for an overnight period (~16 hours) and harvested by centrifugation

using a Beckman Avanti J-25 I equipped with a JLA – 10.500 rotor at 3500 x *g*, 4°C for 12 minutes. The bacterial cell pellets were resuspended in ~35 ml of Buffer A and either flash frozen in liquid nitrogen and stored at -80°C or immediately used.

2.6 Disruption of bacterial cell walls

Bacteria were lysed using a Soniprep sonicator (Sanyo). Thoroughly resuspended bacterial pellets contained within a 50 ml Falcon tube were secured inside a beaker filled with ice and a small amount of water. The beaker was placed on the moveable stage within the sonicator cabinet and positioned such that the probe was immersed in ~ 5 cm of resuspended cell solution. Pulses of 15 μ m amplitude sonication were applied to the sample in cycles of 30 seconds, followed by 30 seconds of no sonication to allow the sample to cool. This process was repeated until a total of 10 minutes had elapsed. Immediately afterwards the lysate was clarified by centrifugation using a Beckman Avanti J-25 I equipped with a JA 20 rotor at 39 000 x *g*, 4°C for 20 minutes to remove cell debris.

2.7 Purification of 4RepCT and 4RepCT^{3Aha}

An AKTA prime in conjunction with AKTA prime view software (Amersham Pharmacia) was used to purify 4RepCT and 4RepCT^{3Aha}. Clarified lysate was loaded onto a Ni IMAC column (GE life sciences) pre-equilibrated with 6 column volumes of Buffer A at a speed of 2 ml/min and the flow through collected. When the entire sample had been loaded, the column was washed with Buffer A to remove loosely bound proteins; until the UV 280 nm absorbance of the column eluate returned to baseline. Upon achieving a stable baseline, 4RepCT or 4RepCT^{3Aha} was eluted from the column by the gradual application of elution buffer also known as Buffer B (see Appendix 7.1) (0-100%) using an 80 ml linear elution gradient. Elution fractions were collected during the entirety of the elution gradient and peaks in the UV 280 nm trace analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE). Peaks found to contain either 4RepCT or 4RepCT^{3Aha} were pooled.

2.8 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE)

A BioRad mini protean 3 gel kit was used to cast gels (12 or 15% (w/v) acrylamide) and analyse samples. Samples to be analysed were combined with 4 x SDS loading buffer (12 µl of sample to 4 µl SDS loading buffer) and incubated at 90°C for 5 minutes, subsequently 10 µl of each boiled sample was loaded into separate gel wells. All samples

were analysed against a BioRad precision plus pre-stained protein ladder.

2.8.1 Staining and de-staining of SDS PAGE gels

Gels were transferred to a microwaveable plastic box containing Coomassie Blue stain solution and microwaved at 75% power for 20 seconds. The box was removed from the microwave, sealed and placed onto an orbital rocker for ~20 minutes. The stained SDS PAGE gel was removed from the stain solution, transferred to another plastic box containing de-stain solution and placed on an orbital rocker for ~ 1 hour. During this time period, the de-stain solution was replaced every 20 minutes.

2.8.2 SDS PAGE gel visualisation

De-stained SDS PAGE gels were visualised using a Geneflow GBox (Syngene) in conjunction with GeneSnap software. Brightness, contrast and focus were controlled within the software and images exported as TIF files

2.9 Dialysis of purified 4RepCT/^{3Aha}

Elution fractions containing 4RepCT/^{3Aha} were pooled and transferred to an appropriate length of pre-soaked Spectra/Por ® dialysis tubing (Spectrum Labs). The open end of the tubing was sealed with a dialysis

tubing clip of appropriate width, and transferred to a beaker containing 2L of dialysis buffer and a magnetic stirrer. Finally the beaker was incubated at 4°C with gentle stirring for an overnight period (~16 hours).

2.10 Assessing protein concentration

A Nano Drop spectrophotometer set to detect absorbance at 280 nm was used to assess the concentration of purified protein. 2 µl of sample was carefully pipetted onto the pedestal of the spectrophotometer and analysed. All samples were blanked against the buffer in which the protein was dissolved, typically 20 mM Tris pH 7.5.

3. Demonstrating Incorporation of L-azidohomoalanine (L-Aha) into 4RepCT

3.1 Introduction

This chapter describes how the non-canonical amino acid (NCAA) L-Aha was incorporated into the miniature spideroin 4RepCT and how it was subsequently detected through click chemistry mediated conjugation with fluorophores. The effect of incorporation regarding protein functionality is also discussed.

3.1.1 Non-canonical amino acids

Out of the twenty proteinogenic amino acids, there are few that are useful for chemical conjugation (typically lysine, cysteine, glutamate and aspartate); and targeting these residues usually results in a multiply labelled protein. Man-made or non-canonical amino acids (NCAAs) have expanded the number of amino acid building blocks from which a protein can be synthesised. Introduction of NCAAs has permitted the study of nascent protein expression and imaging of protein populations^{1,2}. In addition, unique chemical functionalities can be introduced into the protein sequence, permitting more specific methods of downstream chemical conjugation such as Staudinger ligation and 'click chemistry'^{3,4}.

The introduction of NCAs into proteins is not a new technique. The methionine analogue, selenomethionine, was first incorporated into proteins produced in *E. coli* in the 1950's; a development that led to great progress in protein crystallography^{5,6}. Since then, methods for the incorporation of NCAs into proteins have improved and can be divided into residue specific incorporation or site specific incorporation.

3.1.2 Site specific incorporation of NCAs

This method aims to incorporate NCAs at a specific residue position within the primary sequence. Early attempts used an *in vitro* system to achieve incorporation of NCAs^{7,8}. Here the amber nonsense codon (TAG in DNA), instead of terminating translation, was used to encode for the desired NCA (chemically loaded onto complementary tRNA). If a single amber codon is introduced and all other amber stop codons mutated out, a single NCA is incorporated in only one position (Figure 3.1). This technique has also been shown to work well *in vivo*, where synthetic tRNA and mRNA were introduced into *Xenopus* oocytes using microinjection⁹. This permitted the interrogation of neuroreceptor mechanisms and gating through the incorporation of NCAs into nicotinic acetylcholine receptors^{10–14}.

Despite the incorporation of many NCAs into various proteins, this technique has drawbacks such as low target protein yield, but most critical, was the difficulty associated with synthesising NCA loaded tRNAs¹⁵. To overcome these problems, expression of orthogonal

tRNAs and aminoacyl tRNA synthetases (aaRSs) that recognise the NCAA in an *in vivo* system was required¹⁶. Therefore genes encoding the above, and the protein of interest (containing the amber codon), are co-expressed in a heterologous host in growth medium containing the desired NCAA to produce the modified protein (Figure 3.1). As a result many different NCAs, including those with azide functional groups, have been incorporated using the amber nonsense codon¹⁷.

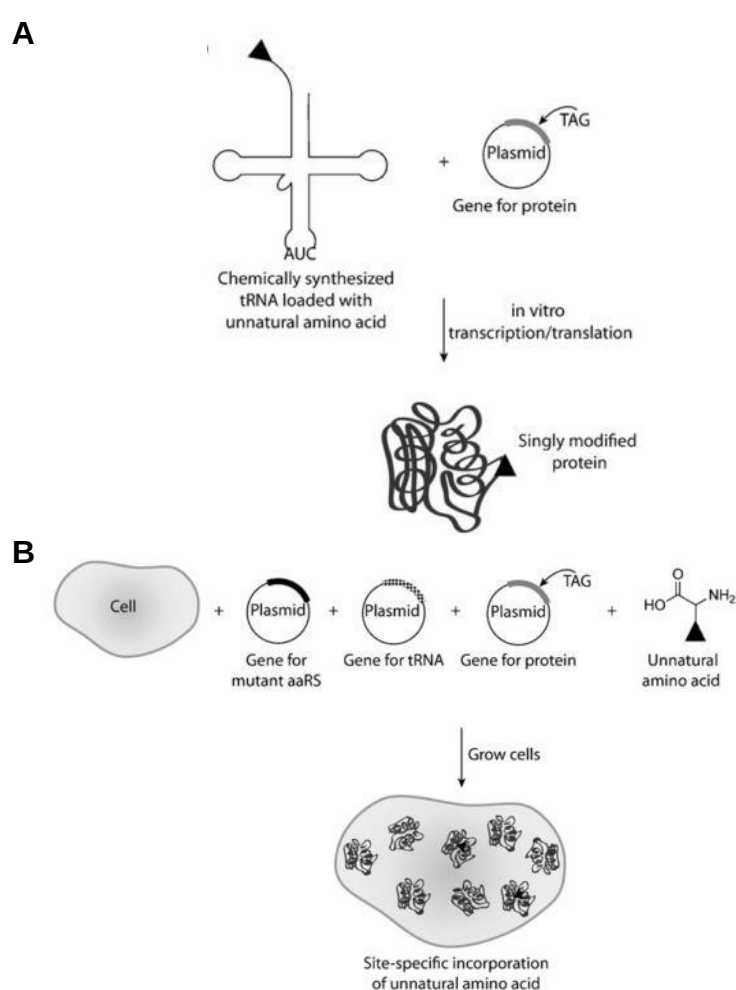


Figure 3.1: Site specific incorporation of NCAs using the amber nonsense codon

Site specific incorporation in an *in vitro* setting using synthetic tRNA (A). Site specific incorporation in an *in vivo* setting, co-expressing protein, tRNA and aaRS (B)¹⁸

3.1.3 Residue specific incorporation of NCAAs

When compared to site specific incorporation, residue specific incorporation is a much simpler way to incorporate NCAAs. This method typically uses NCAAs that are surrogates for canonical residues, for example methionine. Therefore the host translational machinery (aaRSs) is able to tolerate the NCAA (because it is structurally similar) and replace all of the target residues with the desired NCAA^{4,19}. However structural similarity is no longer a requirement, since plasmid encoded tRNAs have expanded the choice of NCAAs that can be incorporated²⁰. The efficiency of the method is enhanced if the expression host is made auxotrophic for the residue to be substituted; only incorporating the NCAA when the host is denied the natural residue (Figure 3.2).

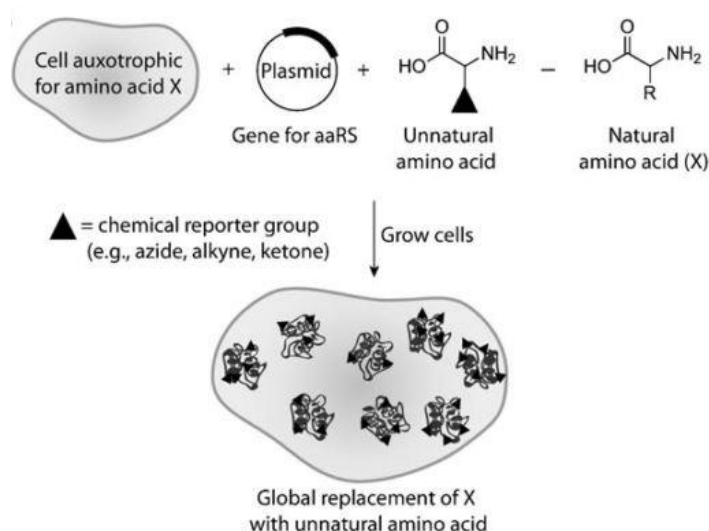


Figure 3.2: Residue specific incorporation of NCAA.

Growing an auxotrophic host in media devoid of the natural amino acid results in the incorporation of the NCAA analogue¹⁸.

Residue specific incorporation of NCAAs has led to several developments within protein engineering. Fluorinated NCAAs have been shown to stabilise hydrophobic structures within proteins and control protein-protein interactions without perturbing natural function^{21–24}. Furthermore, incorporation of NCAAs (4-aminotryptophan) produced a red shifted variant of green fluorescent protein (GFP) that classical mutagenesis could not achieve²⁵.

However, more pertinent to this project was the incorporation of the methionine analogue L- Aha and its subsequent modification (Figure 3.3)^{3,26}. This residue has a bio-orthogonal azide functional group that permits highly selective conjugation chemistry and after incorporation, will only constitute a small portion of the protein owing to the low frequency of methionine residues. Through the substitution of methionine residues in 4RepCT for L-Aha (forming 4RepCT^{3Aha}), it was the aim of this project to utilise the azide groups to functionalise the protein with various ligands.

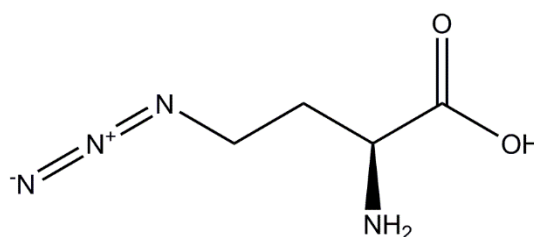


Figure 3.3: L-azidohomoalanine.

The non-canonical amino acid and methionine surrogate with an azide functional group

3.1.4 Click Chemistry

Click chemistry is a chemical reaction approach first coined by Barry Sharpless in 2001²⁷. In order for a reaction to be labelled as a 'click reaction', a strict set of criteria must be met. Firstly the reaction must be modular, wide in scope, attain high yields, stereospecific and produces inoffensive by-products. Secondly the reaction must be simple and ideally tolerate aqueous solvent. Finally, the reaction must have a high thermodynamic driving force $>20 \text{ kcal mol}^{-1}$. In summary, click reactions are simple, benign and run to completion very rapidly, yielding a single product²⁷. A number of click reactions exist, however copper catalysed azide-alkyne 1,3-dipolar cycloaddition (CuAAC) is considered the quintessential click reaction (Figure 3.4). Here Cu(I) catalyses the reaction via the formation of a copper acetylide with terminal alkynes, making them reactive towards azides; ultimately yielding a 1-4 disubstituted 1,2,3-triazole approximately seven orders of magnitude faster than an uncatalysed reaction²⁸. Consequently, CuAAC has been employed in a variety of studies from organic synthesis to protein modification^{29,30}.

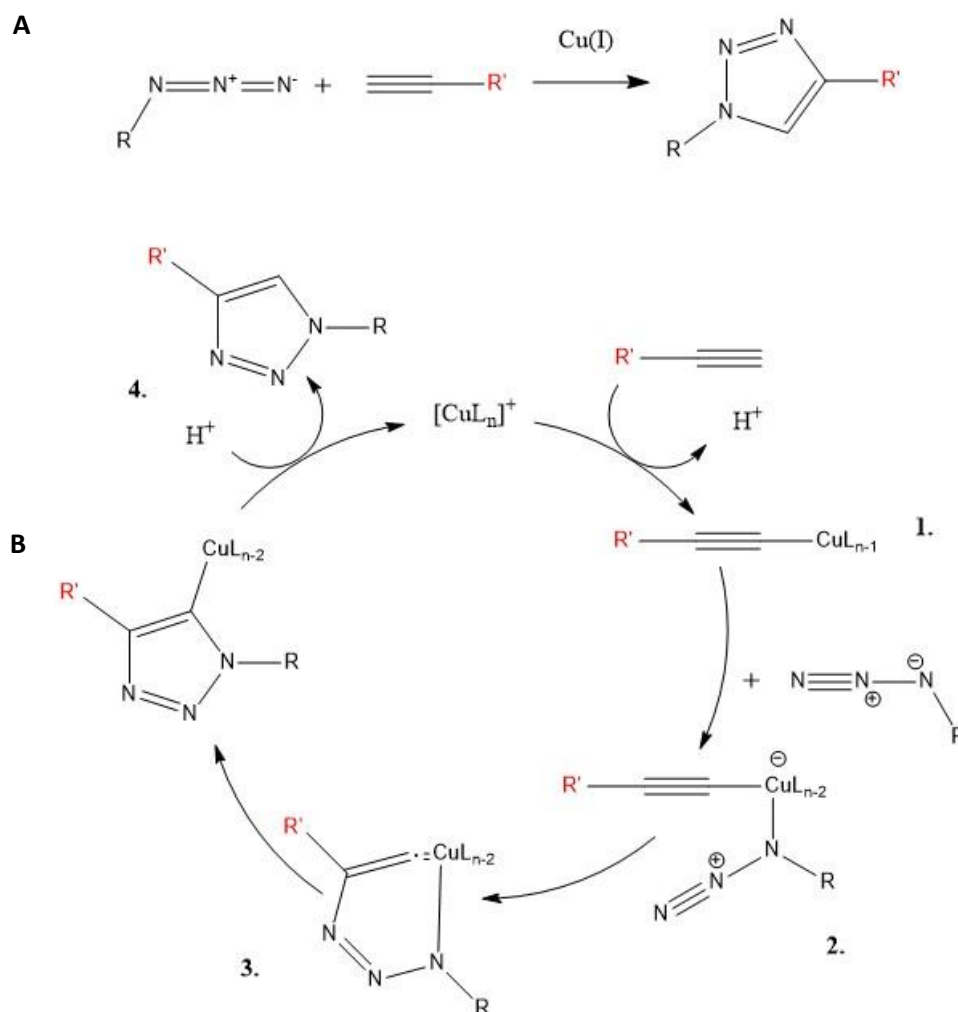


Figure 3.4: Copper catalysed azide alkyne cycloaddition (CuAAC) general reaction and proposed mechanism.

The general cycloaddition reaction scheme between azides and terminal alkynes in the presence of Cu(I) as a catalyst (A). The proposed mechanism; (1) formation of Cu acetylide (2) azide replaces a ligand and binds to Cu (3) formation of six membered metallacycle (4) protonolysis releases triazole product (B). Figure adapted from ²⁸.

However CuAAC does have its limitations. The toxicity of copper is well known and is reflected in attempts to use CuAAC in living systems that report deleterious effects on viability ^{18,31,32}. In response to this problem, copper free click reactions were developed that employ a range of ring-

strained alkynes (cyclooctynes) in a so called strain promoted azide alkyne cycloaddition (SPAAC) (Figure 3.5)^{33–36}. Whilst addressing the issue of copper toxicity, the reaction kinetics of SPAAC did not match that of CuAAC initially³⁶. However subsequent refinement of the cyclooctyne group, including the incorporation of electron withdrawing fluorine, resulted improved reaction rates³⁴. Consequently several studies have employed SPAAC to label proteins and sugars within living systems^{34–38}. Within this chapter CuAAC and SPAAC were utilised to conjugate 4RepCT^{3Aha} with fluorophores; and are later used to functionalise 4RepCT^{3Aha} with bioactive ligands.

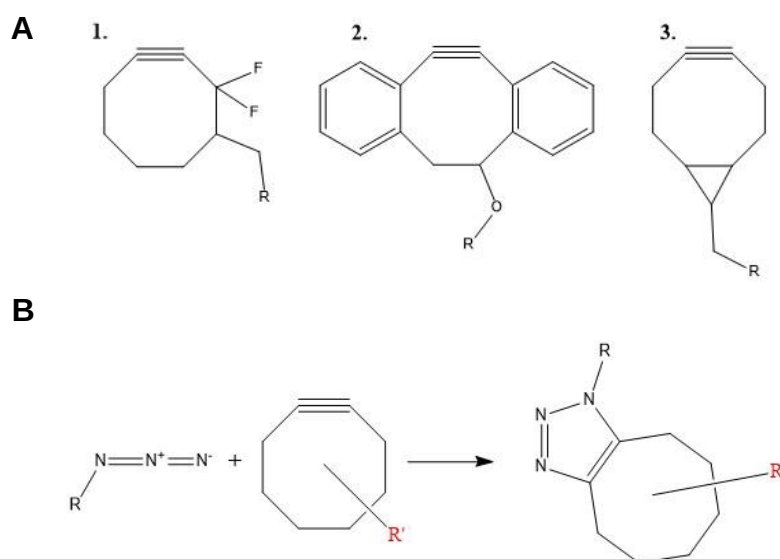


Figure 3.5: Examples of cyclooctynes and the general reaction scheme for strain promoted azide alkyne cycloaddition.

Some examples of cyclooctynes (A): (1) difluorinated cyclooctyne, DIFO; (2) dibenzocyclooctynol, DBO; (3) bicyclononyne, BCN. The general reaction scheme between an azide and a cyclooctyne to give a triazole product (B).

3.1.5 NCAs and the functionalisation of silk proteins

To date, the site specific or residue specific incorporation of NCAs into spider silk proteins has not been explored, but has been attempted for silk worm silk ³⁹. Here three methionine analogues were separately incorporated into the silk of *B. mori* by dietary introduction. This functioned similarly to residue specific incorporation but with greatly reduced efficiency, with the incorporation of homopropargylglycine (Hpg), homoallylglycine (Hag) and Aha at 23%, ~23% and 13% respectively. Furthermore the authors noted that incorporation of the analogues negatively impacted cocoon production. However the recombinant spider silk protein eADF-4 has been modified to harbour bio-orthogonal azide functional groups. Here Hardy and co-workers conjugated azidopropylamine to carboxylic acid groups of the protein via EDC NHS coupling or to the N terminal residue with NHS coupling alone ^{40,41}. It was shown in both studies that the azido groups were available for conjugation, permitting the functionalisation of the silk protein with DNA and glycopolymers. To this end, it was demonstrated that mammalian cell binding could be improved with glycopolymer functionalisation. DNA functionalised eADF-4 was shown capable of forming ribbon and raft fibril structures with the influence of temperature; driven by DNA hybridisation.

3.1.6 4RepCT miniature spidroin

As described in Chapter 1, 4RepCT is a miniaturised version of a MaSp1 spidroin, cloned from the nursery web spider *Euprosthenops australis*⁴². 4RepCT is expressed, in *E. coli*, and purified as a soluble 39 kDa fusion protein; a hexa-his tag and thioredoxin are N terminally fused to the spidroin, aiding purification and solubility respectively. However upon enzymatic (thrombin) removal of thioredoxin with gentle agitation, 4RepCT spontaneously self-assembles into water insoluble fibres⁴³. Consequently, 4RepCT is arguably one of the easiest spider silk proteins to express, purify and produce macroscopic silk structures with. As a fusion protein, 4RepCT has 6 methionine residues excluding the N terminal methionine which is normally removed after translation⁴⁴. Half (3) of the methionine residues are within thioredoxin and the other half located in the C terminal domain of 4RepCT (Figure 3.6).

MAHHHHHHKETAAAKFERQHMDSSGLVPQGSMSDKIIHLTDDSFDTDVLKADGAILVDF
WAEWCGPCKMIAPILDEIADEYQGKLTVAKLNIQNPGTAPKYGIRGIPTLLLFKNGEVAATK
VGALSKGQLKEFLDANLAGTDDDDKLVPRGSGNSGIQGGQGGYGGGQGGYGGGAGSSAAA
AAAAAAAAAGGQGGQGGYGGQSGGSAAAAAAAAAAAAAAAAAGRGQGGYGGQSGGN
AAAAAAAAAAAAAAAAAGGQGGQGGYGRQSQGAGSAAAAAAAAAAAAAAAAAGSGQGGYGGQ
GQGGYGGQSSASASAAASAATVANSVSRLSSPSAVSRVSSAVSSLVSNQGVNMAALPNIISNI
SSVSASAPGASGCEVIVQALLEVITALVQIVSSSSVGYINPSAVNQITNVVANAMAQVMG

Figure 3.6: 4RepCT fusion protein primary sequence.

Methionine residues are highlighted in red, cysteine in green. Enterokinase D, Lys-C and thrombin cleavage site is shown double underlined (introduced by Dr Philip Bardelang). Thioredoxin is upstream of the thrombin cleavage site and 4RepCT is downstream. The non-repetitive C terminal domain is shown in bold underline. The methionine before the his tag is removed after translation.

These residues were targeted for substitution with L-Aha since they are not in a position to interfere with the cysteine loop needed for C terminal dimerization ⁴⁵. Furthermore they do not appear to be buried residues since two of them are found on the C terminal end of helix 5 and the other residue is found on the N terminal end of helix 3 (Figure 3.7)⁴⁶. In order to achieve incorporation of L-Aha, the 4RepCT gene was placed under the transcriptional control of a T5 promoter, and was expressed in DL41 *E. coli* (methionine auxotroph).

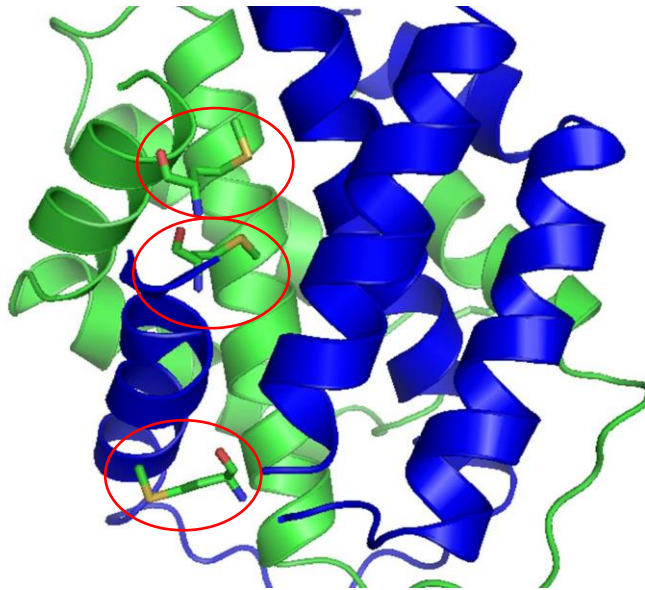


Figure 3.7: Structure of the C terminal dimer

C terminal domain chains are distinguished by colour (one green, one blue). Methionine residues are shown in stick form and circled.

3.2 Methods

See General Methods for transformation of methionine auxotroph DL41 *E. coli*, expression of non L-Aha containing 4RepCT, expression of L-Aha containing 4RepCT (4RepCT^{3Aha}), lysis, purification, SDS PAGE, dialysis and assessment of protein concentration.

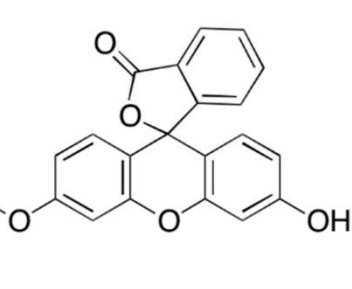
3.2.1 Fibre formation

Fibres were made by incubation of 4RepCT or 4RepCT^{3Aha} with thrombin (5 units), Lys-C (Promega) (2 µg) or Enterokinase (5 units). Cleavage was achieved at a fusion protein concentration of 1-2 mg/ml, 1 mM potassium phosphate pH 7.5 and a total volume of 1- 3 ml. The reaction was contained in sealed tubes, incubated at 30°C with gentle rocking for 4 hours.

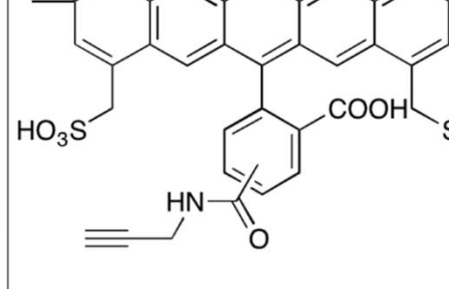
3.2.2 Conjugation of soluble 4RepCT3Aha with fluorophores

Soluble 4RepCT^{3Aha} was conjugated with fluorophores that bore a terminal alkyne or a ring-strained alkyne. Terminal alkyne fluorophores (Figure 3.8); FAM (3'-hydroxy-6'-(2-propyn-1-yloxy)-3H-spiro[2-benzofuran-1,9'-xanthen]-3-one)) (synthesized by Inderpal Kaur Sehmi). Alexa Fluor 594 ® (life technologies). Ring-strained alkyne fluorophore (Figure 3.8); Fluorescein bicyclononyne (synthesized by Francesco Zamberlan) 4RepCT^{3Aha} (1mg/ml) was labelled using 0.1 mM FAM, Alexa Fluor 594 ® or Fluorescein

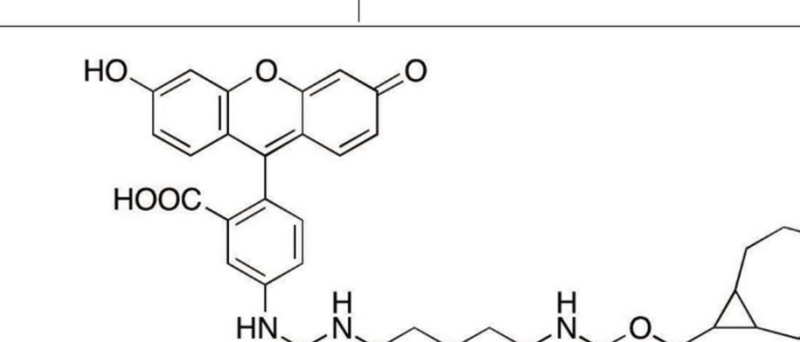
A



B



C



A: Green fluorophore FAM (excitation maximum 490 nm, emission maximum 525 nm) with terminal alkyne used in CuAAC. B: Red fluorophore Alexa Fluor 594[®] (excitation maximum 590 nm, emission maximum 617 nm) with terminal alkyne used in CuAAC. C: Green fluorophore fluorescein (excitation maximum 490 nm, emission maximum 525 nm) bicyclononyne with ring strained alkyne used in SPAAC.

3.2.3 Conjugation of 4RepCT3Aha fibres with fluorophores

Preformed 4RepCT^{3Aha} fibres of ~ 1cm in length were labelled with FAM alkyne or Alexa Fluor 594[®] as above, however 5.0 mM of Cu[I] was used in order to avoid inconsistency of catalyst concentration along the fibre structure. Reactions were protected from light and incubated at room temperature with gentle rocking for 4 hours. After the incubation period, fibres were transferred to 15 ml Falcon tubes and washed three times with 5 ml 20 mM Tris pH 7.5 and incubated overnight in 10% (v/v) dimethyl sulfoxide (DMSO) milli-Q water at room temperature with gentle rocking

3.2.4 Production of multiple fluorophore labelled fibre

Separate aliquots of 4RepCT^{3Aha} were labelled with FAM or Alexa Fluor 594[®] as described above. Each of the labelled aliquots was dialysed separately against 20 mM Tris pH 8.0 to remove DMSO and click reagents (THPTA, CuSO₄, and sodium ascorbate). After dialysis, the labelled proteins were combined (1:1) in the same tube. Fibre formation was initiated with thrombin (10 units) and treated as outlined in 'fibre formation'.

3.2.5 Fluorescence imaging

Labelled fibres were placed onto glass slides of 1.5 mm thickness and imaged using a Nikon Eclipse Ti fluorescence microscope with FITC (467-498 excitation 513-556 emission) and Texas Red (542-582

excitation 604-644 emission) filter cubes. Silks were observed under 60 X (Nikon Apo TIRF NA 1.49 objective) and 4 X (Nikon Plan Fluor NA 0.13 objective) magnification.

3.2.6 Tensile strength measurements

Fibres were removed from solution and mounted directly onto cardboard windows (all fibres tested were not dried). Once straightened, the fibres were glued into place using cyanoacrylate super glue (LOCTITE, Henkel Corporation). Glue was allowed to dry and the fibre-mounted cardboard window fixed into the grips of the tensile tester (Mecmesin MultiTest 2.5 i) equipped with a 2N load cell. The sides of the cardboard window were carefully cut, and tensile test initiated. Fibres were pulled vertically at a speed of 5 mm/min until the fibre broke (failed) and the load at failure recorded. Temperature and humidity during testing was not controlled; tests were carried out under ambient laboratory temperature and humidity.

3.2.7 Scanning electron microscopy (SEM)

Adhesive carbon pads were fixed atop aluminium SEM stubs, and were used to secure fibre samples on top of the stub. Once secure, fibre samples were coated with a fine layer of platinum and transferred into the sample carousel of the SEM (FEI Quanta 650). Images were taken at 5.00 kV.

3.3 Results

3.3.1 L-Aha was successfully incorporated into 4RepCT

Using the methionine auxotroph *E. coli* DL41, the NCAA L-Aha was incorporated residue specifically, substituting methionine as outlined previously. Incorporation of L-Aha did not seem to affect protein expression since yields of ~20 mg/L were attained. Once purified, the presence of L-Aha was verified by ‘click chemistry’ conjugation of soluble 4RepCT^{3Aha} with alkyne tagged fluorophores via copper catalysed azide alkyne cycloaddition (CuAAC (FAM and Alexa Fluor 594[®])) or strain promoted azide alkyne cycloaddition (SPAAC (fluorescein bicyclononyne)) (Figure 3.9). Analysis of the fluorophore 4RepCT^{3Aha} reaction by SDS PAGE revealed fluorescent bands corresponding to the mass of 4RepCT; indicating that a successful click reaction occurred and confirmed the presence of L-Aha.

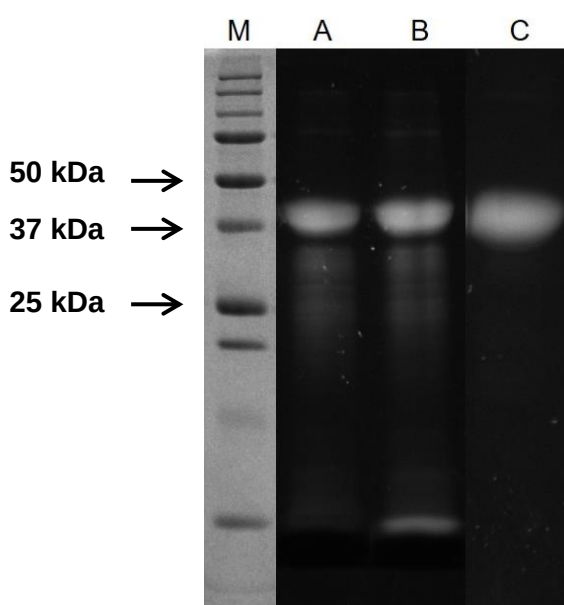


Figure 3.9: A composite of SDS PAGE gels that show successful conjugation of alkyne tagged fluorophores to 4RepCT^{3Aha}.

All fluorophore conjugated 4RepCT^{3Aha} ran to ~39 kDa. Protein. 4RepCT^{3Aha} conjugated with FAM (lane A), Alexa Fluor 594[®] (lane B) and fluorescein bicyclononyne (lane C).

3.3.2 Incorporation of L-Aha did not affect fibre self-assembly

Purified 4RepCT^{3Aha} could still be processed into fibres upon enzymatic removal of thioredoxin. No alterations were required for the fibre assembly process and macroscopic fibres appeared within the same incubation period previously reported ⁴³. SDS PAGE analysis of digestions demonstrated that thrombin, enterokinase D and Lys-C successfully removed thioredoxin. Removal of thioredoxin with thrombin and enterokinase D resulted in a band at ~15 kDa (corresponding to the mass of thioredoxin with hexa-histidine tag). However digestion with Lys-C did not produce a band at 15 kDa because Lys-C cleaves at multiple sites within thioredoxin. No band corresponding to 4RepCT^{3Aha} without the thioredoxin tag was observed in any digestion. This is because once thioredoxin is removed insoluble fibres form and fall out of solution, making them undetectable by SDS PAGE (Figure 3.10).

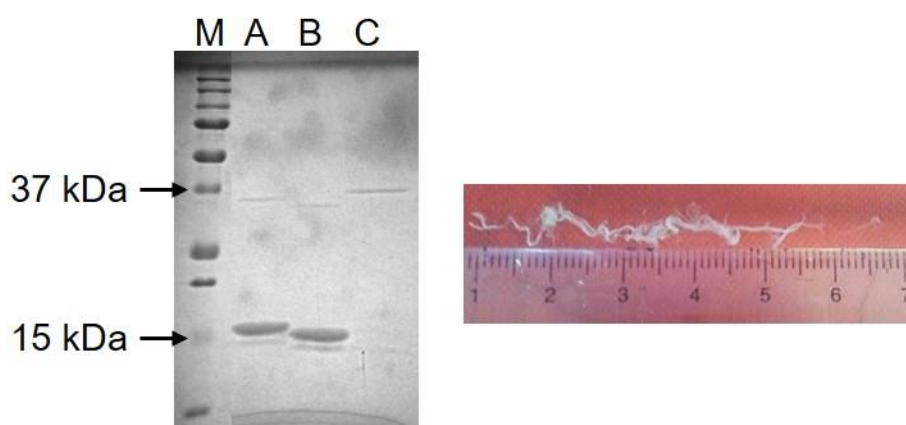


Figure 3.10: A 15% SDS PAGE gel showing the enzymatic digestion of 4RepCT^{3Aha} and a resulting fibre.

Protein marker (lane M), bands corresponding to 37 kDa (representing undigested 4RepCT^{3Aha}) and 15 kDa (representing his-tagged thioredoxin) marked by an arrow. Pictured left; 4RepCT^{3Aha} was digested by thrombin (lane A), enterokinase D (lane B) and Lys-C (lane C). Right panel; a fibre of approximately 6.5 cm in length made from thrombin digestion of 4RepCT^{3Aha}.

3.3.3 Preformed macroscopic 4RepCT^{3Aha} fibres were conjugated with fluorophores

Preformed 4RepCT^{3Aha} fibres were labelled with FAM or Alexa Fluor 594[®]. 4RepCT (no L-Aha) was used as a control to detect nonspecific binding of fluorophore to the silk fibre surface. After the incubation period, fibres were washed vigorously with buffer and 10% (v/v) DMSO to remove non-specifically bound fluorophore and excess labelling reagents. To the naked eye, 4RepCT^{3Aha} fibres retained a deep colouration after washing, whereas control fibres reverted to the colour of un-labelled fibres. Visualisation with excitation of fluorophores under identical conditions (exposure time and light intensity) of labelled and control fibres at 60X magnification revealed their fibrillar structure. 4RepCT^{3Aha} fibres labelled with either FAM or Alexa Fluor 594[®] displayed uniform fluorescence throughout the fibre structure, whereas control fibres did not display any fluorescence (Figure 3.11). This result suggests that covalent conjugation is the reason for sustained fluorescence, not nonspecific binding of fluorophore to the silk fibre surface.

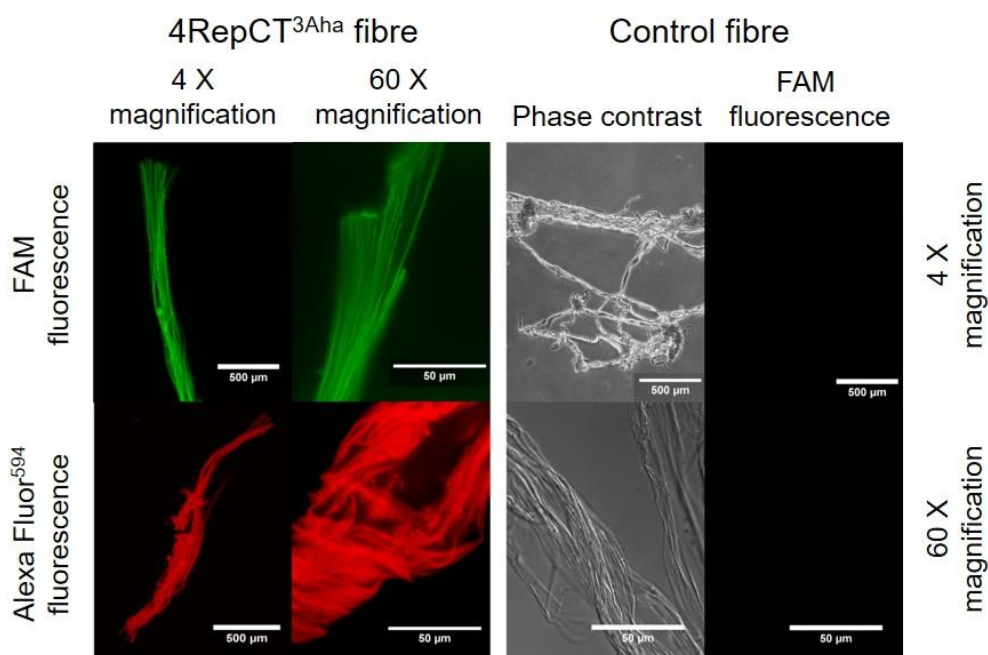


Figure 3.11: Fluorescence microscope images of fluorophore conjugated 4RepCT^{3Aha} and 4RepCT control fibre.

The labelled 4RepCT^{3Aha} fibres in the left hand panel display strong and uniform fluorescence. At 60 x magnification the fine fibrillar structure of both FAM and Alexa Fluor 594[®] labelled fibres is revealed. Control fibre, located in the right hand panel also display the fibrillar structure but do not give any fluorescent signal.

3.3.4 Soluble 4RepCT^{3Aha} labelled with fluorophore assembles into fibres

A 1 ml volume of pure soluble 4RepCT^{3Aha} was labelled with FAM as described previously and the excess labelling reagents removed by dialysis. The removal of thioredoxin as initiated through the addition of thrombin to labelled dialysed protein. The digestion mixture was incubated for the same period as previously described, again with gentle agitation. After incubation, macroscopic yellow coloured fibres were present in the reaction vessel (Figure 3.12). Apart from their colour, the physical appearance of the fibres was similar to unlabelled

4RepCT^{3Aha} fibres. Fibres were removed to fresh tubes and thoroughly washed to remove non-specifically bound fluorophore. As with preformed fibres, the control fibre (no L-Aha) readily lost colour and fluorescence after several washing steps (Figure 3.12).

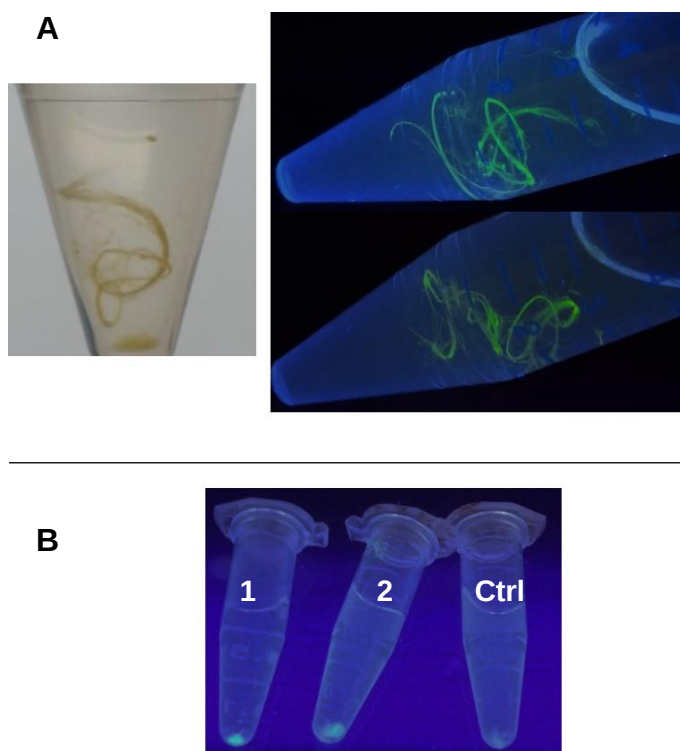


Figure 3.12: Macroscopic fluorescent fibres processed from soluble 4RepCT^{3Aha} labelled with FAM and fibre pellets after washing.

Fluorescent fibres made from FAM labelled 4RepCT^{3Aha} in white light (W. L.) and under ultra violet (U.V.) light (A). FAM conjugated 4RepCT^{3Aha} fibre pellets clearly remain fluorescent after washing indicating successful conjugation (1 & 2). Control fibre pellet (Ctrl) does not retain fluorescence after washing (B).

3.3.5 Dual labelled fibres were made by combining soluble FAM conjugated 4RepCT^{3Aha} with soluble Alexa Fluor 594[®]

4RepCT^{3Aha} was labelled with FAM and Alexa Fluor 594[®] in separate reaction tubes and dialysed separately. Subsequently, FAM labelled and Alexa Fluor 594[®] labelled 4RepCT^{3Aha} were mixed (1:1 v/v) in the same tube and fibre formation initiated by addition of thrombin. The digestion was incubated as previously described and, after incubation a macroscopic fibre (of ~ 4 cm in length) was present. Unlike singularly labelled fibres which had a green or red appearance to the naked eye, the dual labelled fibre appeared orange. Fluorescence microscopy analysis revealed that green and red fluorescence was displayed when excitation wavelengths were changed to excite only FAM or only Alexa Fluor 594[®] (Figure 3.13). This demonstrated that both fluorophores were present in the same fibre. Furthermore the images show that fluorescence was not localised to 'hot spots'; indicating that FAM and Alexa Fluor 594[®] share even distribution throughout the fibre.

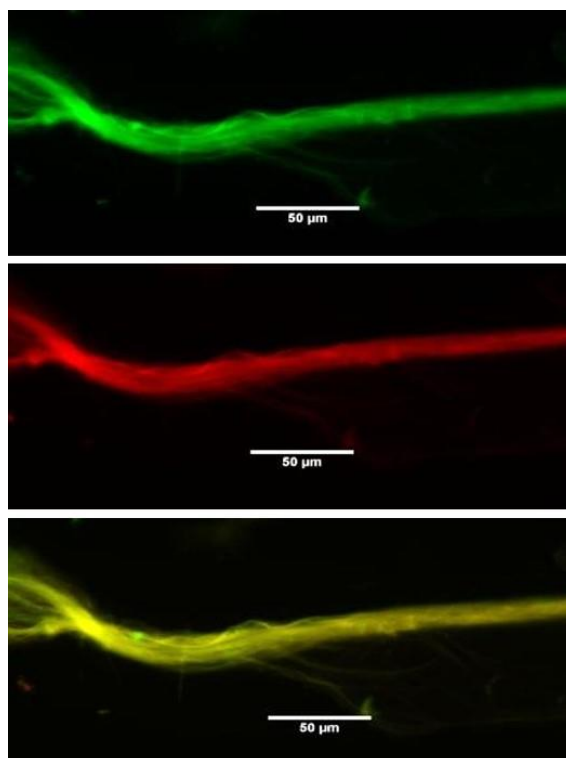


Figure 3.13: 4RepCT^{3Aha} fibre labelled with both FAM and Alexa Fluor 594[®] fluorophores

Fibre excited with 467 – 498 nm light (A), 542 – 582 nm light (B) and a composite image of the both fluorescent signals (C) highlighting even distribution of fluorophore throughout the fibre. Scale bars represent 50 μm

3.3.6 Modification of 4RepCT^{3Aha} with fluorophore did not impact fibre tensile strength.

Unconditioned 4RepCT^{3Aha} fibres were loaded into a tensile testing rig and pulled until the fibres failed. Unconditioned fibres made from FAM modified 4RepCT^{3Aha} were subjected to the same test in order to determine whether conjugation affected tensile strength. Using identical draw speeds, replicates of fibres were tested and fail point loadings recorded. Failed fibres were collected, observed under SEM and their diameters measured (Figure 3.14).

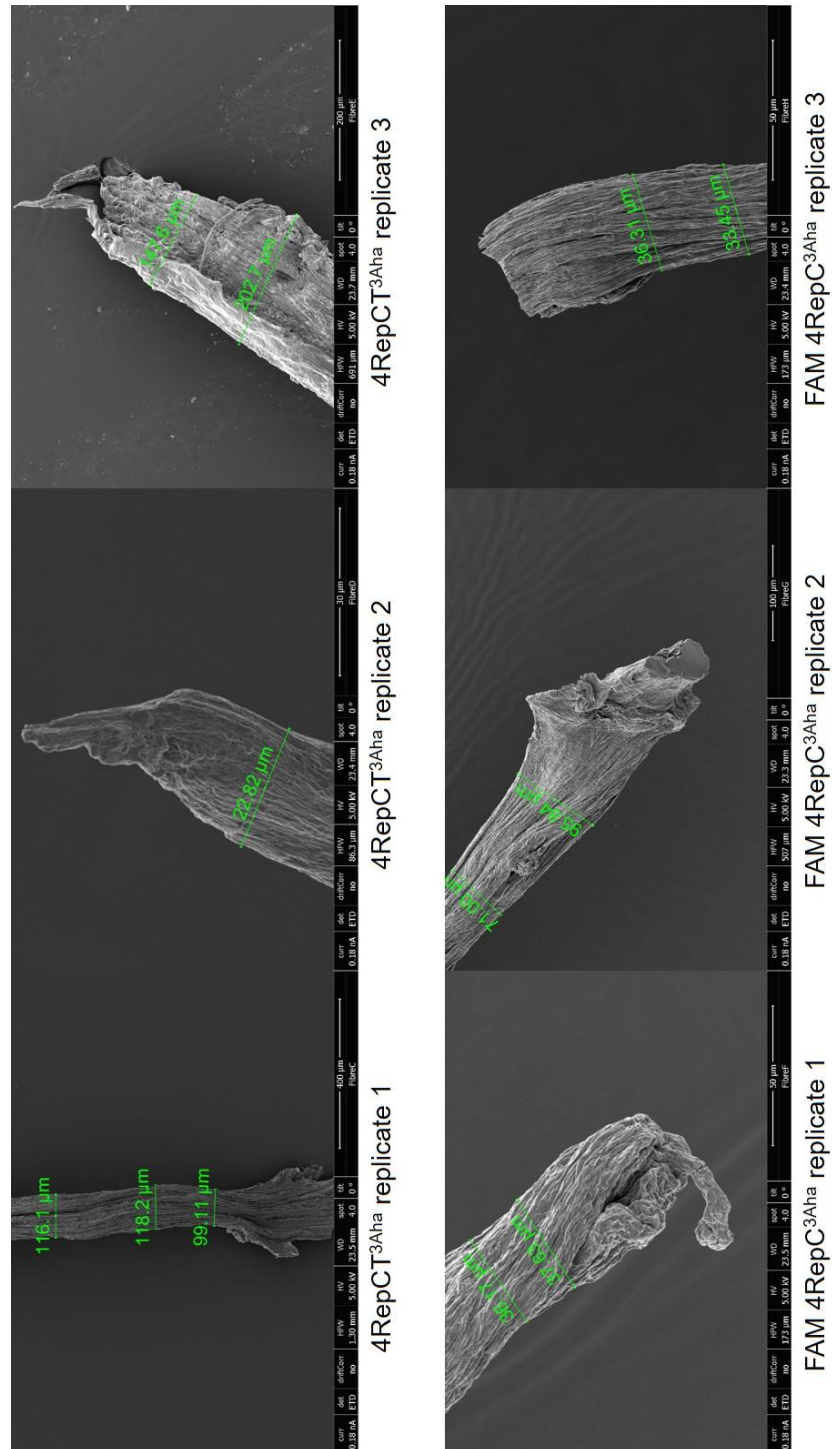


Figure 3.14: SEM images of fibres after tensile testing.

Platinum coated fibres observed using SEM. The diameters of the fibres were measured manually (green lines) in order to calculate cross sectional area.

In addition, SEM revealed that the morphology of the fibres (across both types) was similar; with all fibres displaying tightly packed fibres

with lengthwise striated patterns of fibrillar bundles. Using the assumption that the cross sectional shape of the fibres was a circle, cross sectional area in metres squared was calculated. Finally the tensile strengths of each fibre (and fibre type) were calculated using equation 3.1 (Table 3.1), and shown to be not significantly different from one another when analysed by a two tailed unpaired t-test (Figure 3.15). However since some of the fibres adopt a more flattened cross sectional shape rather than a circle, the calculated cross sectional areas are not accurate. Therefore, as with previous works, the calculated tensile strengths should be interpreted with caution ⁴³.

$$\text{Tensile strength (Pa)} = \frac{\text{Load of fibre at fail point (N)}}{\text{Cross sectional area (M}^2\text{)}}$$

Equation 3.1: Calculation of tensile strength

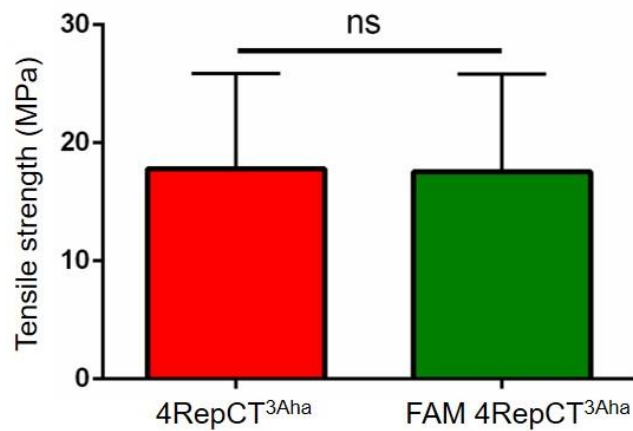
Tensile strength is expressed in Pascals, fibre loadings are recorded in Newtons and cross sectional area was expressed in metres squared.

Fibre type	Replicate	Load at fail (N)	Cross sectional area (M ²)	Tensile Strength (MPa)
4RepCT ^{3Aha}	1	0.07	2.08 x 10 ⁻⁹	33.65
	2	0.003	4.09 x 10 ⁻¹⁰	7.34
	3	0.05	4.04 x 10 ⁻⁹	12.38
FAM 4RepCT ^{3Aha}	1	0.035	1.11 x 10 ⁻⁹	31.53
	2	0.07	3.84 x 10 ⁻⁹	18.23
	3	0.003	1.06 x 10 ⁻⁹	2.83

Table 3.1: A table showing fibre loadings, cross sectional area and tensile strength

Cross sectional area of each fibre was calculated after tensile testing.

A graph comparing the tensile strength of 4RepCT^{3Aha} and FAM conjugated 4RepCT^{3Aha} fibres



A graph comparing cross sectional areas of 4RepCT^{3Aha} and FAM conjugated 4RepCT^{3Aha} fibres

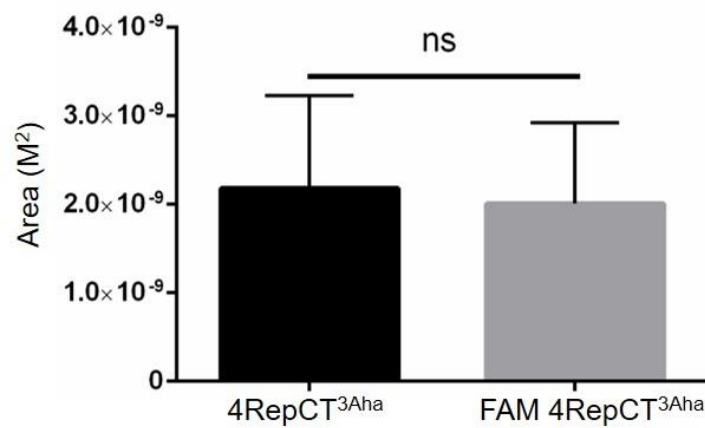


Figure 3.15: Comparisons of tensile strength and cross sectional area between 4RepCT^{3Aha} fibres and FAM conjugated 4RepCT^{3Aha} fibres.

The tensile strengths of labelled and unlabelled fibres were not significantly different (A). The average cross sectional areas of each fibre type were not significantly different (triplicate data $p = >0.05$).

3.4 Discussion

3.4.1 Overview

It was the aim of this chapter to determine whether L-Aha could successfully substitute methionines in the primary sequence of 4RepCT, and if this had any effect on fibre assembly. More critically, were the newly incorporated azide functional groups available for conjugation and how would conjugation affect fibre formation. Utilising molecular and visual techniques it was shown that 4RepCT^{3Aha} could be conjugated with fluorophores with no apparent effect on fibre assembly.

3.4.2 Detection of L-Aha in soluble 4RepCT^{3Aha} and fibres

Following expression and purification, the presence of L-Aha was verified by CuAAC or SPAAC mediated conjugation of fluorophores (Figure 3.8). This confirmed that the methionine analogue, L-Aha, was successfully incorporated into 4RepCT using the existing translational machinery. Owing to the bio-orthogonal nature of azides and alkynes and their high specificity for one another in 'click reactions', the alkyne tagged fluorophores were an ideal probe for L-Aha detection.

Therefore, using this approach, false positives are avoided since cross reactivity is not a concern. Furthermore, the successful conjugation of soluble 4RepCT^{3Aha} with fluorophores also informed that azide functional groups were exposed as opposed to being buried within the protein. However from this data alone, it cannot be said that the L-Aha

residues within the C terminal domain of 4RepCT are exposed and able to conjugate. While in soluble form 4RepCT is fused to thioredoxin which also has three methionines substituted for L-Aha. Consequently, given just this data, it is unclear whether fluorescent banding (Figure 3.8) is a result of conjugation with L-Aha residues in 4RepCT, thioredoxin or both. However combining the structure of dimerised C terminal domains (Figure 1.5) with the L-Aha residue positions suggests that these residues will not be buried. This is supported by the fact that fluorophores were successfully conjugated to premade 4RepCT^{3Aha} fibres (Figure 3.10). Here thioredoxin is completely removed from the system, therefore only L-Aha residues within the C terminal domain are present. In addition, the capability to wash away fluorophore from control silk fibres highlights the appropriateness of alkyne tagged fluorophores as a detection method and the stability of the triazole bond between fluorophore and fibre. This drives home the absence of cross reactivity and that any interactions between fibre and fluorophore were transient; non-specific hydrophobic interactions for example.

3.4.3 4RepCT^{3Aha} and fluorophore labelled 4RepCT^{3Aha} fibre formation

Purified 4RepCT^{3Aha} was shown capable of forming macroscopic water insoluble fibres upon enzymatic removal of thioredoxin. This suggested that L-Aha appeared as methionine and did not perturb C terminal dimerization required for fibre formation^{46,47}. Fibres were created within the same incubation period and appeared physically similar to

those previously described^{43,48}. This suggested that the oligomerisation of 4RepCT^{3Aha} spidroins and their organisation throughout the fibre structure was similar to that of non L-Aha containing 4RepCT fibres. The ability to assemble fibres from fluorophore labelled 4RepCT^{3Aha} suggested several things. The first is that methionine residues do not play a critical role in C terminal dimerization, unlike those present in the cysteine loop⁴⁵. Whilst L-Aha is an agreeable analogue of methionine; once conjugated to a fluorophore, its chemical nature is altered, its steric bulk increased and can therefore no longer be considered an analogue. This change approximates a mutation, and since no deleterious effect on fibre formation was observed, it was assumed that methionines have no functional role. The second is that further support is given to the notion that azide groups are exposed, and not buried in the dimerization interface. One would expect that if this was the case, then dimerization would be hindered by the gain in steric bulk and an impact on fibre formation would be apparent. Lastly the observation that, like preformed fibres, control fibres lost fluorescence indicates that fluorophore isn't permanently trapped within the fibre structure during formation. Consequently fluorescence is only retained by successful conjugation with L-Aha, meaning that false or over labelling through the trapping of fluorophore did not occur.

3.4.4 Multiply labelled fibre

Through the blending of soluble 4RepCT^{3Aha} labelled with different fluorophores, it was shown that a dual-labelled fibre was produced; an

outcome that has not been seen in other studies. This result demonstrated the potential to generate silk fibres with multi-functionality. Interestingly at the blend ratio used, the uniformity of the different fluorescent signals throughout the fibre suggested that spidroin labelled with FAM was not prevented from interacting with spidroin labelled with Alexa Fluor 594[®] (and vice versa), allowing the formation of one multiply labelled fibre as opposed to two mono labelled fibres. Therefore, using this technique, tuneable (by altering blending ratios) multifunctional silk fibres with evenly distributed functional ligands could be created. Consequently this method of functionalisation of 4RepCT^{3Aha} fibres demonstrates a high level of customisation, given that multiple functional ligands can be incorporated into the same fibre in ratio dependent quantities. Furthermore, by blending numerous functionalised proteins (i.e. more than two), the potential exists to create silk fibres with many functional ligands. Therefore generating a silk material with complex ligand groups, that could perhaps work synergistically or antagonistically

3.4.5 The effect of modification on fibre morphology and tensile strength.

Under the scrutiny of SEM, fibres processed from 4RepCT^{3Aha} and FAM modified 4RepCT^{3Aha} were analysed (Figure 3.13). It was observed that the morphology (smoothness, appearance of fibrillar bundles) of the unmodified and modified fibres were very similar. This finding suggested that the packing or alignment of FAM modified spidroins

within the fibre is unaffected, and therefore generates fibres that closely resemble unmodified 4RepCT^{3Aha} fibres. This similarity was also mirrored in the tensile strengths of the fibre types, however as previously outlined the calculated tensile strengths are subject to inaccuracies (Figure 3.14). Whilst the tensile strengths of individual fibres were in some cases very different, the average performance was statistically indistinguishable; each yielding average tensile strengths of ~ 17 MPa. In addition, no statistical difference was observed between cross sectional diameters of fibre types, indicating that tensile strengths were not swayed by larger or smaller cross sectional areas in just one fibre type. Comparing the tensile strength of 4RepCT^{3Aha} fibres (modified or unmodified) to previous 4RepCT studies, it would seem that the very introduction of L-Aha reduces tensile strength⁴³. In the 2007 study, 4RepCT was reported to attain a tensile strength value of ~150 MPa; approximately 5 x greater than the 'highest scoring' of each 4RepCT^{3Aha} fibre type. However in that study, fibres were subjected to 'post-spin' conditioning / annealing where the fibres are repeatedly stretched and relaxed. This process is known to increase silk tensile strengths by homogenising and reducing fibre diameters and influencing extensibility of the fibre^{49,50}. Therefore the reduced tensile strength of both types of 4RepCT^{3Aha} fibres may be explained, since both fibre types were tested 'as self-assembled', and not subjected to a process that arguably artificially increases tensile strength. Consequently the fibres tested yielded tensile strengths that are approximately 5 x lower than those previously reported⁴³.

Whilst modification of 4RepCT^{3Aha} with fluorophores is a convenient method of detecting azide functionality, exploring different routes to modification and studying the impact modification has on the protein; fluorophores themselves are not functional ligands. Therefore labelled 4RepCT^{3Aha} silk fibres, whilst exhibiting fluorescence, do not gain any additional functionality. The next two chapters focus on functionalisation of 4RepCT^{3Aha} with bioactive ligands to gain new functions such as antimicrobial activity, and work to enhance performance within an application such as cell culture.

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4. Levofloxacin functionalised 4RepCT^{3Aha} fibres

4.1 Introduction

This chapter describes how potent antimicrobial activity was attained through functionalisation of 4RepCT^{3Aha} with an antibiotic via a labile linker. The impact of antimicrobial activity regarding the application of silk materials will also be discussed.

4.1.1 Antimicrobial materials

Pathogenic infections from bacteria are a major issue in health care, and many can occur as hospital acquired infections (HAIs). Over half of all HAIs arise from bacterial colonisation of medical devices which constitute an instrument, apparatus, implement, machine, contrivance, implant, *in vitro* reagent, or other similar or related article, as defined by the U.S. Food and Drug Administration (FDA) ¹. In response to this, polymeric antimicrobial materials designed to prevent, combat or eradicate these infections have gained considerable academic and industrial interest. To achieve this, two ‘types’ of synthetic polymer have been created. The first can be classed as biocidal, and kill bacteria either by direct contact with the bacterium or by releasing a payload of low molecular weight antimicrobial compounds ^{2,3}. The second is termed biopassive, and prevents bacteria attaching to its surface and therefore biofilm formation ⁴. Consequently bacteria are easily cleared

from the surface. A vast number of studies have focused on producing antimicrobial synthetic polymers. Therefore comparatively only a few examples are introduced here that demonstrate commonly used techniques or motifs that convey antimicrobial activity.

4.1.2 Biocidal polymers; contact killing

The major component of the bacterial cell membrane is phosphatidylethanolamine, resulting in an overall negative charge. Consequently the majority of contact killing synthetic polymers feature positively charged groups, the most common being quaternary ammonium groups (Figure 4.2 C), that destructively interfere with the bacterial cell wall and membrane ⁵. As such, quaternary ammonium groups have been incorporated into many polymers made from a wide range of monomers including; styrene, 4-vinylpyridine, divinylbenzene, 2-(dimethylamino)ethyl methacrylate, siloxane and polyethyleneimine to name a few ⁶⁻⁹. Despite antimicrobial activity *in vitro*, not all of the polymers were biocompatible, and so would not be applicable for implantation ¹⁰. Furthermore quaternary ammonium containing polymers can over time facilitate fouling of its surface through the build-up of dead bacteria; leading to chronic inflammation. However this problem has been overcome through the advent of a polymer that can release bacterial cells after killing ¹¹.

4.1.3 Biocidal polymers; antimicrobial compound release

These polymers attain antimicrobial activity by releasing covalently or non-covalently bound antimicrobial compounds, including clinically relevant antibiotics, into their immediate environment. A wide range of small molecules have been covalently attached to various polymer materials. These include heterocyclic hydantoin compounds (nylon and polyester materials), acid group containing compounds such as hydroxybenzoic acid (ethylene-co-vinyl acetate) and antibiotics such as levofloxacin (polysiloxane), penicillin and ampicillin (expanded polytetrafluoroethylene) ¹²⁻¹⁶. However in order to exert an antimicrobial effect, the compounds (or chlorine in the case of hydantoin) must be released from the polymer materials into the environment. Levofloxacin and penicillin were attached to their polymers via linkers, allyl glycidyl ether and polyethylene glycol (PEG) respectively (Figure 4.1). Ester bonds were formed between linker and antibiotic, a labile bond that can be hydrolysed by changes in pH or esterase activity. Ampicillin on the other hand was bonded to the PEG linker by an amide bond (Figure 4.1). Therefore by using linkers and forming bond between linker and antibiotic that are susceptible to cleavage, antimicrobial activity was achieved against a wide range of bacteria.

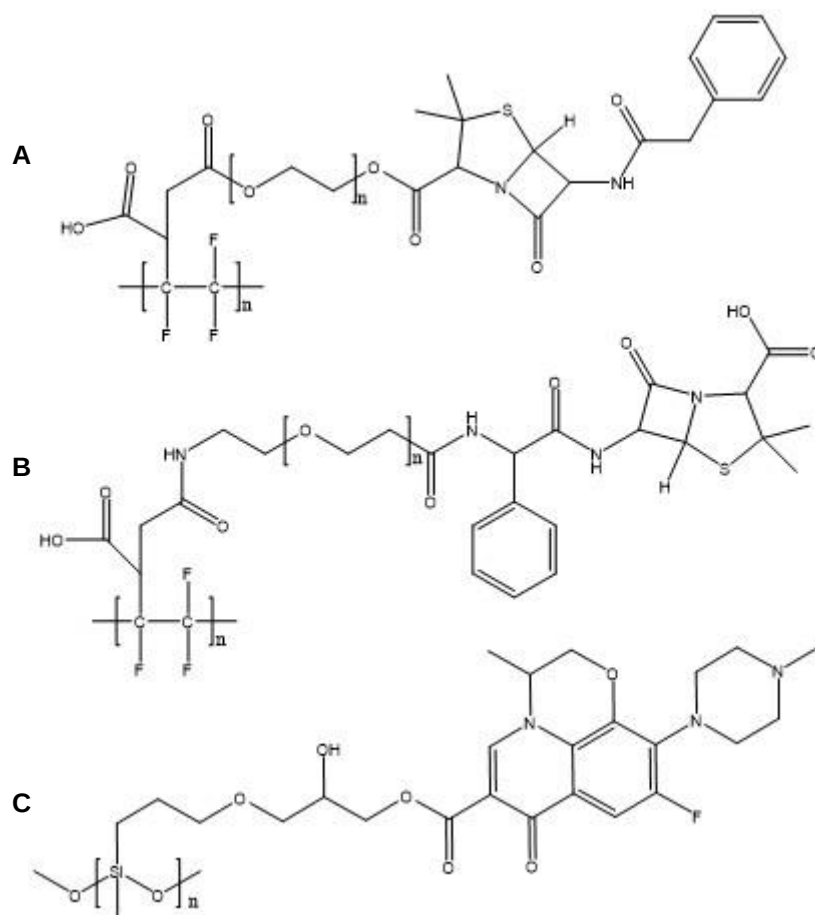


Figure 4.1: Antibiotics conjugated to synthetic polymers via labile linkers

Penicillin conjugated to expanded polytetrafluoroethylene via an ester bonded PEG linker (A). Ampicillin conjugated to expanded polytetrafluoroethylene via an amide bonded PEG linker (B). Levofloxacin conjugated to polysiloxane via an ester bonded allyl glycidyl ether linker (C).

Polymers that non-covalently bind antimicrobial compounds do so by blending the compounds into the polymerisation reaction or by trapping the compounds within structure made from the polymer. Therefore antimicrobial activity of the resulting material arises from the diffusion of the antimicrobial compounds from the polymer structure into the environment. A wide range of compounds and molecules have been used as components of polymerisation or encapsulated into synthetic

polymers, including the widely used antibacterial compound triclosan and the antiseptic chlorhexidine (Figure 4.2) ^{17,18}. Furthermore several studies have successfully incorporated antibiotics into various morphologies of poly lactic-co-glycolic acid (PLGA) such as; wafers loaded with penicillin or tetracycline and microspheres encapsulating doxycycline or rifampicin (Figure 4.2) ¹⁹⁻²¹. However antimicrobial activity of polymer materials with non-covalently bound compounds can vary considerably. The type of polymer, morphology and solvent in which the material is placed can strongly affect the outward diffusion of the compound, and can even render the material useless against bacteria ^{17,22}. However antimicrobial polymers that gain activity through covalent attachment or non-covalent binding of compounds share the same drawback. Unlike polymers that kill bacteria by contact, these polymers lose activity over time as their payloads are released. Consequently there is a risk that if the bacteria are not efficiently despatched with the load of antimicrobial compound, re-colonisation could occur.

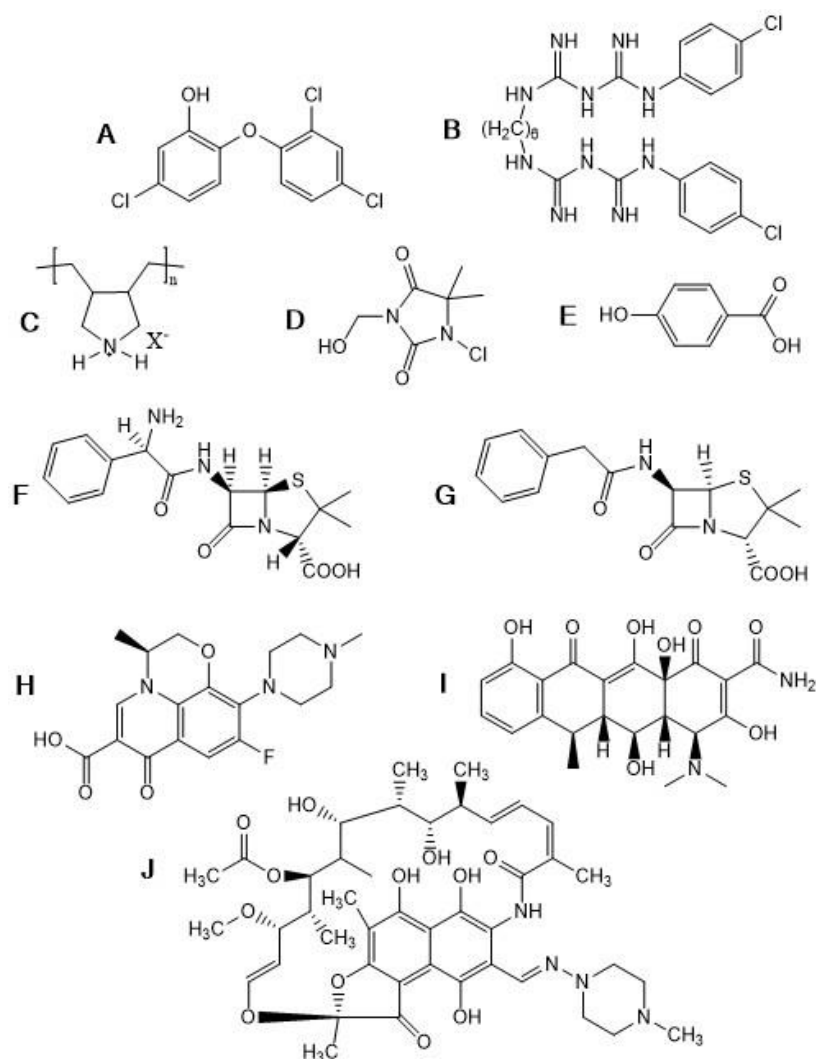


Figure 4.2: Examples of antimicrobial compounds found in biocidal synthetic polymers

Triclosan (A), chlorhexidine (B), example quaternary ammonium (X^- = counter ion ²³) (C), hydroxymethylhydantoin (D), 4-hydroxybenzoic acid (E). The β -lactam antibiotics ampicillin (F) and penicillin (G) effective against Gram positive bacteria. Broad spectrum antibiotics levofloxacin (H), doxycycline (I) and rifampicin (J) effective against Gram positive and Gram negative bacteria including *Mycobacterium tuberculosis* (rifampicin).

4.1.4 Biopassive polymers

As described previously, biopassive polymers exhibit antimicrobial activity through the prevention of bacterial colonisation at its surface (antifouling), the most common polymer being PEG and PEG-

copolymers owing to their low dispersity and biocompatibility^{24,25}.

These biomedically relevant polymers are used to coat surfaces of materials; conveying antifouling activity by resisting the adsorption of proteins²⁶. However PEG polymers have presented some drawbacks, such as the vulnerability of its polyether backbone to oxidative degradation and the production of anti-PEG antibodies even in naïve patients^{27–29}. Alternatives to PEG such as polyoxazoline (POx) demonstrate the same desirable traits as PEG such as antifouling activity and biocompatibility; whilst overcoming some of its pitfalls such as stability^{30,31}. However unlike PEG, POx has not yet been approved by the FDA³². Arguably biopassive polymers are superior to biocidal polymers because they do not require the inclusion of killing groups (quaternary ammonium) or antimicrobial compounds, but still remove the threat of bacterial colonisation at its source. However the lack of FDA approval and low stability raise concerns for the use of these polymers in biomedical applications.

4.1.5 Example biomedical applications of antimicrobial polymers

As mentioned earlier, over half of HAIs are associated with medical devices such as gauzes/meshes and catheters. Gauzes/meshes are routinely used in soft tissue repair, hernia and prolapse surgery, unfortunately bacterial infections are found at 90% of all implantation sites which can delay the healing process and cause severe complications even years after implantation³³. Consequently

antimicrobial polymers have been utilised in order to reduce or eradicate gauze/mesh associated infections, as opposed to the conventional administration of intravenous antibiotics. Meshes made from polypropylene were coated with biocidal degradable polymers poly(D,L-lactic acid) and poly(ε-caprolactone) loaded with ofloxacin or amoxicillin^{33,34}. Both studies observed *in vitro* release of the antibiotics upon degradation of the polymer coating, conferring prolonged antimicrobial activity against *E. coli*. Moreover when used in infection models in rats, signs of bacterial infection were not observed for meshes coated in biocidal polymer³⁴. Similarly catheters are also widely used, primarily to gain access to the vascular system in procedures such as haemodialysis, and are therefore at risk of biofouling. To combat this, biopassive and biocidal polymer coatings containing chlorhexidine, silver compounds and rifampicin have been applied to catheters^{35–38}. As a result these coatings have reduced the risk of bacterial infections and catheter failure caused by thrombosis, however increased cost of coated catheters has deterred their use in the clinic.

Therefore the potential application of antimicrobial polymers and coatings in human medicine is clear. Furthermore since antimicrobial coatings could be applied to many devices (not just gauzes/meshes and catheters), infections from many different surgical procedures could be avoided; resulting in improved morbidity rates and decreased HAI rates.

4.1.6 Drawbacks of synthetic polymers

The manufacture of synthetic polymers has several environmental impacts. Firstly polymer starting materials are derived from non-renewable petro chemical sources, and therefore the polymers themselves are considered as non-renewable materials. Secondly the reactions themselves often require elevated or decreased temperatures, increased reaction pressure and the use of organic solvents. For example the synthesis of poly(trimethylene carbonate) (Figure 4.3), a polymer that shows biomedical application potential, requires temperatures of 130°C and the use of chloroform and hexane for purification ³⁹. The arguably environmentally benign polymers such as polylactic acid (PLA) do not rely on non-renewable feedstocks, since its monomer, lactic acid, can be obtained through fermentation; however polymerisation still requires temperatures of up to 200°C ⁴⁰. Furthermore polymers based on acidic monomers such as PLA and PLGA (Figure 4.3) release acidic monomers into the environment upon breakdown ³⁹. Consequently, if these polymers were implanted into a host, the subsequent release of acidic compounds would have detrimental effects on the surrounding tissues ⁴¹.

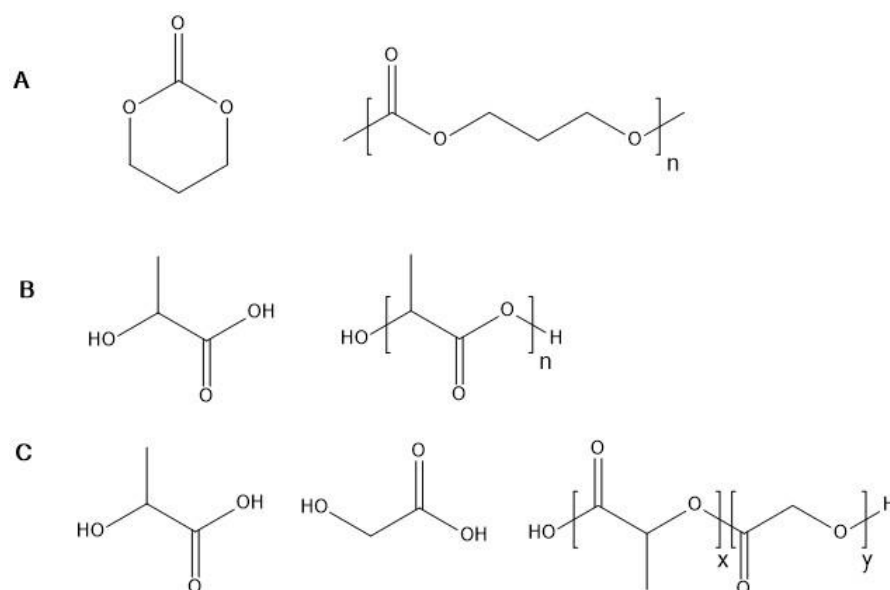


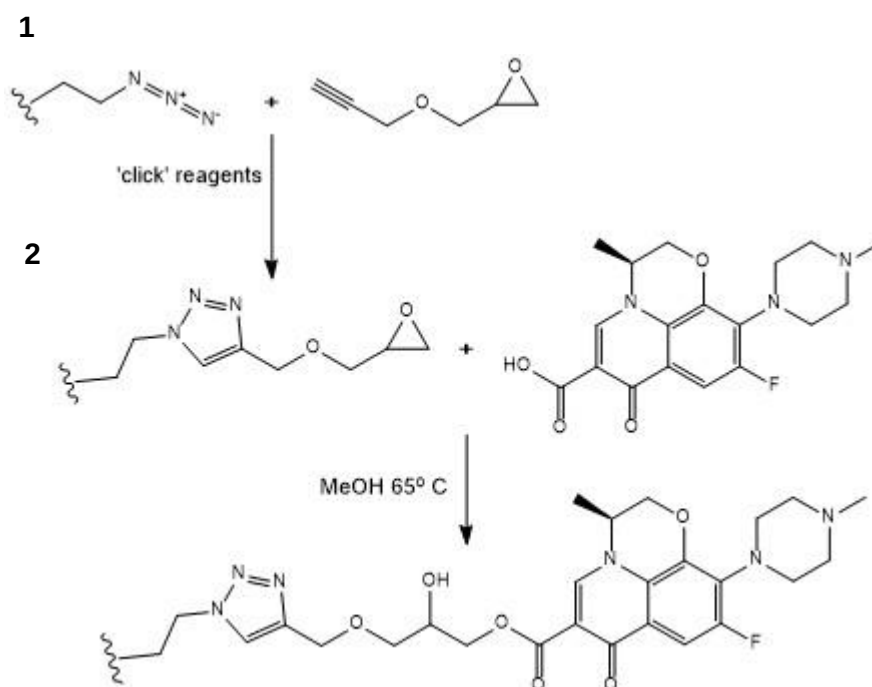
Figure 4.3: Examples of synthetic polymers

Poly(trimethylene carbonate) and its monomer, trimethylene carbonate (A). Polylactic acid and its monomer, lactic acid (B). Poly(lactic-co-glycolic acid) and its monomers, lactic acid and glycolic acid. X represents the number of lactic acid units and Y represents the number of glycolic acid repeats (C).

Therefore an alternative polymer material may be required in order to address these issues. Spider silk, a natural and robust polymer material able to withstand sterilisation by autoclave, offers solutions to the problems outlined here. The production of spider silk using bacterial fermentation is an environmentally benign process, requiring only simple nutrient sources and physiological temperature in order to grow the bacteria efficiently. Furthermore, spider silks are biodegradable and have been shown to be broken down within living hosts over a number of months. However, unlike PLA and PLGA, the breakdown of spider silk releases amino acids or peptides, which can be absorbed by the host.

4.1.7 Antimicrobial silk

As eluded to in section 1.9.2, there are very few studies that have attempted to create antimicrobial spider silk materials. The few that did, elected to create biocidal silk materials by genetically fusing antimicrobial or silver binding peptides with the primary sequence of the silk protein^{42–44}. Whilst these constructs did exhibit antimicrobial activity, zones of inhibition were small, a few mm greater than the size of the material tested. Strangely, attempts to create antimicrobial silk materials using conventional antibiotics have remained unique to silk worm silk research. In one study, large zones of inhibition were produced when silk worm silk was ‘bulk loaded’ with antibiotics by soaking in antibiotic solutions⁴⁵. However, it is reasonable to assume that release of the antibiotics may vary significantly; seeing that release is solely diffusion driven. Therefore conjugation of antibiotic/s to silk proteins via a labile linker can potentially impose an element of control to the release of the antibiotic. In this chapter the azide handles present in 4RepCT^{3Aha} were utilised to functionalise pre-made fibres with levofloxacin via an esterase or pH sensitive linker (Scheme 4.1). Functionalised fibres were tested for activity against a reference strain of *E. coli* (NCTC 12242) grown on solid and liquid medium.



Scheme 4.1: Linker and levofloxacin conjugation to 4RepCT^{3Aha} fibres

Production of levofloxacin functionalised 4RepCT^{3Aha} fibres was a two-step process. CuAAC mediated conjugation of the linker to silk fibres (wavy line). Reaction of levofloxacin with pendant epoxide functional groups producing an ester bond between the linker and antibiotic.

4.1.8 Levofloxacin

Levofloxacin belongs to the fluoroquinolone family of antibiotics and is the S-(-) isomer of ofloxacin (Figure 4.4). This seemingly small difference between the two drugs results in levofloxacin having greater efficacy across a wide range of bacteria than ofloxacin^{46–48}. It is a bactericidal antibiotic that is active against most aerobic Gram positive and negative bacteria, and shows moderate activity towards anaerobic bacteria⁴⁹. As with the other members of the fluoroquinolone family, their therapeutic targets are DNA gyrase and topoisomerase IV^{50,51}. These enzymes bind bacterial DNA; DNA gyrase negatively twists DNA, catalysing separation of daughter chromosomes and

topoisomerase IV un-links daughter chromosomes⁵². Consequently, inhibition of these enzymes results in perturbations of DNA replication, ultimately causing bacterial cell death. In addition the affinity of the quinolones for bacterial protein-DNA complexes reduces off-target effects in eukaryotic cells. Furthermore all of the fluoroquinolones shown in Figure 4 can be ester bonded to the linker in the same way as levofloxacin owing to the single carboxylate group, meaning that different fluoroquinolones could be conjugated if desired.

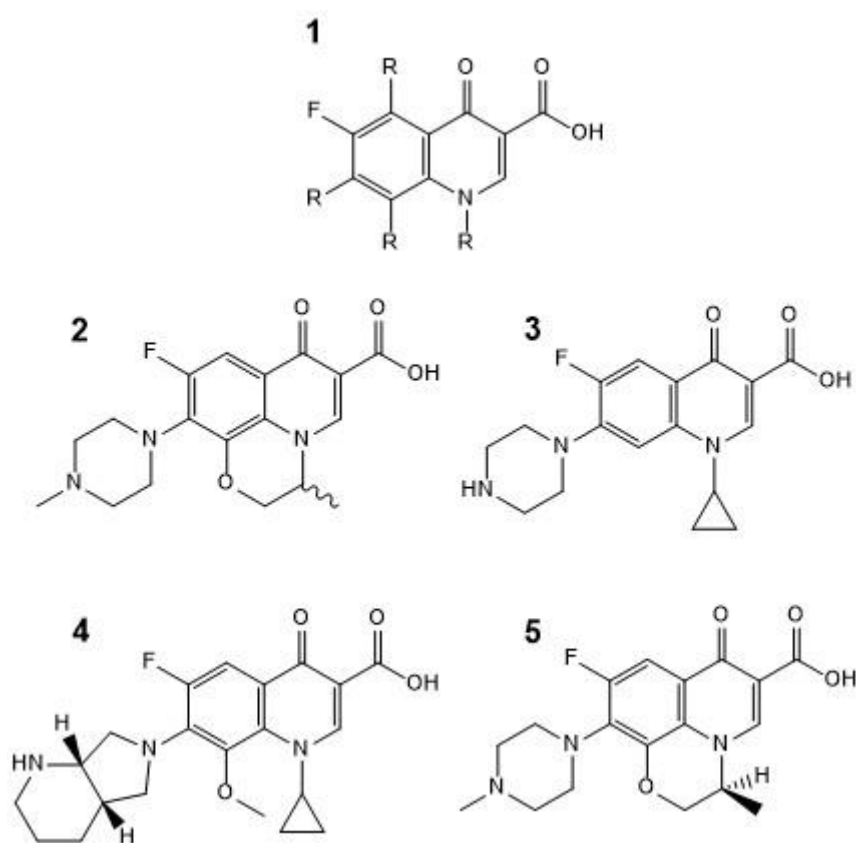


Figure 4.4: Examples of the fluoroquinolone family of antibiotics.

The fluoroquinolones pharmacophore (1). Ofloxacin (2) and ciprofloxacin (3) are second generation fluoroquinolones. Moxifloxacin (4) and levofloxacin (5) are third generation fluoroquinolones. The difference in stereochemistry between 2 and 5 results in greater efficacy for levofloxacin.

4.2 Methods

4.2.1 Conjugation of linker to 4RepCT^{3Aha} silk fibre

The esterase sensitive linker (similar to the linker used in the 2010 study carried out by Kugel and co-workers ¹⁶), glycidyl propargyl ether (2-((prop-2-yn-1-yloxy)methyl)oxirane) was first conjugated to 1 cm long 4RepCT^{3Aha} fibres using CuAAC (Scheme 4.1). Fibres were placed into 1.5 ml Eppendorf tubes that contained 500 µl of reaction solution; 20 mM Tris buffer (pH 7.5), 5 mM sodium ascorbate, 5 mM THPTA, 5 mM CuSO₄ and 9.27 mM linker. The reaction was incubated at room temperature with gentle rocking for 4 hours. After incubation, fibres were removed and washed twice with 20 mM Tris buffer with 1 mM ethylenediaminetetraacetic acid (EDTA) (pH 7.5).

4.2.2 Conjugation of levofloxacin to linker-conjugated fibre

In a 20 ml round bottomed flask, 90.3 mg of levofloxacin was combined with 5 ml of methanol (MeOH) and briefly sonicated to ensure all levofloxacin was dissolved. Washed linker-conjugated fibres were added to levofloxacin MeOH solution and the round bottom flask sealed with a glass stopper and clip. The sealed vessel was heated to 65° C and held using an oil bath for approximately 16 hours (Scheme 1). During this time, the epoxide ring opens and reacts with the carboxylate group of levofloxacin, forming an ester bond. Control fibres (without linker) were also incubated in levofloxacin MeOH solution for the same

time period (termed levofloxacin control fibres). After incubation, fibres were removed from the reaction vessel and washed with 2 ml MeOH four times and twice more with 20 mM Tris (pH 7.5)

4.2.3 Testing antimicrobial activity against *E. coli* NCTC 12242 in liquid culture

Non-functionalised (normal 4RepCT^{3Aha} fibres), washed levofloxacin control fibres and washed levofloxacin-linker conjugated fibres were transferred to 2 ml LB broth in a 15 ml Falcon tube and left to equilibrate at 30°C for 24 hours (each fibre type was in triplicate). After equilibration, 10 µl of an overnight culture of *E. coli* NCTC 12242 was used to inoculate each 15 ml Falcon tube containing 2 ml LB broth, including a blank (LB only in triplicate). All tubes were incubated at 37°C with shaking (180 rpm). Optical densities (OD₆₀₀) were measured every two hours for 16 hours, and a final measurement was taken at 28 hours.

4.2.4 Testing antimicrobial activity against *E. coli* NCTC 12242 using a zone of inhibition assay

A culture of *E. coli* NCTC 12242 was grown with shaking (180 rpm) at 37°C in Mueller Hinton broth (Oxoid) to an OD₆₀₀ of 0.1 and diluted 100 x in the same medium. The diluted culture was spread onto Mueller Hinton agar (Oxoid) plates using a cotton swab. Non-functionalised, levofloxacin control and levofloxacin-linker conjugated fibres (previously soaked for 72 hours in a minimal amount of 50 mM MES (2-(N-

morpholino)ethanesulfonic acid) buffer (pH 5.5)) were placed in the centre of the agar plate. Plates were incubated at 37°C for 120 hours in total.

4.2.5 Statistical analysis

GraphPad Prism 6 was used to analyse data. Cell proliferation numbers were input into the software and bar charts generated. An unpaired two-tailed t-test was used to determine significance between two conditions.

4.3 Results

4.3.1 4RepCT^{3Aha} fibres displayed good chemical stability

During the process of conjugating levofloxacin to the epoxide pendant groups, the fibres were exposed to boiling MeOH for approximately 16 hours. The appearance of fibres was unchanged even after exposure to boiling MeOH. This observation impressed how robust even miniature spidroins are once formed into fibres.

4.3.2 4RepCT^{3Aha} fibres displayed significant antimicrobial activity against *E.coli* NCTC 12242 grown in liquid medium

The growth of bacteria was affected by the presence of all 4RepCT^{3Aha} fibre types (non-functionalised, levofloxacin control and levofloxacin-linker conjugated) and was reflected in the optical density (measured at 600 nm (OD₆₀₀)), a measure of turbidity directly proportional to bacterial growth, of cultures. Figure 4.5 shows growth curves, expressed as OD₆₀₀ vs incubation time, of *E.coli* NCTC 12242 grown in liquid medium (LB broth). Over a 16 hour incubation period, all cultures displayed growth, evidenced by elevated OD₆₀₀. However cultures exposed to each silk fibre type display a growth curve that was shifted to the right when compared to LB alone. This shift indicated that the bacteria took longer to transition to the exponential phase of growth. In addition the growth curves of cultures exposed to all silk fibre types follow a similar profile up until around 10 hours. After this time point, the optical

densities of bacterial culture exposed to levofloxacin-linker conjugated fibres starts to decline, whereas the cultures exposed to other silk fibre types continue to gain in OD₆₀₀.

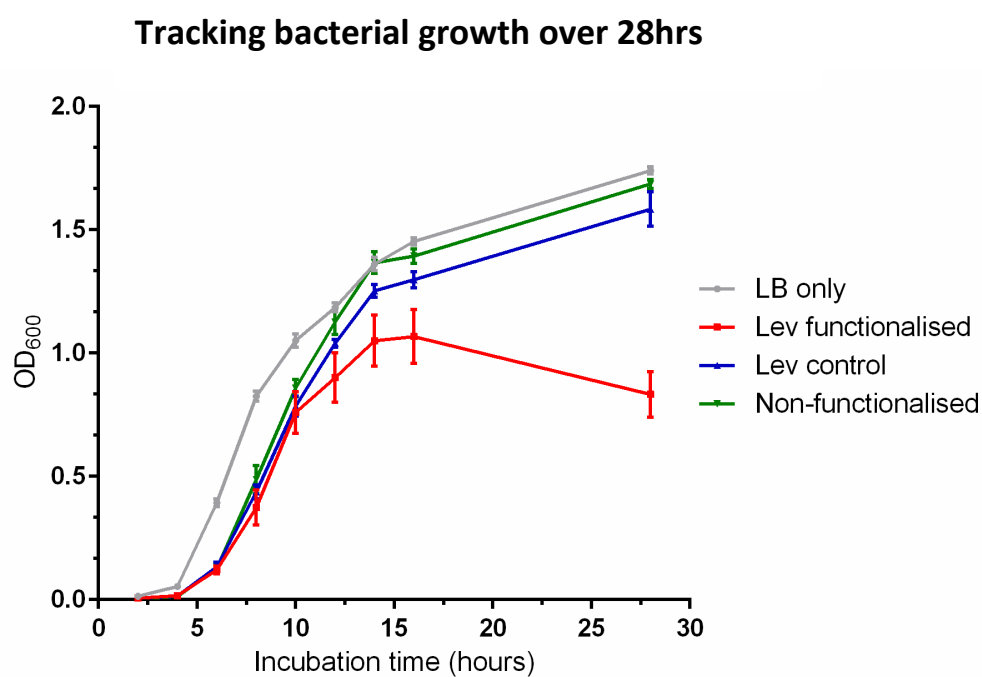


Figure 4.5: Effect of 4RepCT^{3Aha} fibre types on the growth of *E. coli* in liquid medium over 28 hours.

Silk types; levofloxacin-linker conjugated (Lev functionalised), levofloxacin control (lev control) and un-functionalised (non-functionalised). Whilst there was an immediate lag in growth with all silk conditions compared to LB only the Lev Functionalised silk was the only condition to cause a sustained reduction in growth over the time course of the experiment. This was significant compared to LB only and compared to the other two conditions (Lev control and non –functionalised) The Lev control silk did cause a significant reduction compared to LB only until 16hrs possibly due to residual antibiotic on the silk surface. Number of experiments N = 1, number of replicates used in experiment n = 3. Readings were made every 2 hrs up till 16hrs then a final measurement at 28hrs.

The statistical significance of changes in OD₆₀₀ between silk fibre type culture conditions after 10, 12, 14, 16 and 28 hours is shown in Table

4.1. Cultures exposed to all fibre types (including non-functionalised) displayed significantly reduced OD₆₀₀ when compared to LB alone for the 10 hour incubation time point. However cultures exposed to non-functionalised fibres were not significantly different to LB alone after 10 hours incubation (12 hours and onwards). Significant differences between cultures exposed to fibre types (non-functionalised, lev control and lev functionalised) start to occur after 14 hours of incubation, with levofloxacin-linker conjugated fibres having significantly reduced OD₆₀₀ when compared to non-functionalised fibres. Contrastingly, lev control fibres were not significantly different to non-functionalised fibres at 14 hours incubation onwards. After 28 hours incubation however, levofloxacin-linker conjugated fibres had significantly reduced OD₆₀₀ when compared to LB alone, levofloxacin control and non-functionalised fibres. Whereas the OD₆₀₀ for levofloxacin control fibres and non-functionalised fibres were no longer statically different when compared to one another and LB alone.

	Average OD ₆₀₀ (± SD) at each time point					
	8 hrs	10 hrs	12hrs	14hrs	16hrs	28hrs
LB Only	0.83 (±0.04)	1.05 (±0.05)	1.19 (±0.03)	1.36 (±0.05)	1.45 (±0.03)	1.74 (±0.03)
Lev Functionalised	0.37 (±0.12)	0.76 (±0.15)	0.90 (±0.17)	1.08 (±0.20)	1.12 (±0.19)	0.85 (±0.18)
Significance vs LB Only	** (p=0.0037)	* (p=0.0311)	* (p=0.0486)	* (p=0.0446)	* (p=0.0247)	*** (p=0.0006)
Lev Control	0.44 (±0.05)	0.76 (±0.07)	1.04 (±0.03)	1.24 (±0.05)	1.30 (±0.06)	1.58 (±0.12)
Significance vs LB Only	*** (p=0.0004)	** (p=0.0055)	** (p=0.0038)	* (p=0.0458)	* (p=0.0120)	NS (p=0.0958)
Non Functionalised	0.49 (±0.10)	0.86 (±0.06)	1.13 (±0.09)	1.34 (±0.06)	1.39 (±0.05)	1.69 (±0.03)
Significance vs LB only	** (p=0.0052)	* (p=0.0120)	NS (p=0.3367)	NS (p=0.9132)	NS (p=0.1432)	NS (p=0.0907)
Notes: Up to 12hours, there was no significant difference in OD between the lev ctrl, lev functionalised and non-functionalised silks when compared against one another. At 14 hrs and 16 hrs there was a significant reduction in OD ₆₀₀ in the Lev Functionalised compared to Non functionalised with no significant difference to Lev control. At 28hrs Lev functionalised had significantly reduced growth compared to all three other conditions (vs Lev Ctrl ** p=0.003 and vs Non functionalised ***p=0.0008)						

Table 4.1: Changes in average OD₆₀₀ between cultures exposed to different fibre types at 10, 12, 14, 16 and 28 hours incubation.

Differences in OD₆₀₀ between fibre types changed in significance over time. Levofloxacin functionalised fibres, unlike the other fibre types exhibited continued antimicrobial effect, significantly reducing the OD₆₀₀ of the culture past the 16 hour time point

4.3.3 Levofloxacin-linker conjugated fibres inhibited growth of *E. coli* NCTC 12242 on agar plates for 5 days.

Non-functionalised, levofloxacin control and levofloxacin-linker conjugated 4RepCT^{3Aha} fibres were placed in the centre of Mueller Hinton agar plates previously inoculated with *E. coli* NCTC 12242 using a cotton swab and incubated at 37°C. Figure 4.6 shows that after 24 hours incubation, levofloxacin control and levofloxacin-linker conjugated fibres produced zones of growth inhibition (2 cm and 3.5 cm respectively), whereas the non-functionalised 4RepCT^{3Aha} fibre did not. Continued incubation of the plates revealed that after 5 days the zone of inhibition created by the levofloxacin control fibre (seen after 24 hours incubation) had been recolonised by the bacterium. Furthermore bacterial colonies were actually growing on the levofloxacin control fibre itself, an observation also noted for non-functionalised silk fibre. Contrastingly, the levofloxacin-linker conjugated fibre retained a clear zone of inhibition after 5 days of incubation. Additionally the zone was the same size as the one seen after 24 hours incubation, and did not show any signs of recolonization by the bacteria.

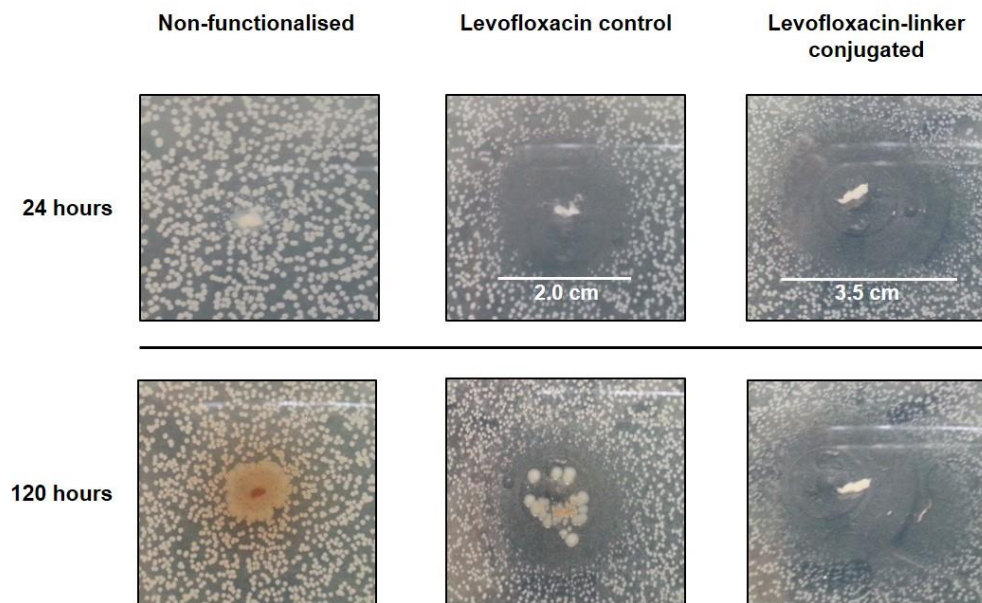


Figure 4.6: The growth of *E. coli* inhibited by levofloxacin-linker conjugated 4RepCT^{3Aha} fibres.

Non functionalised fibre showed no zone of inhibition after 24 hrs or 120 hrs incubation. Levofloxacin control fibre produced a clear zone of inhibition with a 2 cm diameter at 24 hrs however this did not persist to 120 hrs with colonies clearly visible in the zone of inhibition. The levofloxacin linker conjugated fibre produced a clear zone of inhibition with a 3.5 cm diameter at 24 hrs which remained clear at 120 hrs. Experimental repeats N =1, replicates n = 1.

4.4 Discussion

4.4.1 Overview

It was the aim of this chapter to determine whether a bioactive ligand (such as antibiotics) could be conjugated to azide groups within 4RepCT^{3Aha} fibres and retain bioactivity after subsequent release.

Utilising microbiological techniques it was shown that 4RepCT^{3Aha} fibres functionalised with levofloxacin displayed antimicrobial activity.

4.4.2 Bacteriostatic and bactericidal activity of 4RepCT^{3Aha} fibres in liquid medium

It was observed that all 4RepCT^{3Aha} fibre types initially affected the growth of *E.coli* in liquid medium. *E.coli* inoculated into LB medium alone transitioned to the exponential phase of growth at around 5 hours. Whereas exponential growth of cultures exposed to silk fibres was observed to occur after 8 hours incubation, a delay of approximately 3 hours when compared to LB alone. This delay is described in Figure 4.5, and shows that the growth curves of cultures exposed to silk fibres are shifted to the right; meaning that these cultures had longer lag phases. An interesting finding was that all silk fibre types, including non-functionalised fibres, produced very similar growth curves until 10 hours incubation. This suggested that even normal (non-functionalised) 4RepCT^{3Aha} silk fibres exhibit some form of transient antimicrobial activity (causing a prolonged lag phase) that

fades after 10 hours incubation. This transient activity could be considered to be bacteriostatic as opposed to bactericidal, a property previously described for native silk fibres taken from *Tegenaria domestica* (*T. domestica*) webs⁵³. However in contrast to this body of work, *T. domestica* silk was shown only to inhibit the growth of the Gram positive bacterium, *Bacillus subtilis* and not the Gram negative bacterium *E. coli*. Furthermore incubation of native silk with proteinase-k caused a reduction in antimicrobial activity, suggesting that the antimicrobial agent was proteinaceous. The authors speculated therefore that glycoproteins, that coat the native silk fibres, could be responsible for the antimicrobial activity observed. However, the transient antimicrobial activity of 4RepCT^{3Aha} fibres cannot be attributed to glycoproteins. 4RepCT^{3Aha} does not contain any glycoproteins since it is a single recombinant protein expressed in *E. coli*, a host that does not post-translationally modify proteins. Therefore the bacteriostatic activity demonstrated by non-functionalised 4RepCT^{3Aha} must be caused by something else. One potential reason may be that bacteria find it difficult to adhere to dragline silk fibres, an observation previously reported for dragline silk from *N. clavipes* where *E. coli* and *Pseudomonas aeruginosa* (*P. aeruginosa*) do not adhere well to dragline fibres. Despite this finding, no significant difference was observed between liquid cultures of *E. coli* exposed to dragline and control cultures⁵⁴. Consequently the conclusion was drawn that dragline silk fibres do not actively inhibit bacterial growth, but are resistant to bacterial adherence. Therefore it is unclear at this point why

non-functionalised 4RepCT^{3Aha} silk fibres seem to transiently deter bacterial growth. However resistance to bacterial adherence, similar to the activities of biopassive polymers may well be an influencing factor.

After the 10 hour incubation time point though, the growth curves associated with each fibre type start to differ and separate from one another. From this point, cultures exposed to levofloxacin-linker conjugated fibres linger around an OD₆₀₀ of ~1.0, whereas cultures exposed to levofloxacin control and non-functionalised fibres continued to gain in OD₆₀₀, indicating continued bacterial growth. As shown in Table 4.1, the differences in OD₆₀₀ between fibre types only became significant after 14 hours incubation, with cultures exposed to levofloxacin-linker conjugated fibres displaying significantly reduced OD₆₀₀ to non-functionalised fibres and LB alone. This indicated that non-functionalised fibres were no longer inhibiting bacterial growth, allowing the culture to proliferate similarly to LB alone. Cultures exposed to levofloxacin control fibres however were not statistically different to non-functionalised fibre, but were when compared to LB alone. However, considering the increase in OD₆₀₀ in comparison to levofloxacin-linker conjugated fibres and the fact that levofloxacin control fibres were almost not significantly different to LB alone ($P = 0.0458$), the data suggested that the antimicrobial activity was deteriorating. This decline in activity continued and, at the 28 hour incubation time point (Table 4.1), levofloxacin fibres were statistically indistinguishable from LB alone and non-functionalised fibres. Therefore, early antimicrobial activity of levofloxacin control fibres was likely to be caused by small

amounts of levofloxacin that remained absorbed to the silk surface after washing. However the amount of antibiotic left over was not enough to overwhelm the growth of bacteria indefinitely, evidenced by the gain in OD₆₀₀ relative to early incubation time points. Levofloxacin-linker conjugated fibres however did not permit culture density to increase much past ~1.0, and at 28 hours incubation actually reduced the OD₆₀₀ of the culture to approximately 0.8. In comparison to the other fibre types and LB alone, levofloxacin-linker conjugated fibres had a highly significant impact on bacterial growth (Table 4.1). This indicated that the loading of levofloxacin and subsequent release from these fibres was greater than that of levofloxacin control fibres; since levofloxacin was covalently attached to the fibre via the linker and so remained after washing. However bacterial growth was not completely abolished by these fibres evidenced by the densities of the cultures rising to ~ 1.0 over a 16 hour incubation. A potential reason for this lies with the breakdown of the ester bond connecting levofloxacin to the former epoxide group of the linker. As mentioned earlier, this bond is susceptible to cleavage by esterase or acidic conditions. Esterase enzyme are secreted by bacteria into the extra cellular environment and acidic by products are produced by *E.coli* as part of aerobic metabolism⁵⁵. Consequently sufficient quantities of levofloxacin will only be released when the density of the culture reaches a critical point. After this point, a sufficient drop in pH or sufficient amounts of esterase would catalyse the efficient breakdown of the ester bond, releasing the antibiotic into the medium. This model explains why the OD₆₀₀ of

cultures exposed to levofloxacin-linker conjugated fibres initially rose to ~ 1.0 and remained stable for a while. At this point, levofloxacin was released from the linker efficiently; initially stopping the culture from growing further and eventually reducing the culture density, indicating bacterial death.

4.4.3 Sustained antimicrobial activity

The antimicrobial activity of the levofloxacin-linker conjugated fibres was also tested by means of a zone of inhibition assay (Figure 4.6).

The results demonstrated a similar trend to that observed in Figure 4.5 and Table 4.1, where levofloxacin control fibres demonstrated an initial burst of antimicrobial activity, generating a 2 cm zone of inhibition.

However, unlike the data gathered from growth in liquid medium, non-functionalised fibres did not display obvious antimicrobial activity.

Despite this, it was worth noting that the non-functionalised fibre did not appear to have bacteria growing on its surface, presumably because of the difficulty Gram negative bacteria have adhering to silk⁵⁴. The initial activity of the levofloxacin control fibre, as with liquid medium, can be attributed to levofloxacin left behind after washing. Consequently, levofloxacin absorbed to the silk surface is rapidly released into the immediate environment causing a zone of inhibition to form. However, continued incubation saw repopulation of the zone with bacterial colonies. This indicated that, similar to liquid medium growths, the amount of levofloxacin released was insufficient to maintain a clear

zone. Therefore the antibiotic was exhausted, giving way to bacterial recolonization. Contrastingly, the levofloxacin-linker conjugated fibre demonstrated a 1.5 cm larger zone of inhibition that remained clear for 5 days. The larger zone diameter suggested that the loading of levofloxacin was once again higher than that of levofloxacin control fibre. Moreover the fact that the zone remained clear for 5 days reinforces the notion of increased loading, and suggested that levofloxacin was being continually released via breakdown of the ester bond.

In summary, the data gathered from the two experiments identified that the biocidal antimicrobial activity of fibres was attained in a two-step process. First, the levofloxacin absorbed to the silk fibre surface is rapidly released into the environment. Second, the ester bond between levofloxacin and linker is broken by either esterase or lowered pH, causing a second longer lasting phase of levofloxacin release. This effect could be of value in the treatment of infected wounds for example, where an initial burst of antibiotic followed by a sustained release over a number of days would provide enduring protection against infection. Furthermore the apparent biopassive traits of silk fibres add an extra layer of protection to bacterial colonisation after the antibiotic payload is exhausted. In addition, the ability to produce films from functionalised 4RepCT^{3Aha} (the focus of the next chapter) opens up the potential to create biocidal coatings for medical devices; similar to the synthetic polymer coatings outlined earlier.

4.5 References

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5. Functionalisation of 4RepCT^{3Aha} for use as a cell scaffold

5.1 Introduction

This chapter describes how 4RepCT^{3Aha} has been functionalised with copies of the arginine-glycine-aspartate (RGD) functional peptide. This peptide motif is found in extracellular matrix (ECM) proteins such as fibronectin. Cells recognise and bind these motifs via surface proteins called integrins. Therefore functionalisation of 4RepCT^{3Aha} with RGD peptide should improve the performance of 4RepCT^{3Aha} films and fibres in cell culture. Both CuAAC and SPAAC were employed to conjugate RGD peptide to the silk protein and their impact on cell culture compared. Cell culture with macroscopic fibres demonstrated the potential to make 3D scaffolds from 4RepCT^{3Aha}

5.1.1 Tissue Engineering

Tissue engineering is an interdisciplinary field whose primary objective is to understand how human tissues grow. This understanding is then applied to the production of replacement tissues for implantation to repair or enhance tissue function, hence why tissue engineering is sometimes labelled as regenerative medicine ¹. In order to achieve this, polymeric materials are processed into various 3D morphologies (depending on the application) and used to culture or co-culture specific cell types (or combinations thereof) within the lab. Alternatively

materials free of cells are implanted into a living host, providing a framework for host cells to proliferate in to. The materials used must allow cell attachment, proliferation and extracellular matrix (ECM) deposition in order to facilitate correct tissue growth and more importantly, they must be biodegradable and not illicit an immune or significant pyrogenic response when implanted ².

5.1.2 Materials currently used for scaffolding in Tissue Engineering

Cell scaffolding materials can be made from synthetic polymers such as poly lactic acid (PLA) and poly glycolic acid (PGA), or from natural polymeric materials such as proteins and polysaccharides ³⁻⁶. However in this chapter only commonly used natural protein scaffolds and a few examples of their applications are discussed.

5.1.2.1 Protein based scaffolds

The proteins currently utilised as scaffolds within tissue engineering are ECM proteins extracted from animal or human tissues. The ECM is the 'skeleton' of tissues and is a complex environment composed of several proteins that not only offers structural integrity to the tissue, but conveys information into the cells via cellular binding domains and local delivery of molecular cues ⁷. Therefore, utilising ECM proteins to create tissues *in vitro* is a very logical choice, since it is what cells within the tissue would ordinarily 'see' and respond to. Scaffolds can be made from just

one ECM protein, or a combination of proteins depending on the application. Owing to the complexity of the ECM, the resulting materials can vary greatly in terms of composition complexity. Furthermore, scaffold processing conditions and chemical treatments (such as crosslinking), can be used to alter the mechanical properties of the material; increasing complexity further.

5.1.2.2 Matrigel™

One of the most complex materials used in tissue engineering is Matrigel™. This material is a thermo-responsive hydrogel reconstituted from the basement membrane of mouse tumours and is sometimes referred to as basement membrane extract (BME)⁸⁻¹⁰. BME is comprised of several structural proteins, growth factors and enzymes which allow it to support the growth of several cell lines (Table 5.1). Most notably, BME has been used to culture embryonic stem cells; stem cells that can differentiate into any cell type^{11,12}. Importantly, stem cells grown on this material are able to maintain their stemness, however the reasons why remain unclear.

Components of Matrigel/BME			
Proteins ⁹	Growth Factors ¹⁴	Enzymes ^{15, 16}	Other proteins ^{17, 18}
Laminin	Fibroblast growth factor (FGF)	Matrix metalloprotease (MMP)-2	Transferrin
Collagen type IV	Epidermal growth factor (EDF)	MMP-9	Clusterin
Heparin Sulphate proteoglycan	Transforming growth factor beta (TGF- β)	Urokinase	
Entactin	Platelet-derived growth factor (PDGF)	Tissue type plasminogen activator (TPA)	
	Insulin-like growth factor (IGF)		

Table 5.1: The known components of Matrigel/BME hydrogel scaffold.

Table modified from ¹⁹

More commonly BME is used for *in vitro* assays within cancer biology and the production of *in vivo* human tumour models (summarised in Table 5.2), but has also been shown to facilitate the regeneration of cardiac tissue in rats ¹³. Therefore BME would appear to be a versatile and biologically relevant mimic of the ECM, making it highly appropriate for tissue engineering.

Common applications of Matrigel / BME					
<i>In vitro</i>			<i>In vivo</i>		
Assay / used to study	Brief description	Used for	Assay / application	Brief description	Used for
Invasion ²⁰	Cancer cells migrate across a layer of BME on top of a filter into a chamber containing attractant	Characterisation of invasive activity ²¹ , isolation of invasive subpopulations ²² , identifying therapeutic targets ²³	Tumourgraft / xenograft ^{28,29}	Human tumour biopsy coinjected with BME into mice	Creation of human based cancer models in animals quicker and more cost effectively ¹⁹
3D culture	Cancer cells cultured on BME hydrogel	Predict malignant potential ²¹ or to define stage of cancer progression ^{24,25}			
Dormancy	Dormant cancer cells cultured on BME hydrogel	Identification of mechanisms that regulate dormancy and potential therapeutics ²⁷			
Clonogenic	Culturing cells on BME hydrogels by limiting dilution	Identify stem cell populations and subpopulations with different malignant potentials			

Table 5.2: Uses of Matrigel/BME in cancer biology

However, owing to the highly complex nature of BME and variability between batches highlights the fact that this material is very poorly defined ³⁰. Consequently some studies have warned that caution should be used when interpreting cellular activity data while using BME ¹⁴. Furthermore BME only forms soft hydrogel structures of low elastic modulus and stiffness (BME is a visco-elastic material)³¹. Adherent cells can sense the stiffness of their environment and respond by adopting different morphologies or by further differentiation ³². Therefore, since Matrigel/BME is a soft material, it is limited in terms of how it can influence cells mechanically. Despite these apparent drawbacks Matrigel/BME is an effective material on which to culture cells. Consequently some have argued that it may be hard to find a more defined replacement that is able to achieve the same results.

5.1.2.3 Collagen

Collagens are produced by fibroblasts and are the most abundant fibrous ECM proteins. They primarily provide structural stability but also provide cell adhesion points and support chemotaxis and cellular migration⁷. Generally collagen I is the most commonly used collagen in tissue engineering owing to its relative abundance (isolated from skin and bone)³³. In addition to collagen being a major constituent of the ECM, collagen is biocompatible, non-immunogenic and non-antigenic, however in order to achieve this, collagen has to be digested with pepsin^{34,35}. Consequently, collagen scaffolds have been used extensively within cell culture and tissue engineering. Unlike Matrigel/BME however, collagen has been processed into several morphologies such as gels, sponges and fibres (formed by electrospinning); each with their own advantages and disadvantages. Gels of various shapes and thicknesses have been prepared from collagen as early as 1962 and were subsequently widely used to culture cells *in vitro*^{36,37}. Many cell types including, chondrocytes, epithelial cells, keratinocytes, mesenchymal stem cells, cardiomyocytes, fibroblasts and smooth muscle cells have been cultured on collagen gel scaffolds *in vitro*; subsequent implantation of seeded scaffolds regenerated damaged tissues such as cartilage and tendon *in vivo*^{36,38–41}. However whilst collagen gels provide a useful scaffold on which to grow cells *in vitro* and *in vivo*, there is little control over the final properties of the gel. This disadvantage results in collagen gels that are too weak for surgical manipulation and loads

found *in vivo*, necessitating the use of other materials for reinforcement⁴². In addition, the lack of stiffness also causes issues *in vitro*, where gels are contracted by the cells grown on them. Contraction increases the concentration of collagen and reduces the transparency of the gel, making visual inspection difficult^{40,43}. Creating stiffer gels by increasing collagen concentration or by cross-linking can address these issues, however additional problems arise relating to cellular movement and potential cross-linker toxicity^{35,44,45}.

Collagen sponges on the other hand are stiffer than gels (but not rigid enough to be useful in major weight-bearing tissues) and have been employed in some circumstances to mitigate the contractile problem^{40,46}. Similarly to gels, collagen sponges have permitted the culture of a wide variety of cells, including those outlined for gels⁴⁷. However because of the porosity of the sponge scaffolds, optimised conditions for engineering different tissues was achieved by continuous perfusion of the scaffold with medium; avoiding problems such as hypoxic centres and poor distribution of nutrients around the tissue. This technique was used to improve cellular viability and activities such as haematopoiesis, osteogenesis and mucosa formation^{48–50}. As with collagen gels, sponges seeded with chondrocytes have also been implanted into living hosts to regenerate damaged tissues such as cartilage⁵¹. Bone regeneration has also been investigated however, sponges are implanted unseeded. Instead sponges are loaded with bone morphogenic protein (BMP) which is subsequently released into the

implantation site, encouraging surrounding cells in the tissue to invade the scaffold and differentiate into bone ⁵².

Finally collagen can be processed into non-woven fibrous mats by a process known as electrospinning. Here collagen solution is pushed through an electrified needle and collected on a grounded collection plate. If the collection plate is spinning, aligned fibrous mats can be attained, whereas if the collection plate is static, randomly distributed fibre mats are generated ^{53,54}. These collagen scaffolds provide a close mimic to the ECM and similarly to gels and sponges, have been used to successfully culture a variety of cells and regenerate tissue *in vivo* ^{55,56}. However a notable drawback of electrospun collagen is its rapid degradation in culture, necessitating the use of cross linking agents to extend the lifetime of the scaffold ^{54,57}. Furthermore during the process of electrospinning, collagen is first dissolved in hexafluoroisopropanol (HFIP) which can denature the protein extensively.

5.1.2.4 Gelatin

Gelatin is the denatured form of collagen and is extracted from collagenous material either by acidic pre-treatment or alkali pre-treatment. Due to the similarities between gelatin and collagen, gelatin has become an attractive natural polymer for tissue engineering. Morphologies including gels and electrospun fibres have been processed from gelatin and have been utilised in similar ways to the same morphologies made from collagen ^{55,58}. However, gelatin does

not gel at 37°C or above but remains liquid. Instead gelation occurs at around room temperature or lower, and is only altered through the addition of cross linkers or when combined with synthetic polymers ⁵⁹.

Therefore from the few naturally derived proteinaceous cell scaffolding materials introduced here; materials made from single ECM proteins are more defined and customisable in terms of morphology. However, all of the materials described are obtained from animal sources. Consequently variability between batches and immunogenicity, although clinically rare will always be concerns when using these materials ^{60,61}. A potential solution to the immunogenicity problem would be to isolate material from human tissue; however this raises the more dangerous prospect of disease transfer ⁶². Therefore the reproducible production of scaffold proteins recombinantly could remove these concerns and additionally generate potential for scaled-up production.

5.1.3 Recombinant spider silk as cell scaffolds

As described in section 1.10.2, favourable biocompatibility, biodegradability and superior mechanical properties of recombinant spider silk proteins (in comparison to the protein scaffolds discussed earlier) make them attractive candidates for use in tissue engineering and regenerative medicine. Furthermore because the majority of these proteins are made using *E. coli*, batch to batch variability is minimised and production can be scaled up to industrial quantities ⁶³. Several scaffold morphologies made from different recombinant spider silk proteins have already been shown to be capable of supporting

mammalian cells^{64–66}. However a common drawback associated with spider silk scaffolds is the lack of cellular adhesion, leading to poor cell culture. Considering cell scaffolding materials are meant to facilitate the growth of cells, this issue needed to be overcome in order for silk scaffold materials to be useful. Consequently ECM elements, such as RGD peptides, have been introduced into the silk scaffolds either by genetic manipulation or chemical conjugation, resulting in improved cellular adhesion and proliferation^{67,68}. Alternatively, synthetic glycopolymers have been used to mimic glycoproteins within the ECM and improve cellular adhesion of mouse 3T3 fibroblasts on silk films⁶⁹. Here the glycopolymers were conjugated to silk films in a two-step chemical process. Firstly, aspartate and glutamate residues were conjugated with azidopropargylamine using EDC NHS coupling, resulting in azide-presenting silk films. Secondly, alkyne tagged glycopolymers were reacted with the azide groups in the presence of CuSO₄ (CuAAC) yielding glycopolymer conjugated to silk films via a triazole.

Therefore with the capability to improve cellular adhesion and proliferation on silk scaffolds, it was the aim of this chapter to use click chemistry to functionalise 4RepCT^{3Aha} with RGD peptides. The functionalised silk protein was then tested to determine its performance in cell culture.

5.2 Methods

5.2.1 Conjugation of RGD peptide to 4RepCT^{3Aha}

For CuAAC conjugation, 20 x 100 µl reactions were set up. Each contained; 60 µl of 32 µM 4RepCT^{3Aha}, 24 µl of 20 mM Tris buffer pH 7.5, 5 µl of 50 mM THPTA, 5 µl of 50 mM sodium ascorbate, 1 µl of 50 mM CuSO₄ and 5 µl of 5 mM RGD (Peptides International, U.S.A.) peptide with terminal alkyne (Figure 5.1A). Reactions were incubated at room temperature for 2 hours with gentle shaking. After incubation all reactions were pooled and dialysed against 20 mM Tris pH 8.0 buffer to remove excess reactants

For SPAAC conjugation, 20 x 100 µl reactions were set up. Each contained: 50 µl of 95 µM 4RepCT^{3Aha}, 40 µl of 20 mM Tris buffer pH 7.5 and 10 µl RGD peptide with ring-strained alkyne (Figure 5.1B). Reactions were incubated at room temperature for 2 hours with gentle shaking. After incubation all reactions were pooled and dialysed against 20 mM Tris pH 8.0 buffer to remove excess reactants

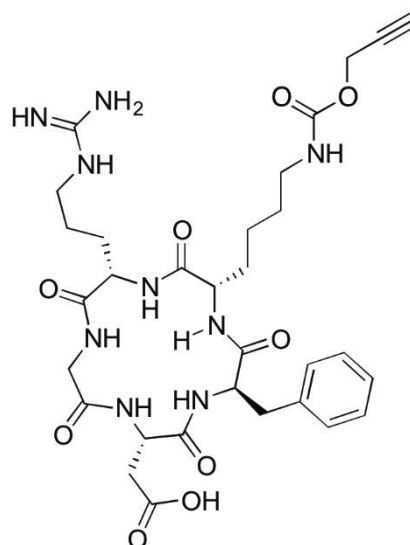
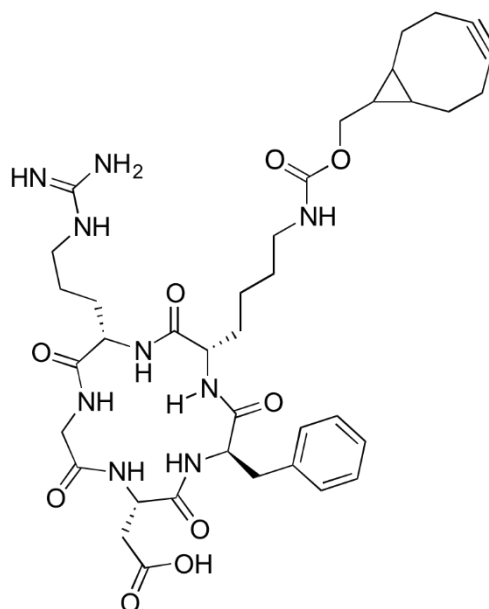
A**B**

Figure 5.1: Cyclic RGD peptides used to functionalise 4RepCT^{3Aha}

The cyclic peptide has two additional residues to RGD, a D-phenylalanine required for cyclisation of the peptide and a lysine. Here the ε amino group of the lysine has been modified to carry either a terminal alkyne (A) or a ring strained alkyne, bicyclononyne (B), via a carbamate bond.

5.2.2 Making films from cyclic RGD conjugated 4RepCT^{3Aha}

Following overnight dialysis, soluble 4RepCT^{3Aha} and 4RepCT^{3Aha} functionalised with cyclic RGD peptide (4RepCT-RGD) was used to construct films within a 24 well non-tissue culture plastic (NTCP) well plate (Falcon). All films were made in sterile conditions by pipetting 200 µg of soluble protein into wells and evaporating off the solvent with sterile air. After film formation, each of the films were soaked in 1 ml 70% (v/v) ethanol for 10 mins to ensure sterility of the film and removed by aspiration. All films were subsequently washed three times with 1 ml sterile phosphate buffered saline to remove traces of ethanol. Finally 1 ml of Dulbecco's modified eagle medium (DMEM) (Gibco) was added into the wells and the plate transferred to an incubator set at 37°C 5% CO₂ to allow the media to warm up

5.2.3 SPAAC conjugation of RGD peptide to premade 4RepCT^{3Aha} fibres

4RepCT^{3Aha} fibres were made as described in section 3.2.1 . Fibres were placed into an Eppendorf tubes containing 180 µl 20 mM Tris pH 7.5 and 20 µl of 5 mM RGD peptide. Reactions were incubated at room temperature for 2 hours with gentle shaking. After incubation, fibres were removed from the reaction tube and washed with 20 mM Tris pH 7.5 buffer. Fibres were sterilised by autoclave (120°C, 15 minutes) and again with 70% (v/v) ethanol.

5.2.4 Cell culture

A vial of commercially immortalised mesenchymal stem cells (CiMSCs) (Japanese Collection of Research Bioresources) or MSCs expressing green fluorescent protein (GFP) (MSC GFP) were rapidly thawed and subsequently cultured in T75 flasks (Corning Costar). Cells were maintained in DMEM containing 10% (v/v) fetal bovine serum (Sigma Aldrich) and 1% (v/v) penicillin-streptomycin (Sigma Aldrich) and incubated at 37°C 5% CO₂. Once confluent, cells were passaged using trypsin/EDTA (0.25%/0.02% v/v) (Sigma Aldrich) and their culture continued no further than 8-10 passages.

5.2.5 Culture of MSC GFPs on cyclic RGD conjugated 4RepCT^{3Aha} fibres

RGD conjugated fibres were tested against unmodified 4RepCT^{3Aha} fibres. Sterile fibres were placed into separate wells of a NTCP 24 well plate. 1 ml of DMEM was added to the wells, and the plate incubated at 37°C 5% CO₂ to allow the media to warm up. MSCs GFP were harvested from a single T75 flask and counted using a Neubauer improved haemocytometer. 25 000 MSCs GFP were added into the wells containing fibres, and the plate returned to the incubator for 4 hours. After incubation fibres were transferred to a fresh NTCP plate containing pre-warmed DMEM and returned to the incubator for 24 hours.

5.2.6 Visualisation of MSC GFPs grown on RGD 4RepCT^{3Aha} fibres

After 24 hours incubation, the fibres within the well were inspected using phase contrast and fluorescence microscopy at 4 x magnification (Leica DMRBE) and velocity software (Improvision UK). Images of the fibres were taken with phase contrast, and then under fluorescence conditions (using 400 nm light to excite the GFP) to identify the location of cells growing along the fibres. Cells observed to be attached to the fibres were then counted manually.

5.2.7 Culture of CiMSCs on RGD conjugated 4RepCT^{3Aha} films

RGD conjugated films were tested against unmodified 4RepCT^{3Aha} films, NTCP as a negative control and tissue culture polystyrene (TCP) as a positive control (Figure 5.2). CiMSCs were harvested from a single T75 flask and counted using a Neubauer improved haemocytometer. Wells containing pre-warmed DMEM were seeded with 25 000 CiMSCs, as shown in Figure 5.2, and returned to the 37°C 5% CO₂ incubator for 24 hours.

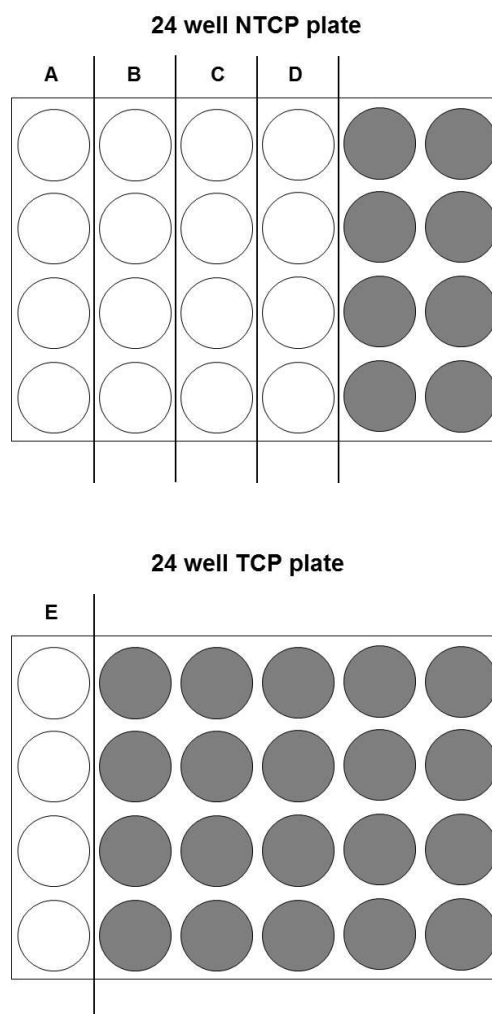


Figure 5.2: Layout of conditions within 24 well plates used to culture CiMSCs.

Top image represents a non-tissue culture plastic (NTCP) 24 well plate used to culture CiMSCs on 4RepCT^{3Aha} films. Column A is the negative control, CiMSCs grown on NTCP plastic. Column B contains unmodified 4RepCT^{3Aha} films. Column C contains RGD modified 4RepCT^{3Aha} films. Column D represents a blank, DMEM media alone. Bottom image represents tissue culture plastic (TCP) 24 well plate. Column E represents the positive control, cells grown on tissue culture plastic. Greyed wells represent wells that were not used.

5.2.8 Quantification of cellular proliferation

After the incubation period, wells were inspected and images taken by phase contrast microscopy using 4 x magnification (Leica DMRBE) and velocity software (Improvision UK). Cellular proliferation was assessed

by PrestoBlue™ assay (Invitrogen), an assay that quantifies live, metabolically active cells. Here old DMEM in all wells (including the blank- DMEM only, no cells) was aspirated and replaced with fresh DMEM containing PrestoBlue solution at 1X concentration. Plates were returned to the incubator for a further 2 hours, after which 200 µl of DMEM PrestoBlue from each well was aspirated and transferred to a black 96 well plate (Corning Costar). The fluorescence of the aspirates was determined using a TECAN infinite 200 plate reader and TECAN i-control 1.10.4.0 software using an excitation wavelength of 535 nm and an emission wavelength of 615 nm. A standard curve was made by incubating increasing numbers of CiMSCs with DMEM + PrestoBlue for 2 hours (plotted in GraphPad Prism 6 (Figure 5.3)). An exact copy of the data was used to quantify cell numbers in Microsoft Excel 2010. Total cell numbers in each well were calculated from the trend line of the standard curve using the following command; =
IFERROR(TREND(X AXIS cell range, Y AXIS cell range, RFU number cell),""). This command utilised the equation of the straight trend line to calculate total cell number of the well. Cellular proliferation was calculated by subtracting the initial seeding density (25 000) from the total cell number calculated from the standard curve trend line. A calibration curve was not constructed for each experiment which may contribute slight inaccuracies in calculated cell numbers. However using a premade calibration curve is common practice in cell culture due to cell, space and practicality constraints.

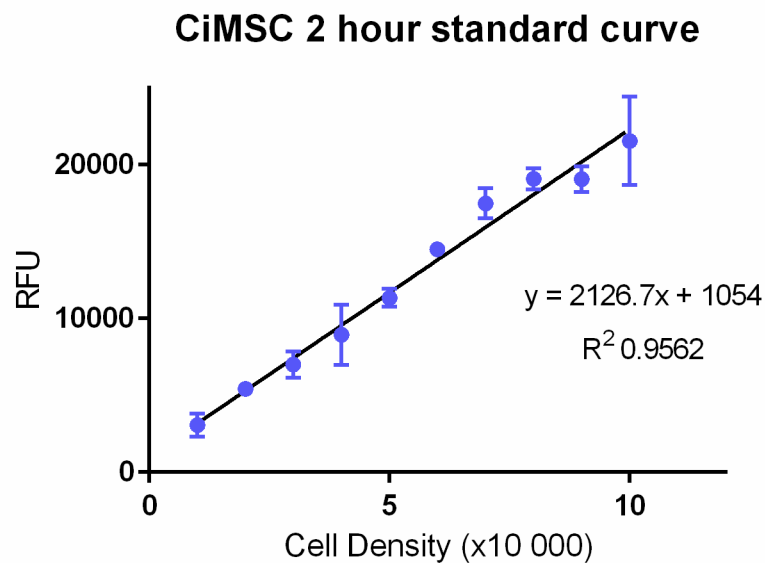


Figure 5.3: A standard curve of CiMSCs of increasing density incubated with DMEM PrestoBlue for 2 hours

Cell numbers, displayed on the x axis, range from 10 000 up to 100 000 and increase in increments of 10 000. The relative fluorescence absorbance units (RFU) are displayed on the y axis and increase linearly with increasing cell numbers. This generated a reliable standard curve with a well-fitting trend line with an R^2 value of 0.9562. The equation representing the trend line is displayed bottom right. Error bars represent standard error of mean. Experimental repeats $N=1$. Number of replicates per data point $n = 3$.

5.2.9 Statistical analysis

GraphPad Prism 6 (GraphPad Software Inc, U.S.A.) was used to analyse data. Cell proliferation numbers were input into the software and bar charts generated. An unpaired two-tailed t-test was used to determine significance between two conditions.

5.3 Results

5.3.1 RGD functionalised 4RepCT^{3Aha} films and fibres displayed good thermal and chemical stability

After the formation of 4RepCT^{3Aha} and RGD modified 4RepCT^{3Aha} films, they were washed with 20 mM Tris buffer (pH 7.5) and did not show any obvious signs of resolubilisation. Following washing the films were sterilised with 70% (v/v) ethanol which again did not have any obvious deleterious effects on the films cast within the wells and to the naked eye, remained intact and uniform in appearance. Films remained bound to the polystyrene surface and their appearance unperturbed even after incubation at 37°C with DMEM. RGD functionalised fibres were also unaffected by sterilisation with 70% (v/v) ethanol, but most notably remained intact after autoclaving, showcasing the remarkable thermal stability previously demonstrated⁷⁰. As a result this indicated that both films and fibres were highly water insoluble and could therefore withstand prolonged culture conditions.

5.3.2 CiMSCs were confluent and displayed an adherent morphology after 24 hours culture on RGD functionalised 4RepCT^{3Aha} films

After 24 hours incubation, each of the wells containing cells was inspected using phase contrast microscopy (Figure 5.4). CiMSCs cultured on NTCP (negative control) were not confluent and displayed low numbers of sparsely distributed adherent cells. The few adherent

cells displayed the typical morphology of adherent fibroblastic cells i.e. flattened and needle-like. However single cells with a rounded morphology were also observed along with clumps of rounded cells, indicating a lack of adhesion to the NTCP surface. Unmodified 4RepCT^{3Aha} films yielded similar results with a very low confluency monolayer of cells and few displaying an adherent morphology. In addition to the cells, dark debris-like objects were observed along with clumps of rounded cells. In contrast, cells cultured on TCP (positive control) were approximately 60-70% confluent after 24 hours incubation. The vast majority of cells displayed adherent fibroblastic morphology, however some rounded cells were observed on top of adherent cells. CiMSCs cultured on RGD functionalised 4RepCT^{3Aha} films however were approximately >95% confluent after 24 hours incubation. Similarly to cells cultured on TCP, the vast majority of cells adopted an adherent morphology, rounded non adherent cells (light white spheres) were not observed however dark spots, similar to unmodified 4RepCT^{3Aha}, were observed. Therefore the identity of the dark spots was thought to be aggregates of silk protein since they are only found in wells containing silk film.

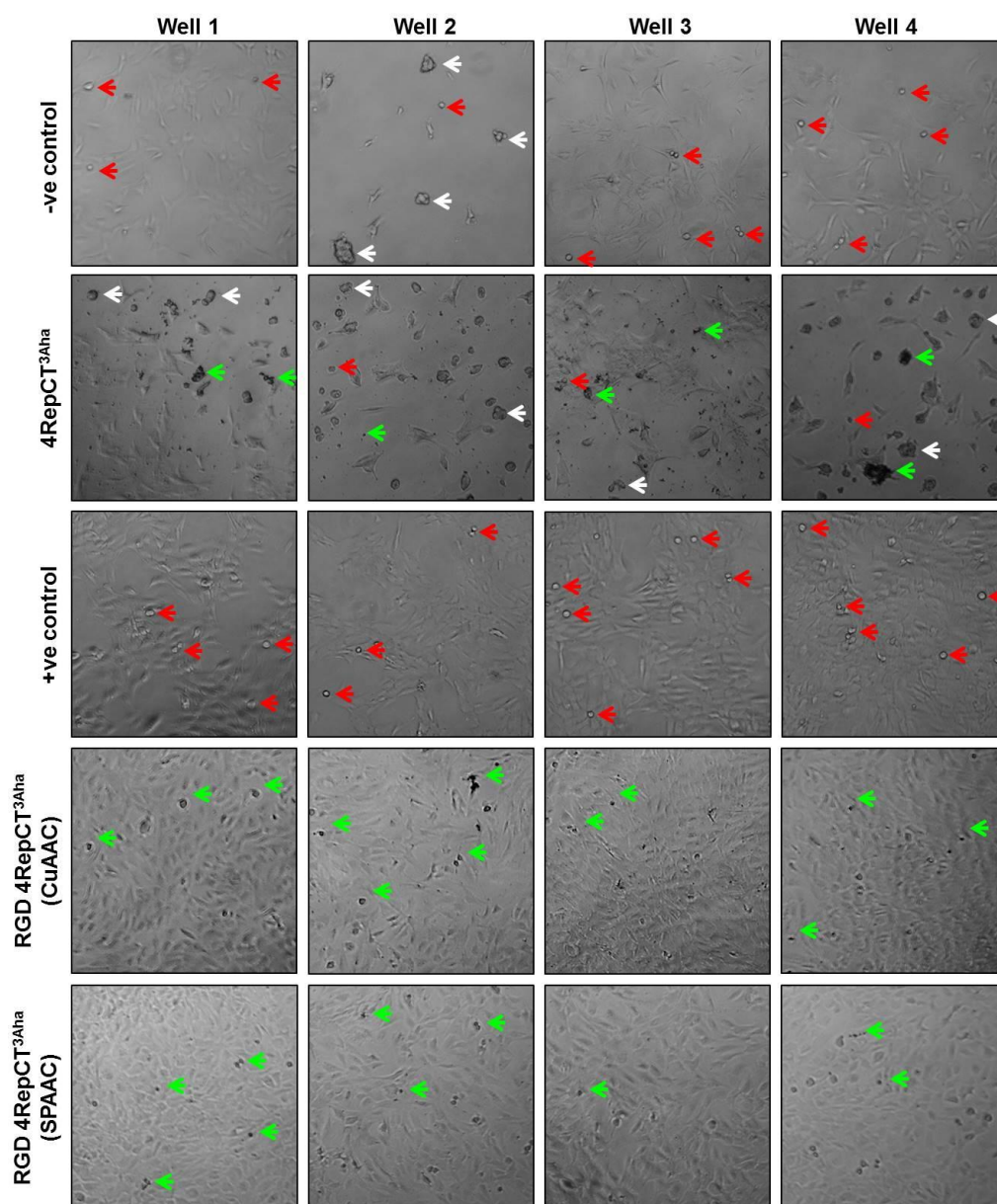


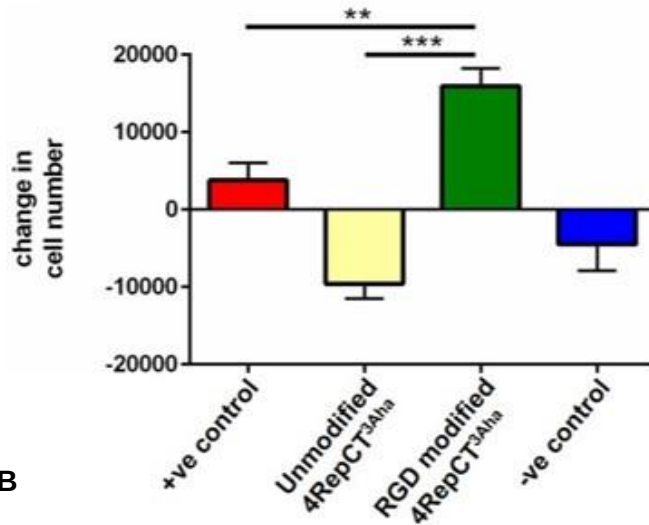
Figure 5.4: CiMSCs after 24 hours growth on NTCP, TCP, unmodified 4RepCT^{3Aha} film and RGD modified 4RepCT^{3Aha} film.

Single non-adherent cells (red arrows), clumps of non-adherent cells (white arrows) and dark debris like structures (green arrows). Large gaps are present in negative control and unmodified 4RepCT^{3Aha} film condition. Smaller gaps are present in the positive control. Cells grown on RGD 4RepCT^{3Aha} are very tightly packed, with only very small gaps between cells visible. Experimental repeats N = 1, replicates per condition n = 4.

5.3.3 RGD peptide conjugated to 4RepCT^{3Aha} using CuAAC and SPAAC resulted in significantly improved cellular proliferation.

After 24 hours of incubation the proliferation of CiMSCs was assessed using the PrestoBlue assay for each culture condition. Figure 5.5 shows that the negative control and the unmodified 4RepCT^{3Aha} film conditions consistently had negative changes in cell number after 24 hours. This indicated that cells were dying in these conditions, with 4RepCT^{3Aha} yielding cell numbers approximately 50% of the initial seeding density and the negative control losing up to approximately 60%. Contrastingly cells grown on TCP, the positive control, and RGD 4RepCT^{3Aha} films demonstrated gains in cell number. The positive control demonstrated gains in cell number between 15 – 60%, whereas RGD functionalised 4RepCT^{3Aha} films demonstrated gains of 63 – 118%. The gains in cell number observed for RGD conjugated films, made by CuAAC and SPAAC, were significantly higher than those observed for the positive control.

A CiMSC proliferation after 24 hours incubation



B

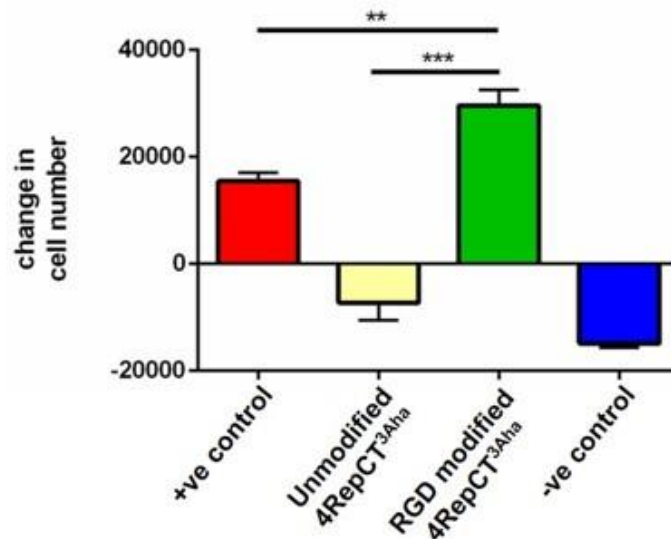


Figure 5.5: Proliferation of CiMSCs after 24 hours culture on RGD modified 4RepCT^{3Aha}

Cell growth is supported on tissue culture plates (+ve control) whilst the normal plastic plates (-ve control) show a loss of cells as expected. The growth of cells on CuAAC (A) and SPAAC (B) modified 4RepCT^{3Aha} silk films is significantly increased compared to the +ve control for both methods (CuAAC $p=0.0091$ and SPAAC $p=0.0058$). The unmodified silk films did not support the growth of cells in either experiment with a significant reduction in cell number compared to modified (CuAAC $p=0.0001$ and SPAAC $p=0.0002$). Data is the mean of $n=4$ replicates $\pm$ standard error of the mean. Experimental repeats $N=1$.

5.3.4 There was no significant difference in cellular proliferation between RGD modified 4RepCT^{3Aha} films made by CuAAC and SPAAC

RGD modified 4RepCT^{3Aha} films made by CuAAC and SPAAC were tested against one another to determine if method of conjugation had any effect on the proliferation of cells (Figure 5.6). Under identical conditions to the previous experiments, 25 000 CiMSCs were seeded into negative control wells, positive control wells and wells containing RGD 4RepCT^{3Aha} film made by either CuAAC or SPAAC. After 24 hours incubation, the change in cell numbers, depicted in Figure 5.6, followed a similar pattern to that observed in Figure 5.5. The negative control and unmodified 4RepCT^{3Aha} film once again demonstrated a loss of cells; approximately 20 – 50% respectively of the initial seeding density. Whereas a gain of approximately 50% over the initial seeding density was observed for the positive control. As observed in Figure 5.5, RGD modified 4RepCT^{3Aha} films yielded higher gains in cell number; with both CuAAC and SPAAC conjugation methods seeing initial cell densities double (~104% and ~134% respectively). Although RGD 4RepCT^{3Aha} films made using SPAAC gave slightly higher cell numbers, there was no significant difference when compared to RGD 4RepCT^{3Aha} films made using CuAAC. However, both modified films again showed a significant gain in cell number when compared to the positive control.

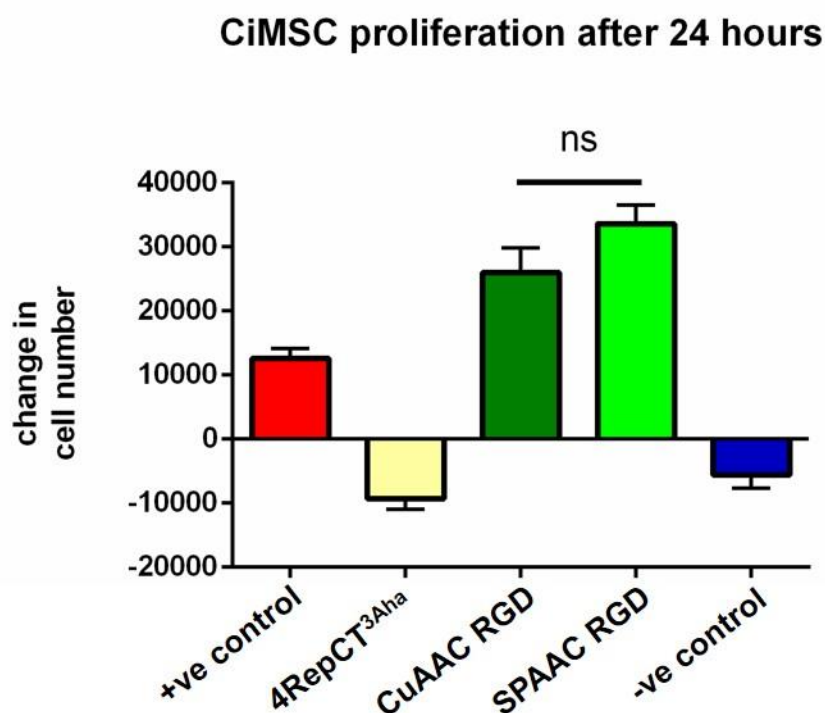


Figure 5.6: Comparison of the performance between RGD modified 4RepCT^{3Aha} films made using CuAAC and SPAAC.

The positive and negative controls responded as expected with an increase and loss in cell number respectively over 24hrs. Cell growth is significantly increased on the CuAAC RGD and SPAAC RGD modified silk films compared to +ve control (CuAAC $p = 0.0312$, SPAAC $p = 0.0031$). There is no significant difference in cell number between the CuAAC and SPAAC although there is a ~30% reduction in cell number in the CuAAC compared to SPAAC. Data is the mean of $n=4$ replicates $\pm$ standard error of the mean. Experimental repeats $N = 1$.

5.3.5 4RepCT^{3Aha} fibres functionalised with RGD peptide have greater numbers of adherent cells than unmodified fibres after 24 hours culture.

After 24 hours incubation, both RGD functionalised and unmodified fibres were observed using phase contrast microscopy and fluorescence microscopy (Figure 5.7). Both fibre types were seen clearly under phase contrast and, when excited with 400 nm light, the

adherent cells expressing GFP were observed to follow the architecture of the silk fibres. Unmodified 4RepCT^{3Aha} fibres did not display many adherent cells after the 24 hour incubation period, giving a total number of 54 cells across all three replicates. RGD functionalised fibres on the other hand displayed 153 adherent cells across all three replicates, suggesting that RGD functionalisation improved cellular adhesion. Furthermore, flattened needle-like cells were observed only on the RGD functionalised fibres; a morphology that again suggested that cells were more able to adhere to RGD functionalised fibres.

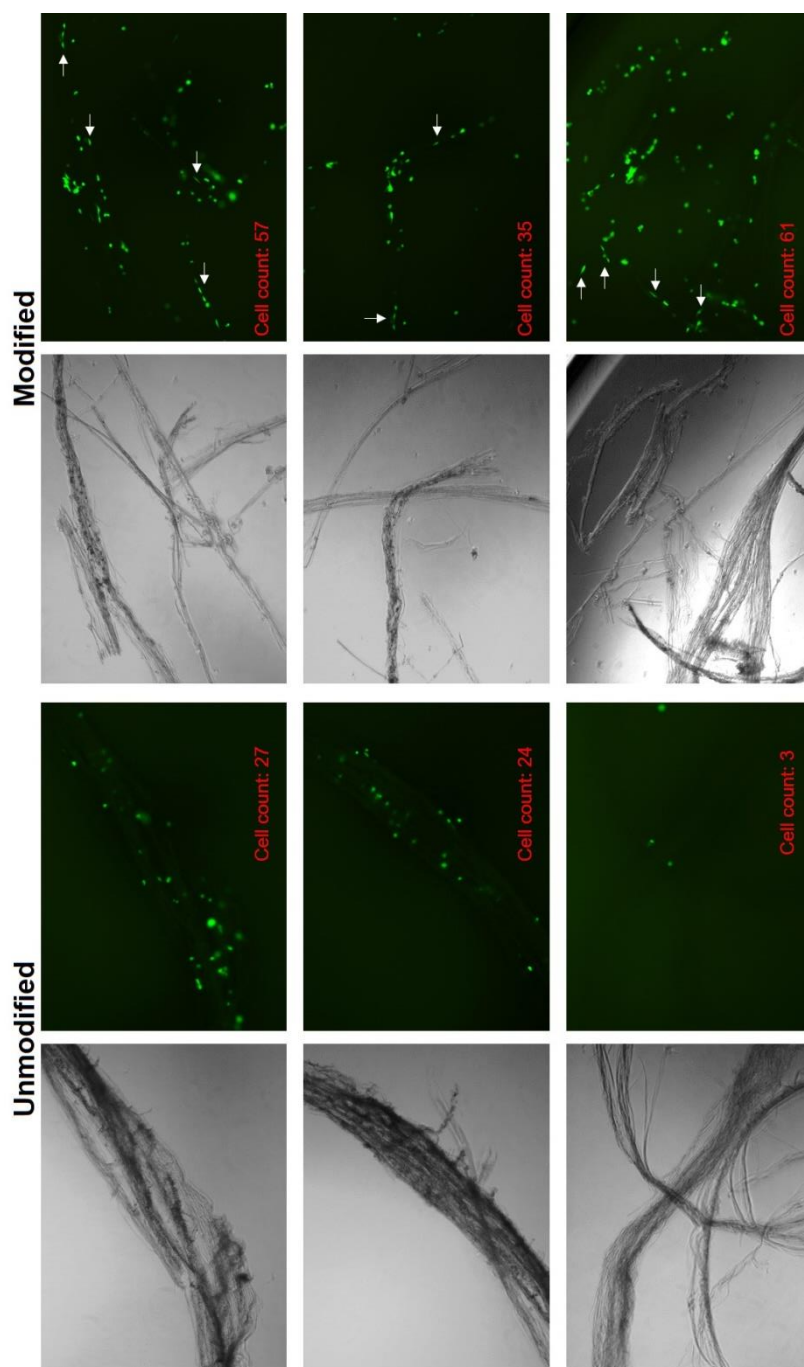


Figure 5.7: MSC GFP cells cultured for 24 hours on unmodified and RGD functionalised 4RepCT^{3Aha} fibres.

Phase contrast and fluorescent images show CiMSC adherence on modified and unmodified silk fibres. The cell number per field of view is increased in the modified silk compared (average = 57) to unmodified (average = 18). Fluorescence is GFP expression by the cells excited at 400nm. Experimental repeats N = 1, replicates per silk type (unmodified or modified) n = 3.

5.4 Discussion

5.4.1 Overview

It was the aim of this chapter to improve the performance of 4RepCT^{3Aha} silk protein in cell culture through functionalisation with the well-known extracellular matrix peptide adhesion motif, RGD. Utilising routine cell culture techniques, light microscopy and a fluorescence based cell quantification assay it was shown that the performance of 4RepCT^{3Aha} was greatly improved by functionalisation, such that tissue culture polystyrene was outperformed.

5.4.2 The thermal and chemical stability of 4RepCT^{3Aha} films and fibres impact on application

The robust stability of films and fibres made from 4RepCT^{3Aha} and RGD modified 4RepCT^{3Aha} permit two useful attributes for its use as a scaffolding material. The first is that films and fibres can be rigorously sterilised to ensure that infectious microorganisms, typically seen in animal derived scaffolds, are eliminated; minimising time and resource costly infections⁷¹. The second is that 4RepCT^{3Aha} scaffold materials persist under cell culture conditions, unlike other proteinaceous scaffolds that are rapidly degraded during cell culture⁵⁴. Therefore cells grown on 4RepCT^{3Aha} materials could be cultured for an extended period of time, without concern of scaffold degradation. This would be especially useful when attempting to create a synthetic tissue *in vitro*, a

process that involves the culture or co-culture of different cell types over a prolonged period of time^{72,73}. However, this does not mean that 4RepCT^{3Aha} materials will persist indefinitely. Previous studies that have implanted silk materials into living hosts observed that degradation occurred after several months, suggesting that degradation is mediated by enzymatic digestion and immune cell activity^{65,74,75}.

5.4.3 Unmodified 4RepCT^{3Aha} performs poorly as a cell culture scaffold

In the experiments performed in this chapter, unmodified 4RepCT^{3Aha} was shown to poorly support the adhesion of CiMSC and MSC GFP cells. Figure 5.4 demonstrated that unmodified silk films did not allow adequate cellular adhesion, evidenced by the presence of individual rounded cells, clumps of rounded cells and cells growing on top of one another. Consequently the lack of cellular adhesion, and therefore survival signals, resulted in the death of cells cultured on the unmodified silk film⁷⁶. The loss of cells directly contributed to the low confluency of cells and the subsequent net loss of cells observed after quantification by PrestoBlue assay (Figure 5.4 and 5.5 respectively). These observations were very similar to those for the negative control and therefore suggest reasons as to why cellular adhesion was so poor for unmodified 4RepCT^{3Aha}. Cells in the negative control wells were cultured on non-tissue culture polystyrene (NTCP), meaning the cells were presented with a hydrophobic surface. The lack of charge meant

that the cells find it hard to adhere to the plastic surface, a problem that is overcome (to make tissue culture polystyrene (TCP)) by chemical treatment ⁷⁷. Therefore, because of the hydrophobicity of silk proteins in general and the fact that they contain very few charged residues may be why unmodified 4RepCT^{3Aha} films did not allow cells to adhere well. Interestingly the same observation was not made in a 2010 study that used 4RepCT films to culture primary fibroblasts ⁶⁶. Here they concluded that films supported the attachment and growth of these cells, evidenced by the increase in cell number over a period of up to 11 days. However, the images of cells grown on films after just 1 day of incubation display a culture of very low confluency (similar to results shown in this chapter). Furthermore, considering the initial seeding density of 15 000 cells/cm² (4781 cells per well of a 96 well plate), a density not too dissimilar than the one used in this study (13 157 cells/cm²), and the images shown of cells growing on films after 1 day, it would appear that most of the cells initially seeded are lost. Despite this initial loss, the continued culture of cells seeded onto 4RepCT films resulted in their proliferation. However this is not evidence that 4RepCT films support cellular adhesion, since it was shown that the fibroblasts produced and deposited collagen into their extracellular space from day four onwards. Consequently, collagen deposits may have in fact been responsible for cellular adhesion, not 4RepCT. Therefore the conclusion that films support cellular adhesion may be slightly misleading. Instead a more fitting conclusion may have been that whilst

4RepCT films did not allow the initial seeding of cells to adhere well, the proliferation of cells was facilitated upon continued culture.

5.4.4 Functionalisation of 4RepCT^{3Aha} with RGD peptide

It was demonstrated that films made from RGD functionalised 4RepCT^{3Aha} harboured significantly more cells than films made from unmodified 4RepCT^{3Aha} after 24 hours (Figure 5.5 and 5.6). Since RGD peptide is known to improve cellular adhesion on various surfaces, including spider silk, we can conclude that both CuAAC and SPAAC successfully conjugated RGD peptide to 4RepCT^{3Aha} ^{67,68,78}.

Furthermore the presence of RGD peptide did not obviously interfere with the fabrication of the films which appeared identical to films made from unmodified 4RepCT^{3Aha}. Consequently, because of the presence of RGD peptide, more cells were able to adhere upon seeding ultimately producing higher cell counts. Importantly, because incubation was only over 24 hours, the cells would not have time to produce and deposit enough collagen to alter cellular adhesion as seen in other studies ⁶⁶. Meaning that increased cellular adhesion was a direct result of RGD functionalised alone. Images of the CiMSCs after 24 hours display a high confluency monolayer which informed on the amount and the distribution of RGD peptide. As highlighted in section 3.1.6 soluble 4RepCT^{3Aha} contains six L-Aha residues, three in the thioredoxin solubility tag and three in the C terminal domain of 4RepCT. Therefore one 4RepCT^{3Aha} monomer has the potential to be functionalised with six RGD peptides, unlike previous attempts to modify spider silk

proteins with RGD peptides that only permit one peptide per silk monomer⁶⁷. Consequently the potential to a 6:1 ratio of peptide to silk protein monomer ensures that many copies of the functional peptide are presented to the cells. Regarding distribution of the peptide across the film surface, if distribution of RGD was for example concentrated in patches throughout the film, one would expect to see patterning of adherence and non-adherence in the monolayer⁷⁹. Consequently the lack of any patterning is therefore highly suggestive that RGD peptide was evenly distributed across the film.

5.4.5 The impact of copper on cell culture

The toxicity of copper including Cu(I) is well known and has been shown to decrease cell viability and impact on differentiation and gene expression^{80,81}. The fact that CuAAC requires Cu(I) to catalyse the cycloaddition reaction between terminal alkynes and azides therefore raised concern. However, as shown in Figure 5.4, 5.5 and 5.6, CiMSCs grown on RGD films made using CuAAC were able to proliferate well. Furthermore, despite a 30% difference in cell number after 24 hours, there was no significant difference in cellular proliferation between RGD 4RepCT^{3Aha} films made using CuAAC and the copper free method, SPAAC (Figure 5.6). This suggested that dialysis of RGD functionalised 4RepCT^{3Aha} and the subsequent washing steps (after film formation) were able to remove copper to acceptable levels. In order to conjugate the RGD peptide to 4RepCT^{3Aha} 500 μ M of copper was used, however

previous studies have shown that 100 μM copper was enough to significantly reduce cell viability⁸⁰. Therefore because CuAAC made RGD films performed similarly to SPAAC made RGD films, we can assume the copper content of functionalised films was below 100 μM . However, it may be possible that trace amounts of copper were left behind, therefore contributing to the ~30% difference in total cell number between SPAAC made RGD 4RepCT^{3Aha} films and CuAAC made RGD 4RepCT^{3Aha} films. From these results it appears that functionalised 4RepCT^{3Aha} made using CuAAC or SPAAC are both appropriate for use in cell culture. However the flexibility to be able to choose either CuAAC or SPAAC broadens the repertoire of potential ligands and potentially the applications. For example, if it was desired to functionalise 4RepCT^{3Aha} with a ligand that chelated metals, CuAAC would not be appropriate since the ligand would chelate the copper ions. Therefore, in this case, SPAAC would be a superior option allowing the conjugation of the metal chelating ligand metal free.

5.4.6 RGD functionalised 4RepCT^{3Aha} fibres

The performance of RGD functionalised 4RepCT^{3Aha} fibres was tested in cell culture. Mesenchymal stem cells were once again used however for these experiments cells expressing GFP (MSC GFP) were used allowing for fluorescence microscopy analysis after incubation. The fluorescence images taken of RGD modified and unmodified fibres after 24 hour cell culture all display dots of green signal (Figure 5.7). Since

the cells used in this experiment express GFP, these green dots are individual cells. Therefore both RGD modified and unmodified fibres have cells attached to them after 24 hours incubation. However the fluorescence images of RGD modified fibres show more cells attached to the surface of the fibres. This indicated that the conjugation of RGD peptide to premade 4RepCT^{3Aha} fibres using SPAAC was successful and moreover the peptide was accessible to the cells. Consequently this allowed for more cells to adhere to the RGD modified fibres as opposed to the unmodified fibres, therefore yielding a greater cell count after 24 hours. In addition, cells with a flattened needle-like morphology were only present in the RGD modified fibre group (similar to RGD modified films), indicating that cells were able to adhere more readily. However considering that 25 000 cells were used in the initial seeding, cell numbers after 24 hours are very low. The reason for this is the low surface area of the fibres meaning that the majority of the cells will not 'find' the surface of the fibre during seeding. Consequently cell numbers could not be quantified using the PrestoBlue™ assay since this method is insensitive for low cell densities. This was an anticipated outcome, and therefore necessitated the use of fluorescent cells in order to perform manual cell counts after the incubation period. Previous studies that utilised fibres to culture cells mitigated for the low surface area by creating non-woven meshes out of fibres by mechanical chopping⁶⁶. In addition, their 'normal' fibre condition resembled a cobweb-like appearance within the well, having multiple branching points and therefore resembling a non-woven mesh itself. As a result both 'normal'

and non-woven mesh fibre types would have much greater surface area in comparison to the fibres used in this chapter; increasing the chances of a cell 'finding' the scaffold surface upon seeding. Furthermore scaffolds were not transplanted into fresh wells after seeding, providing cells with repeated chances to find and adhere to each of the scaffolds over the seven days incubation. Our approach on the other hand provided cells with a small window of opportunity to adhere to the fibres. In this way the effects of RGD functionalisation on adherence after initial cell contact are tested, and not the ability of cells to grow on the scaffold after a prolonged period of time. However, in order to improve cell numbers after 24 hours incubation and subsequently allow the quantification of cells by PrestoBlue™ (or similar) assay, the surface area of the fibres must be increased. Creating non-woven meshes by mechanically chopping the fibres into smaller pieces would be a quick and simple way of doing so, and would not impact greatly on the experimental procedure. Even so the potential of RGD functionalised 4RepCT^{3Aha} fibres as a 3D cell culture scaffold is clear, and owing to the strength and elasticity of spider silk fibres, mechanical stimuli could be used to direct the differentiation of cells. Previous studies have shown that when scaffolds seeded with MSCs are placed under tensile load (static or cyclic) the differentiation of tenocytes, cardiomyocytes and osteocytes can be induced⁸². Therefore, because of their tensile strength and elasticity, fibres seeded with cells could be subjected to static and cyclic stretching regimens, which could be used to direct cellular differentiation.

In summary, the functionalisation of 4RepCT^{3Aha} with RGD peptide improved the performance of both films and fibres in cell culture. In both cases, better cellular adhesion to the silk architectures in a relatively short incubation period was observed. These results demonstrated that RGD functionalised 4RepCT^{3Aha} shows great potential as a cell culture scaffold both in 2D and 3D culture, permitting not only better cellular adhesion, but the opportunity to influence cellular differentiation without the strict requirement of growth factors. Furthermore because of the high degree of biocompatibility, biodegradability, lack of immunogenicity and risk of disease transfer, functionalised 4RepCT^{3Aha} materials could be used to produce tissues *in vitro* which are later implanted into hosts.

5.5 References

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6. General Discussion

6.1 Overview

It was the aim of this project to incorporate the manmade amino acid L-Aha into the primary sequence of 4RepCT in place of all L-Met residues producing 4RepCT^{3Aha}. This was desired in order to allow highly efficient functionalisation of 4RepCT^{3Aha} using biorthogonal CuAAC or SPAAC 'click chemistry' reactions with ligands bearing alkynes. This technique has until now, not being demonstrated in the field of silk research. Instead previous studies have striven to functionalise recombinant silk proteins using genetic fusion of the silk protein with functional peptides, or chemical conjugation of functional molecules using classical conjugation approaches. The results presented in chapters 3, 4 and 5 clearly show that this primary aim has been achieved, demonstrating the successful conjugation of 4RepCT^{3Aha} (soluble protein and fibres) with alkyne-modified fluorophores, antibiotic with acid sensitive linkers and cyclic peptides containing the RGD motif. In addition it has been demonstrated that the broad spectrum antibiotic levofloxacin could be temporarily conjugated via a labile linker, and later released under the correct environmental conditions (lower pH or via enzymatic cleavage). This approach, although common in the generation of antimicrobial synthetic polymers, has not been utilised in the functionalisation of recombinant silk proteins with drug molecules. In place of a labile linker that controls the release of the ligand, previous

studies have relied on the diffusion driven release of drug molecules from the silk structure which was previously soaked in a solution containing a high concentration of the ligand. Furthermore it has been shown that functionalisation using click chemistry did not compromise the biocompatibility of 4RepCT^{3Aha}. Both CuAAC and SPAAC functionalised silk scaffolds were very well tolerated by human cells (chapter 5); with both methods of conjugation outperforming controls. Consequently, it has been demonstrated that functionalised silk materials, produced using click chemistry, can be used in living hosts without issue.

6.2 Sustainability and potential scale up

In chapter 1 the impracticalities of farming spiders were introduced along with the solutions recombinant silk production provided. Various hosts have been shown capable of expressing spider silk proteins, however not all of them have the potential to be scaled up or have other associated drawbacks (as summarized in Table 1.1). For example, production of genetically engineered potato and tobacco plants, whilst easily scalable, would require large areas of land to produce industrial quantities of silk protein. Unfortunately with the ever-increasing global population, large areas of land for the production of non-food stuffs may be a luxury that will not be afforded in the future; therefore undermining the sustainability of this method. On the other hand, bacterial fermentation using hosts such as *E. coli* is a scalable process, and

does not require agricultural amounts of land to achieve industrial quantities. Consequently bacterial fermentation has been adopted by spin out companies in order to produce dragline silk at annual tonne scales ^{1,2}. In addition bacterial fermentation as a process has a low environmental impact. A previous study concluded that, in comparison to petrochemical approaches, energy consumption and CO₂ emissions associated with fermentation were greatly reduced in the production of the desired chemical ³. Moreover the components required to make liquid medium in which the bacteria grow can be obtained from renewable waste biomass, meaning that the process does not burden the demands on food further ⁴. Therefore the combination of these attributes mean that the production of spider silk protein can be sustainably scaled up using bacterial fermentation with minimal cost to the environment.

6.3 Quantification of functional ligands

Whilst successful conjugation of all the ligands was detected (fluorescent protein bands after SDS PAGE, antimicrobial activity and improved cellular adhesion when compared to controls), the extent of conjugation *i.e.* how many copies of ligand were attached to the protein could not be determined. Mass spectrometry, a common method in protein analysis can provide accurate measurements of sample mass, and therefore should allow the quantification of the number of conjugated ligands to soluble protein. However both matrix-assisted

laser desorption ionisation (MALDI) and electrospray ionisation time of flight (ESI-TOF) mass spectrometry methods did not yield any results for 4RepCT, 4RepCT^{3Aha} or fluorophore functionalised 4RepCT^{3Aha}, despite fresh samples of adequate concentration (80 – 100 μ M) and purity. This suggested that 4RepCT itself may not ionise sufficiently due to the lack of ionisable amino acids in its structure, therefore preventing the flight of protein molecules to the detector and ultimately the attainment of an accurate mass. Consequently any differences in mass as a result of conjugation were not detected, thus preventing the quantification of conjugated ligands to soluble 4RepCT^{3Aha}. The conjugation of ligands to pre-made fibres presented an additional barrier to quantification. As demonstrated in chapter 4 and 5, fibres are chemically and thermally stable, and remained as insoluble fibres after extensive methanol exposure and heating; ruling out mass spectrometry analysis completely. Gravimetric analysis of the functionalised fibres may offer an alternative method of quantification. However since the changes in mass would be extremely small this method would more than likely produce inaccurate results, compounded by the differences in mass between control and experimental fibres. In addition the fibres would have to be dried in order to detect changes in mass, making handling very challenging, since the fibres become brittle and have a tendency to stick to surfaces. Consequently the samples used in this quantification method would be very difficult to recover and use in the desired experiment, therefore undermining the requirement to quantify the ligand load of the sample.

The number of ligands present in a 4RepCT^{3Aha} film however may be able to be quantified by an enzyme linked immunosorbent assay (ELISA). Using an antibody specific for a ligand conjugated to the silk, the assay could quantify the number of ligands when compared to a standard curve. However, ligands that are buried within the film would avoid detection and, given the size of an IgG antibody (~150 kDa), it would be unlikely that multiple antibodies would be able to bind a single 4RepCT spidroin, therefore producing inaccurate results. Furthermore some ligands of interest such as drug molecules may not have specific antibodies, meaning that and ELISA could not be performed.

Therefore it seems that the only viable method that would allow the quantification of ligand load is analytical high performance liquid chromatography (HPLC). This method has been used to detect the release of ligands from materials over time, total loading can be calculated after the ligand is no longer being released ⁵. By using standards of known ligand concentration, the concentration of released ligand can be calculated. However, ligands would need to be conjugated to the silk material via labile linkers in order to facilitate ligand release and prolonged analysis would be required in order to confirm that release has ceased. After this point, the data can be combined and used to back calculate how much ligand was present on the silk material at time zero.

6.4 The effect of functionalisation on 4RepCT^{3Aha}

Despite the problems associated with quantification, the use of appropriate controls in the experiments such as unmodified soluble 4RepCT^{3Aha}, fibres and films, indicated successful click chemistry mediated conjugation had occurred. Moreover, as shown in chapter 3, the functionalisation of soluble 4RepCT^{3Aha} with fluorophores demonstrated that azide handles within the C terminal domain were accessible to conjugation (Figure 3.9), but more importantly that conjugation did not prevent the formation of fibres (Figure 3.12). However the fluorophores used to functionalise 4RepCT^{3Aha} in chapter 3 are relatively small molecules and do not carry a charge; attributes that may contribute to their lack of impact on fibre formation. Conversely, functionalisation of 4RepCT^{3Aha} with ligands of increasing size or charge would, intuitively, negatively impact or even prevent fibre formation entirely. The conjugation of very large or bulky ligands to soluble 4RepCT^{3Aha} may, above a given threshold, disrupt the alignment and packing of the spidroins within the fibre by steric hindrance. This outcome could also be possible when ligands bearing many charged groups are conjugated, however electrostatic repulsion may be responsible instead of steric hindrance. Therefore it is reasonable to expect a number of limits regarding ligand properties, such as size and charge, when conjugating to soluble 4RepCT^{3Aha} without compromising fibre formation. Film formation on the other hand may not be affected in the same way, if at all, owing to the fact that they

are formed by deposition of soluble protein (through evaporation of solvent) and do not require the tight packing of spidroin monomer. However, as shown in chapters 3, 4, and 5 ligands can be conjugated to pre-made fibres using CuAAC and SPAAC, therefore circumventing any potential issues the desired ligand may cause to fibre formation. Unfortunately this method of functionalisation may not permit all of the azide handles to be conjugated, since the structure of the fibre itself may limit accessibility. Consequently it may only be possible to conjugate ligands to azide groups on the surface of the fibre or indeed film using this method; therefore the theoretical maximum ligand load may not be achieved. Despite this potential decrease in ligand load, limitations on ligand properties are avoided, permitting the conjugation of large ligands or even other proteins to pre-made fibres or films. Similar to previous studies, functional proteins including enzymes could be conjugated to 4RepCT^{3Aha} structures using click chemistry; broadening the potential applications of functionalised 4RepCT^{3Aha} materials as biosensors or diagnostic tools.

6.5 Multifunctional 4RepCT^{3Aha}; impact on application

Another significant advantage offered by 4RepCT^{3Aha} is the ability to create multiply functionalised silk materials. In chapter 3, the mixing of soluble 4RepCT^{3Aha} monomers functionalised with two different fluorophores resulted in the production of a dual-labelled fibre; a result that has not being demonstrated previously. Furthermore the mixing of

differentially labelled 4RepCT^{3Aha} did not prevent fibre formation or induce the formation of separate fibres *i.e.* generation of individual green and red fibres after thrombin digestion. Consequently this demonstrated the potential to not only create multifunctional 4RepCT^{3Aha} fibres (or other morphologies including films), but to have an additional layer of control over the ultimate ligand load of the silk material. The amount of ligand loading in the final product could be reduced by mixing ligand conjugated 4RepCT^{3Aha} with un-labelled 4RepCT^{3Aha}. By using defined mixing ratios, the ligand concentration could be diluted accordingly. This may become useful when bioactive ligands with very high potency are conjugated to the silk material (growth factors for example), but are required only in very low concentrations in order to exert its affects.

Alternatively, several 4RepCT^{3Aha} solutions functionalised with different bioactive ligands could be mixed together in defined ratios in order to create silk materials with multiple functionalities. These multifunctional silk materials could be useful in a wide variety of applications (depending on the combination of ligands) especially within biomedicine and life science. Synergistic pairings or combinations of ligands could be employed to tackle problems such as antimicrobial resistance, or provide a more complex environment in which to culture specific cell types. As outlined in chapter 4, antimicrobial synthetic polymer coatings are being developed to try and reduce the frequency of medical device associated HAIs in patients that have undergone surgery. However, with the ever increasing threat of antimicrobial resistance, coatings that

contain numerous biocidal elements may be required in the future. Therefore 4RepCT^{3Aha} films functionalised to possess regimens of antibiotics, or antibiotics in combination with inhibitors of resistance associated proteins (such as ampicillin and sulbactam), could provide a route to novel prophylactic coatings for medical devices. Chapter 5 described how RGD functionalised 4RepCT^{3Aha} films and fibres could be utilised as cell scaffolds within tissue engineering. However the RGD peptide motif is only one of many functional motifs and biological cues present in the ECM. Therefore in order to permit 4RepCT^{3Aha} to support the growth of specific cell types, complex tissues or govern cellular differentiation (as the ECM does), a higher level of functional complexity is required. The mixing of 4RepCT^{3Aha} functionalised with different ECM elements such as growth factors, enzymes and cell binding motifs in optimised ratios could fulfil this requirement. Furthermore the generation of multi-functional fibres could also be utilised to create 3D cell scaffolds, a highly complex environment where the presence (or absence), diffusion rates and diffusion gradients of ECM elements influence cellular activity.

Different types of bioactive ligands could also be combined in order to create silk materials, tailor made to meet the requirements of the application. For example a combination of antibiotics and cell binding motifs, like those used in this work, could be employed in order to create novel smart wound dressings or indeed medical device coatings. In this particular example the conjugated antibiotic would serve to prevent potential infection, whereas the cell binding motif would

facilitate or even enhance the healing process. Zeplin *et al* concluded that silicone implants coated in non-functionalised recombinant spider silk protein reduced post-operative inflammation and fibrotic capsule formation around the implant ⁶. Therefore 4RepCT^{3Aha}, with the addition of appropriate functional ligands, could serve to improve the outcome of implantation further.

6.4 Future work involving 4RepCT^{3Aha}

The functionalisation of 4RepCT^{3Aha} permitted its use as a drug delivery vehicle capable of releasing antibiotic payloads, or as a cell scaffold that better supported the adherence and growth of human cells.

However in order to better assess the performance of functionalised silk materials in these applications, more in-depth experiments focused on the materials use as a drug delivery vehicle or cell scaffold are required.

6.4.1 Development of other 4RepCT^{3Aha} morphologies

Since the first introduction of 4RepCT in 2007 morphologies such as fibres, films, foams, non-woven meshes and combinations of mesh and film have been described ⁷. However, unlike the other major miniature spidroin ADF, hydro/gel structures have not been described for 4RepCT ⁸. These morphologies, like fibres and films, have their advantages and disadvantages and, depending on the desired application, a particular morphology may be considered more appropriate because of its

physical characteristics. Therefore in order to broaden the potential applications in which functionalised silk materials are relevant, it would be favourable to be able to create a number of different morphologies.

Gel or gel-like morphologies would prove useful in both drug delivery and cell scaffolding applications. Consequently in order to further the work shown in this thesis, it would be logical to process 4RepCT^{3Aha} into similar structures. Several methodologies may be employed to process 4RepCT^{3Aha} into gel based morphologies including; chemical crosslinking (using glutaraldehyde for example), increased concentration or by using metal ions as co-ordination centres^{8,9}. However differences in gel characteristics such as stiffness, porosity and hydration may arise from each method, therefore it may be necessary to optimise gelation methods to the desired application.

6.4.2 Development of antimicrobial spider silk materials

In order to be effective against a variety of bacterial pathogens, 4RepCT^{3Aha} could be functionalised with various antibiotics or antimicrobial compounds (such as those outlined in chapter 4). Existing morphologies (fibres and films) could be processed from functionalised 4RepCT^{3Aha} and subsequently tested for activity. In addition attempts to coat medical devices with functionalised 4RepCT^{3Aha}, such as meshes and gauzes, could be made with the view to address the issue of medical devices associated HAIs¹⁰. The ability of 4RepCT^{3Aha} to coat devices could be assessed by SEM, and characteristics such as film

thickness measured by focused ionised beam scanning electron microscopy (FIB-SEM). Following *in vitro* antimicrobial tests, SEM could also be employed to determine if silk films are vulnerable to bacterial colonisation. If *in vitro* tests are positive, testing in animal models would be able to provide information about the performance of the coating *in vivo*. Antimicrobial 4RepCT^{3Aha} gels could also be created and their differences in stiffness and porosity could be used to control the release of biocidal compounds. As a consequence this would allow for the customisation of release profiles, and perhaps mitigate potential toxicity issues associated with some therapeutics.

6.4.3 Development of 4RepCT^{3Aha} based cell scaffolds within tissue engineering

4RepCT^{3Aha} was functionalised with RGD peptide using CuAAC and SPAAC. Whilst these different conjugation methods were not significantly different to one another regarding cellular proliferation, the absolute cell numbers were indeed different; with SPAAC yielding more cells after 24 hours. To determine whether this difference in cell number is caused by trace amounts of copper catalyst left over from RGD conjugation, the metal content of the functionalised films would need to be quantified. To accomplish this, energy dispersive X-ray (EDX) analysis could be employed, a technique previously utilised to detect the presence of metals in a proteinaceous sample ¹¹.

Instead of making films using solely RGD functionalised 4RepCT^{3Aha}, hybrid films could be made by mixing non-functionalised and RGD functionalised 4RepCT^{3Aha} in controlled ratios. Films with decreasing RGD concentration could then be tested cell culture in order to ascertain the minimum RGD concentration required for good cellular adhesion and proliferation. In addition films displaying RGD gradients and patterns could be created using this principle (Figure 6.1).

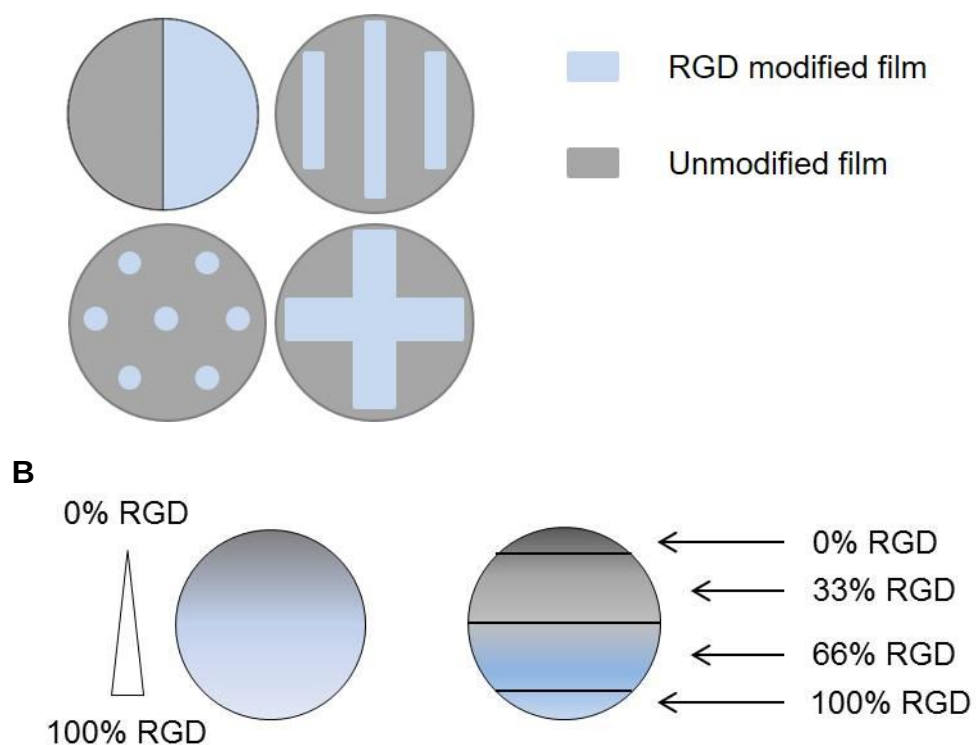


Figure 6.1: Examples of patterns that could be created with RGD functionalised 4RepCT^{3Aha} films.

A few examples of distinct patterns that could be created with RGD modified 4RepCT^{3Aha} films (A). Simple shapes like bars, stripes and islands of RGD functionalised film surrounded by unmodified film are shown; however other more complex iterations could be made. Continuous or step gradients of RGD peptide (B). These films would be made through the blending of RGD modified and unmodified 4RepCT^{3Aha} followed by controlled deposition and drying onto the desired surface

Patterned or gradient films could then be used to study the migration of cells on the silk scaffold. Step gradients and patterns could be created using template moulds and accurate pipetting interspersed with several drying steps (to form the films). Films that display a continuous gradient may be more challenging to create, however with the advent of modern printing technologies capable of printing bio surfaces, one can imagine the development of an appropriate method ^{12,13}.

Different functional elements from the ECM such as the laminin derived peptide motif, IKVAV, could be used to functionalise 4RepCT^{3Aha}.

IKVAV functionalised materials could then be tested in a similar manner to the RGD functionalised films shown in chapter 5. Following this, the complexity of the functionalised silk film could be increased by mixing RGD and IKVAV functionalised 4RepCT^{3Aha} in known quantities. The various RGD: IKVAV combinations could then be tested in cell culture with several cell lines. Finally gel morphologies of mono or multi functionalised 4RepCT^{3Aha} could be used to create 3D cell culture environments. Previously, poor cellular adhesion has been described for spider silk gel structures; however functionalisation with cell binding peptide motifs could work to circumvent this issue ¹⁴.

6.5 References

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7. Appendix

The compositions of buffers, stock solutions, media, SDS PAGE gel components and click reagent compositions are included here

7.1 Buffers

	Composition			
Buffer	Components	Concentration	Amount	Notes
Buffer A	Tris buffer NaCl Imidazole Distilled water	20 mM 400 mM 12 mM	2.42 g/l 23.37 g/l 0.816 g/l 1L	Adjusted to pH 7.5 with conc HCl
Buffer B	Tris buffer NaCl Imidazole Distilled water	20 mM 400 mM 700 mM	2.42 g/l 23.37 g/l 47.65 g/l 1L	Adjusted to pH 7.5 with conc HCl
MES buffer	(2-(N-morpholino)ethanesulfonic acid) (MES) Distilled water	50 mM	9.76 g/l	Adjusted to pH 5.5 with conc HCL
PBS	NaCl KCl Na ₂ HPO ₄ KH ₂ PO ₄ Distilled water	137 mM 2.7 mM 10 mM 1.8 mM	8 g/l 0.2 g/l 1.44 g/l 0.24 g/l	Adjusted to pH 7.4 with conc HCl
Tris buffer	2-amino-2-(hydroxymethyl) Propane-1,3-diol (Tris) Distilled water	20 mM	2.42 g/l	Adjusted to pH 7.5 with conc HCl
Tris buffer	2-amino-2-(hydroxymethyl) Propane-1,3-diol (Tris) Distilled water	1.5 M	181.17g/l	Adjusted to pH 8.8 with conc HCl. 200 ml made
Tris buffer	2-amino-2-(hydroxymethyl) Propane-1,3-diol (Tris) Distilled water	0.5 M	60.57 g/l	Adjusted to pH 6.8 with conc HCl. 200 ml made
Tris buffer	2-amino-2-(hydroxymethyl) Propane-1,3-diol (Tris) Distilled water	1.0 M	121.14g/l	Adjusted to pH 6.8 with conc HCl. 200 ml made
Tris EDTA buffer	Tris Ethylenediaminetetraacetic acid	20 mM 1 mM	2.42 g/l 0.292 g/l	Adjusted to pH 7.5 with conc HCl

7.2 Stock solutions

Solution	Composition			Notes
	Components	Concentration	Amount	
Biotin	Biotin Distilled water	40 mM	10 g/l	1 ml aliquots
Boric acid	H ₃ BO ₃ Distilled water	0.1 M	6.2 g/l	100 ml made
Calcium chloride	CaCl ₂ -2H ₂ O Distilled water	1 M	147 g/l	100 ml made
Cobalt chloride	CoCl ₂ -6H ₂ O Distilled water	0.2 M	47.6 g/l	100 ml made
Copper chloride	CuCl ₂ -2H ₂ O Distilled water	0.1 M	17 g/l	100 ml made
Glucose	Glucose Distilled water	20% (w/v)	200 g/l	500 ml made
Kanamycin	Kanamycin Distilled water		0.5 g/10 ml	1 ml aliquots
Magnesium Sulfate	MgSO ₄ -7H ₂ O	1.0 M	246.5 g /l	100 ml made
Manganese chloride	MnCl ₂ -4H ₂ O	1.0 M	198 g/l	100 ml made
5 X M9 salts	Na ₂ HPO ₄ KH ₂ PO ₄ NH ₄ Cl NaCl Distilled water	238 mM 110 mM 93.47 mM 42.78 mM	33.9 g/l 15 g/l 5 g/l 2.5 g/l	Autoclaved
1 X M9 Salts	Na ₂ HPO ₄ KH ₂ PO ₄ NH ₄ Cl NaCl Distilled water	47.6 mM 22 mM 18.69 mM 8.56 mM	6.78 g/l 3 g/l 1 g/l 0.5 g/l	Autoclaved
Thiamin	Thiamin-HCl Distilled water	29 mM	10 g/l	1 ml aliquots
100 X Trace elements	EDTA FeCl ₃ -6H ₂ O ZnCl ₂ CuCl ₂ -2H ₂ O CoCl ₂ -6H ₂ O H ₃ BO ₃ MnCl ₂ -4H ₂ O Distilled water	13.4 mM 3.1 mM 0.62 mM 76 µM 42 µM 162 µM 8.1 µM	5 g/l 0.83 g/l 84 mg/l 765 µl 0.1 M stock 210 µl 0.2 M stock 1.6 ml 0.1 M stock 8.1 µl 1.0 M stock 1L	Adjusted to pH 7.5 using 10 M NaOH

7.3 Media

7.3.1 Bacterial growth media

	Composition			
Media	Components	Concentration	Amount	Notes
Luria-Bertani (LB)	Tryptone Yeast extract NaCl Distilled water Kanamycin	N/A	20 g/l of pre-mixed powder to distilled water 1ml aliquot antibiotic	LB Pre-mixed powder (Sigma Aldrich)
M9 minimal media	M9 salts Glucose MgSO ₄ CaCl ₂ Biotin Thiamin Trace elements Kanamycin Distilled water	1 X 0.4% (w/v) 1 mM 0.3 mM 40 µM 29 µM 1 X	200 ml 5 X stock 20 ml 20% stock 1 ml 1M stock 0.3 ml 1M stock 1ml aliquot 1ml aliquot 10 ml 100 X stock 1 ml aliquot antibiotic	Ingredients added to sterile water (767 ml) near blue Bunsen flame

7.3.2 Mammalian cell media

	Composition			
Media	Components	Concentration	Amount	Notes
DMEM general culture medium	DMEM Fetal calf serum Penicillin Streptomycin L-glutamine Non-essential amino acids	N/A 10% (v/v) 100 U/ml 100 µg/ml 1% (v/v) N/A	500 ml 50 ml 5 ml 5 ml 5 ml 5 ml	Media made up in class II safety cabinet. All ingredients 0.22 µm sterile filtered

7.4 Click Reagent stock solutions

	Composition			
Reagent	Component	Concentration	Amount	Notes
Copper sulfate	CuSO ₄ ·5H ₂ O	50 mM	12.48 g/l	10 ml made fresh
Sodium Ascorbate	Sodium Ascorbate	50 mM	9.9 g/l	10 ml made fresh
THPTA	Tris(3-hydroxypropyltriazolylmethyl)-amine	50 mM	21.7 g/l	1 ml aliquots

7.5 SDS PAGE components

Gel type	Components	Amount	Notes
12% (w/v) resolving gel stock	Distilled water 1M Tris pH 8.8 10% SDS (w/v) 30% acrylamide (w/v)	34.0 ml 25.0 ml 1.0 ml 40.0 ml	Protect from light once made
4% stacking gel stock	Distilled water 0.5 M Tris pH 6.8 10% SDS (w/v) 30% acrylamide (w/v)	36.4 ml 15.0 ml 0.6 ml 8.0 ml	Protect from light once made
10 X running buffer	Tris base Glycine SDS Distilled water	30.0 g 144.0 g 10.0 g 1L	Dilute to 1 X before use
4 X loading buffer	100% glycerol 1M Tris pH 6.8 Bromophenol Blue 0.5 M EDTA DTT SDS Distilled water	2.0 ml 1.0 ml Approx 0.5 ml 0.309 g 0.4 g Top up to 5 ml	Mix all ingredients then add SDS powder to avoid frothing

N.B. To prepare the SDS PAGE gel: combine 5 ml of resolving gel stock or 2 ml stacking gel stock with 25 µl 10% (w/v) ammonium persulfate solution and 5 µl tetramethylethylenediamine (TEMED) and pour into gel mould.