Cyclo(RGDfK) Functionalized Spider Silk Cell Scaffolds: Significantly Improved Performance in Just One Click

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Recombinant spider silk has the potential to provide a new generation of biomaterial scaffolds as a result of its degree of biocompatibility and lack of immunogenicity. These recombinant biomaterials are, however, reported to exhibit poor cellular adhesion which limits their potential for use in applications such as tissue engineering and regenerative medicine. In this study, a simple chemical functionalization approach is described that specifically addresses this issue and significantly improves the adhesion of human mesenchymal stem cells (CiMSCs) to a recombinant spider silk biomaterial. This utilizes copper-catalyzed or strain-promoted azide–alkyne cycloaddition (CuAAC/SPAAC) “click” chemistry to covalently attach cyclo(RGDfK) peptides to the azide group of L-azidohomoalanine, a methionine analogue previously site specifically incorporated into the primary sequence of a thioredoxin (TRX)-tagged silk fusion protein, TRX-4RepCT, to give TRX3Aha-4RepCT3Aha. This method is used to produce cyclo(RGDfK) functionalized films and macroscopic fibers. Over 24 h, cyclo(RGDfK) functionalized TRX3Aha-4RepCT3Aha films and 4RepCT3Aha fibers display significantly improved performance in CiMSC culture, yielding far greater cell numbers than the controls. This approach circumvents the previously observed lack of cell adhesion, thus allowing spider silk derived biomaterials to be used where such adhesion is critical, in tissue engineering, regenerative medicine and wound healing.

Tissue engineering is a rapidly advancing interdisciplinary field of research that utilizes both man-made and natural polymeric materials as scaffolds and supports that improve the culture of mammalian cells. Prominent examples of natural materials include the extracellular matrix (ECM) proteins collagen and elastin. Both have been extensively studied and processed into several different morphologies including gels and fibrous mats that support the culture of mammalian cells.[1] However, issues such as batch to batch variation, disease transfer, and immunogenicity have been reported that arise from their animal origin.[2,3] Recombinantly produced spider silk proteins ("spidroins") are non-animal alternatives that combine material properties such as strength with those of biocompatibility and a lack of immunogenicity and pyrogenicity. Despite this, the ability of recombinant spidroins, specifically those based upon dragline silk, to support mammalian cells has previously been shown to be poor because of the lack of interaction between the cultured cells, and the silk material.[4] A common approach to improve cellular adhesion is to decorate the biomaterial with functional ligands, such as peptide based ECM mimics.[5–9] One such peptide is the ECM derived arginine-glycine-aspartate (RGD) peptide which selectively binds both α and β subunits of integrins found on mammalian cell surfaces.[10] This sequence has previously been incorporated, at the genetic level, into the primary sequence of dragline silk spidroins in the form of linear RGD motifs, or the peptide has been chemically conjugated in a non-specific way with reactive amino acid sidechains such as lysine, aspartate, glutamate, tyrosine, and cysteine.[11,12] Incorporation of RGD and of other peptide motifs, using these kinds of strategy, has been shown to improve mammalian cellular adhesion.[13] These approaches are, however, limited by the availability of reactive sidechains within spidroins, and by the highly repetitive and extraordinarily high guanine-cytosine content of the silk, which makes the introduction of RGD motifs difficult. Introducing extrinsic sequences may also change the overall structure of the spidroin and thus affect properties such as solubility or tendency to aggregate, with consequent changes.
in physical, and mechanical properties of the spidroin material. Conventional chemical conjugation that targets reactive amino acid sidechains, avoids problems arising from altering the underlying peptide sequence, and can allow the covalent attachment of a wider range of functional ligands, such as organic or inorganic small molecules. The approach is limited, however, by the availability of reactive residues as well as by practical challenges such as reactive ligand instability (hydrolysis of NHS (N-hydroxy succinimide) esters for example)\textsuperscript{14} Perhaps more significantly, difficulties also lie with making the modification site specific because it requires sidechains to be accessible, and suitably bioorthogonal to the rest of the protein. Cysteine residues are often targeted owing to their low abundance and defined Michael-addition chemistry with maleimide-tagged ligands.\textsuperscript{15} Unfortunately cysteine-maleimide conjugates have been reported to undergo thiol exchange reactions, de-conjugating the ligand from the protein thus raising concerns over longer term conjugate stability.\textsuperscript{16} Moreover, targeting cysteines may not be appropriate if they form stabilizing disulfide bridges with other cysteines. This is a particular problem for dragline spidroins, including TRX-4RepCT (sequence shown in Scheme 1), that have a C-terminal domain. This domain contains a highly conserved cysteine residue that has been shown, through its substitution by site-directed mutagenesis, to be important in stabilizing C-terminal dimerization, and therefore fiber formation.\textsuperscript{17}

We have previously demonstrated the incorporation of the bioorthogonal methionine analogue, l-azidohomoalanine (L-Aha), into the miniature dragline spidroin TRX-4RepCT (giving TRX-L-Aha-4RepCT-L-Aha) using a methionine auxotrophic E. coli strain (DL41).\textsuperscript{18} The azide sidechain of L-Aha facilitates the functionalization of TRX-L-Aha-4RepCT-L-Aha using “click” chemistry. This method overcomes the challenges associated with conventional chemical functionalization approaches, by providing a bioorthogonal, and site-specific sidechain to target, tolerating a wide scope of reaction conditions, and achieving functionalization in one step. In the present study we use our method to functionalize TRX-L-Aha-4RepCT-L-Aha with L-glycine–L-aspartate–D-phenylalanine–L-lysine (cyclo(RGDfK), a peptide that improves cell adhesion. We demonstrate copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC), as shown in previous work with alkyl modified levofloxacin, and we further

Scheme 1. Functionalization of 4RepCT-L-Aha fiber and TRX-L-Aha-4RepCT-L-Aha soluble fusion protein with cyclo(RGDfK) peptide alkynes. RGD\textsuperscript{CuAAC}; cyclo(RGDfK) peptide conjugated via CuAAC. RGD\textsuperscript{SPAAC}; cyclo(RGDfK) peptide conjugated via SPAAC. Top right panel: sequence of TRX-4RepCT fusion protein, methionine residues swapped for L-Aha are in bold, and underlined (M). Bottom left panel: production of fibers from TRX-L-Aha-4RepCT-L-Aha fusion protein and functionalization with RGD\textsuperscript{CuAAC} to give RGD-functionalized fibers (fiber-RGD). Bottom right panel: functionalization of soluble TRX-L-Aha-4RepCT-L-Aha fusion protein with RGD\textsuperscript{SPAAC} or RGD\textsuperscript{CuAAC} to produce RGD-functionalized soluble silk fusion protein (TRX-4RepCT-RGD-CuAAC or TRX-4RepCT-RGD-SPAAC).
extend our study to use copper-free strain promoted azide-alkyne cycloaddition (SPAAC)-mediated conjugation for the first time with this minispidroin. We demonstrate both copper catalyzed and strain promoted (copper free) addition with soluble spidroin protein that retains its thioredoxin solubility tag (TRX3Aha-4RepCT3Aha) and also with spidroin protein (4RepCT-T3Aha) that has self-assembled into fibers following cleavage and removal of the thioredoxin (Scheme 1).

As shown in Scheme 1, the soluble fusion protein TRX-4RepCT, contains six methionine residues, three in the TRX, and three in the 4RepCT. All of these are replaced with l-Aha during the expression of TRX3Aha-4RepCT3Aha giving six azide functional groups per fusion protein molecule. In fiber form, there are three azide groups per protein molecule due to the enzymatic removal of the TRX3Aha solubility tag in order to initiate silk fiber self-assembly. Cyclor(GDFK) peptides (RGD-CuAAC and RGD-SPAAC) were chosen to functionalize soluble TRX3Aha-4RepCT3Aha, and 4RepCT3Aha fibers because of their reported increased stability, binding affinity, and resistance to proteolysis when compared to linear RGD sequences. This results from the favorable conformational rigidity of the cyclic peptide and incorporation of a non-proteinogenic ε-phenylalanine residue. Both cyclor(GDFK) functionalized films and fibers were subsequently shown to perform better than their unfunctionalized counterparts in the support of growth of human mesenchymal stem cells (GIMSCs and GIMSC) respectively.

Fibers assembled from enzymatically digested TRX3Aha-4RepCT3Aha were functionalized with the RGD-SPAAC peptide (to give fiber-RGD) and assessed for their ability to support the adherence and growth of MSC(GFP)s against unfunctionalized 4RepCT3Aha fibers. After a 24-h incubation period, both functionalized and unfunctionalized fibers were observed using phase contrast, and fluorescence microscopy (Figure 1). Both fiber types were seen clearly under phase contrast and when excited with 400 nm light, the GIMSC(GFP)s were observed to follow the architecture of the silk fibers. Manual cell counts after 24 h incubation revealed that the unfunctionalized 4RepCT3Aha fibers retained significantly fewer GIMSC(GFP) cells than their RGD functionalized counterparts (counts across all replicates were 42 (unmodified 4RepCT3Aha) versus 141 cells (cyclo(RGDfK) modified 4RepCT3Aha) respectively, unpaired t test p > 0.0375). Furthermore, the RGD-fiber GIMSC(GFP)s exhibited a more easily observed, flattened needle-like morphology which is indicative of good cellular adhesion.

A volume containing 25 000 GIMSCs (13 157 cells cm−2) was used to seed CuAAC or SPAAC mediated cyclo(RGDfK) functionalized 4RepCT3Aha films (TRX-4RepCT-RGD-CuAAC and TRX-4RepCT-RGD-SPAAC respectively). Seeded films were observed after 24 h to assess film performance. The functionalized films, TRX-4RepCT-RGD-CuAAC, and TRX-4RepCT-RGD-SPAAC were tested individually (Figure 2, left-hand and middle panels) or in parallel (Figure 2, right-hand panel) against unfunctionalized 4RepCT3Aha fibers, a positive control (tissue culture treated polystyrene (TCTP)), and a negative control (non-tissue culture treated polystyrene, (NTCTP)). After a 24-h incubation period, the total number of living cells present on each of the films was quantified using the PrestoBlue assay. The number of cells seeded onto the films (25 000) was subtracted from the total number of cells obtained from the assay to give an increase or decrease in cell number for each film type. Cells cultured on NTCTP and unfunctionalized 4RepCT3Aha films were significantly reduced in number after 24 h incubation.
Conversely, both types of RGD functionalized film resulted in a large, positive change in cell number that was significantly greater than the unfunctionalized films and even the TCTP positive control across each experiment. Interestingly, the TRX-4RepCT-RGDSPAAC films produced greater cell numbers when compared to TRX-4RepCT-RGDCuAAC films across experiments. When tested in parallel, TRX-4RepCT-RGDSPAAC films yielded a higher cell number than TRX-4RepCT-RGDCuAAC; however, this difference was not significant (Figure 2, right-hand panel).

The greatly improved performance of TRX-4RepCT-RGDCuAAC and TRX-4RepCT-RGDSPAAC films was reflected in the confluency and morphology of the CiMSC cultures after 24 h. As shown in Figure 3, the NTCTP, and unfunctionalized TRX3Aha-4RepCT3Aha conditions do not display indications of good cellular adhesion, with rounded cells, clumps of cells, and large areas of empty space. In contrast, TCTP, and wells containing TRX-4RepCT-RGDCuAAC or TRX-4RepCT-RGDSPAAC films displayed CiMSC monolayers of high confluency. Furthermore, the flattened fibroblastic morphologies of the GMSCs in the RGD functionalized film conditions, and in the TCTP, both indicate good cellular adhesion, signalling the ability of the cells to exploit the cyclo(RGDfK) peptides on the film surface. However, gaps in the monolayer were observed in the TCTP, and to a lesser extent, the TRX-4RepCT-RGDCuAAC conditions indicating that slightly fewer cells adhered to the well/film surface when compared to the TRX-4RepCT-RGDSPAAC. These observations are all in agreement with the PrestoBlue assay results shown in Figure 2 that demonstrate cell numbers are higher in the presence of cyclo(RGDfK) functionalized silk than on nonfunctionalised silk, that both types of cyclo(RGDfK) functionalized silk perform better than standard tissue culture plastic, and that the copper free (i.e. strain promoted) functionalized silk film performs best of all.

Water contact angle (WCA) analysis was employed to test for any changes in hydrophobicity following functionalization with the cyclo(RGDfK) peptide. The WCA for scores for unfunctionalized TRX3Aha-4RepCT3Aha films were not significantly different from the functionalized versions TRX-4RepCT-RGDSPAAC and TRX-4RepCT-RGDCuAAC (101.4° versus 95.8° and 99.2° respectively, Figure 4). The values for all the silk films were much more hydrophobic than the reported WCA scores for tissue culture plastic (≈65°) and for silkworm fibroin films (74–85°). Silkworm spidroins share many similarities with spider silk[21,22] so the difference in hydrophobicity could be due to subtle differences between the two in their amino acid composition and/or to differences in the experimental conditions under which these respective films were made.

Scanning electron microscopy (SEM) was used to observe the surface of the cyclo(RGDfK)-functionalized and unfunctionalized films (Figure 5). At relatively low magnification it was apparent that there was a difference between the film types. The unfunctionalized TRX3Aha-4RepCT3Aha films produced the least textured films and displayed small raised areas ≈5–20 µm in size. The difference in hydrophobicity could be due to subtle differences between the two in their amino acid composition and/or to differences in the experimental conditions under which these respective films were made.
≈30–50 µm in size (comparable to the diameter of a mammalian cell) were observed, along with a rougher surface in between the bump/aggregate structures.

The need for Cu(I) to catalyze the “click” reaction between TRX3Aha-4RepCT3Aha and the RGD CuAAC peptide leads to the possibility of copper contamination in the final film. Energy Dispersive X-ray Spectrometry (EDS) was used to test both TRX3Aha-4RepCT3Aha and TRX-4RepCT-RGD CuAAC films for the presence of Cu in order to establish whether the toxicity of residual Cu(I) might explain the difference in cell numbers grown in tissue culture. EDS analysis (shown in Figure S1, Supporting Information) did not signal the detection of either Cu(I) or Cu(II) in the films, indicating that the total Cu content was below the detection limit of EDS (0.1% by weight).

In this study, TRX 3Aha-4RepCT3Aha fusion protein, and 4RepCT3Aha fibers were functionalized with cyclo(RGDfK) peptides, a known integrin ligand that improves cellular adhesion.[24] Functionalization was achieved using CuAAC (films only, yielding TRX-4RepCT-RGD CuAAC) and for the first time in recombinant spider silk research, copper-free strain promoted azide-alkyne cycloaddition (SPAAC) (films and fibers, yielding TRX-4RepCT-RGD SPAAC, and fiber-RGD respectively). Both techniques allowed simple and rapid functionalization of the silk material, however SPAAC permitted functionalization of ≈30–50 µm in size (comparable to the diameter of a mammalian cell) were observed, along with a rougher surface in between the bump/aggregate structures.

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without a Cu(I) catalyst, therefore avoiding the possibility of copper toxicity through contamination of the final silk material (film or fiber).

Both TRX-4RepCT-RGD\textsuperscript{CuAAC} and TRX-4RepCT-RGD\textsuperscript{SPAAC} formed films within the wells of a 24 well UCTP plate and were able to tolerate sterilization (in 70% v/v ethanol) and subsequent washing steps without deformation or detaching from the NTCTP surface. Furthermore, fiber-RGD tolerated both autoclave, and ethanol sterilization. This robustness demonstrates their potential to be used reliably as cellular supports or scaffolds.

Functionalization with RGD peptide motifs had a significant impact on performance, with TRX-4RepCT-RGD\textsuperscript{CuAAC}, TRX-4RepCT-RGD\textsuperscript{SPAAC}, and fiber-RGD having greatly improved numbers of cells when compared to unfunctionalized counterparts after 24-h incubation. MSCs express a variety of integrins on their surface so whilst this result was expected based upon data from other studies,[11,12,25–27] it was not guaranteed because as it was not known whether the cyclo-(RGDFK) peptides would be available for integrin binding after conjugation to fibers or after film formation.[28] As an example, previous studies that used N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride, and NHS to conjugate the GRGDSPC peptide motif (via the N-terminal of the G residue) to acidic residues found in the primary sequence of silk worm fibroin reported no significant difference between functionalized and unfunctionalized films in terms of cell adherence. The authors discussed that their findings may indicate that the RGD-integrin binding mechanism is more complex than previously thought but conceded that the peptide ligand design may have also influenced the result.[29] Their observations may also be explained by their conjugation strategy. Using the N-terminal amine of G to conjugate the peptide to the film, rather than via a longer linker, may have brought the RGD motif too close to the film surface (glycine is ≈0.42 nm in length), making it much harder for integrins to bind due to unfavorable steric interactions.[30]

Our study demonstrates that we have overcome the problem described above because we show significant differences between cyclo(RGDFK) functionalized films versus unfunctionalized films and cyclo(RGDFK) functionalized fibers versus unfunctionalized fibers. This indicates that the cyclo(RGDFK) peptides were successfully conjugated and more importantly,
available for integrin binding. In our case, peptide availability was probably aided by the modified flexible lysine side chain linker (shown in Scheme 1), providing enough distance between the peptide, and film/surface. In addition, the short incubation period employed suggested that the cyclo(RGDfK) peptides alone were responsible for the improved performance and this was not a result of GIMSCs producing and depositing ECM proteins on the film or fiber surface. Moreover, the TCTP positive control was significantly outperformed. This demonstrated that the cyclo(RGDfK) functionalized films provided a superior surface to that of standard tissue culture materials for GIMSCs to adhere to and survive on. This result could be explained by the fact that RGD peptide motifs are naturally occurring ligands of integrin receptors, TCTP do not provide these ligands, and so cannot engage integrin receptors as RGD peptides do. The combination of the robust and stable nature of the silk films and the ability to greatly increase cell adhesion/retention with RGD functionalization, showcases the clear potential use of this silk material within cell culture, and tissue engineering.

Functionalized silk fibers (fiber-RGD) also showed potential as a 3D cell culture biomaterial that could be arranged in a mesh to facilitate the growth of layers of cells. However due to their greatly reduced surface area compared to a silk film, the method of cell seeding described in this study is less efficient. To address this problem, cells could be encapsulated into fibers during the fiber formation process as recently described.[31] However this procedure may not be applicable to all cell types owing to the shear force required to generate fibers, which could influence differentiation.

It is well known that the hydrophobicity of a surface can influence the adhesion of cells.[12] It was anticipated that the RGD functionalized films would be less hydrophobic than unfunctionalized films (due to charged arginine and aspartate residues).[26] WCA analysis of all film types revealed that, on unfunctionalized films (due to charged arginine and aspartate RGD functionalized films would be less hydrophobic than the limited loading capacity of TRX 3Aha-4RepCT3Aha, a soluble protein molecule is likely insufficient to significantly change bulk properties. Furthermore, it is possible that some of the cyclo(RGDfK) peptide was buried during film formation, and therefore unable to change the wettability of the surface tested.

To supplement the WCA findings the films were observed under SEM and were all shown to have textured surfaces (Figure 3). The overall appearance of the films consisted of smoother areas interspersed with bump-like features in the order of 10 s mm in diameter, however some differences were also observed. Unfunctionalized TRX 3Aha-4RepCT3Aha and TRX-4RepCT-RGD 4RepCT-RGD SPAAC produced films that had the smoothest features and were comparable to one another in appearance. In contrast, films made from TRX-4RepCT-RGD 4RepCT-RGD SPAAC displayed a rougher surface with large aggregate structures of comparable size to mammalian cells (>20 mm diameter). These larger aggregate structures may have been produced as a result of interactions between TRX 3Aha-4RepCT3Aha and “click” reagents necessary to catalyze the CuAAC reaction (2.5 mm sodium ascorbate, 0.5 mm copper sulphate and 2.5 mm Tris (3-hydroxypropyltriazolylmethyl) amine (THPTA)) and carried through to film formation. Therefore, these aggregates may have hindered the binding of GIMSCs to the film surface, contributing to lower cell numbers in comparison to TRX-4RepCT-RGD 4RepCT-RGD SPAAC but still providing a significantly superior surface to that of the TCTP.

The use of Cu(I) to catalyze the CuAAC reaction raised concerns of copper ion contamination in TRX-4RepCT-RGD CuAAC films which could exert a toxic effect on the GIMSCs, effecting cell viability, and total cell number. Subsequent EDS analysis of TRX-4RepCT-RGD CuAAC versus unfunctionalized films was performed to detect copper contamination. However, it was revealed that copper content, in both film types, was below detectable levels (0.1% by weight). This indicated that copper was removed by dialysis (post cyclo(RGDfK) conjugation), washing steps involved in film formation, and perhaps aided by using THPTA (as an efficient Cu(I) chelating agent) during the CuAAC reaction. Consequently, copper toxicity does not seem responsible for the lower cell numbers observed for TRX-4RepCT-RGD CuAAC films, instead this appears more likely to have been caused by increased roughness of the TRX-4RepCT-RGD CuAAC film surface.

In conclusion, we have shown copper catalyzed, and strain promoted “click” chemistry mediated functionalization of 4RepCT 4RepCT 4RepCT 4RepCT 4RepCT CuAAC CuAAC CuAAC CuAAC CuAAC 4RepCT-, in soluble and fiber form, with cyclo(RGDfK) peptides. This highly flexible approach yielded functionalized materials that performed significantly better than their unfunctionalized counterparts in GIMSC culture. After 24 h, functionalized materials retained greater cell numbers, and can therefore reduce the time taken to reach confluency; a valuable capability when culturing precious primary cells often used in tissue engineering, and regenerative medicine. In addition, we have previously shown that by blending functionalized soluble silk protein, it is possible to create multi-labeled silk materials.[18] Similarly, multi-labeled TRX 3Aha-4RepCT3Aha films or 4RepCT3Aha fibers, functionalized with a suite of peptides or molecules, could be made simply by blending controlled ratios of soluble functionalized silk protein together. This could be used to produce 2D and 3D silk materials of increasing complexity that more closely mimic tissue specific niches.

**Experimental Section**

**Chemical Synthesis Methods:** Cyclo(RGDfK) peptides (Peptides International, Kentucky, USA) were chemically modified to bear either a propargyl or bicyclononyne (BCN) functional group, giving RGD CuAAC or RGD SPAAC respectively. Expanded chemical synthesis methods relating to the preparation of the functional groups can be found in the supporting information along with information on all the analytical methods used.

**Synthesis of RGD CuAAC:** Propargyl p-nitrobenzyl carbonate (2.1 mg, 4.97 mmol, 1.26 equivalent) was dissolved in 1 mL dimethylformamide (DMF) and the resulting solution was used to dissolve cyclo(RGDfK) (5 mg, 8.3 mmol). Triethanolamine (TEA) was added dropwise until equal molar ratios of Cu(II) were reached. An equal volume of Cu(II) solution (2.5 mm sodium ascorbate, 0.5 mm copper sulphate and 2.5 mm Tris (3-hydroxypropyltriazolylmethyl) amine (THPTA)) was added, and allowed to react for 1 h at room temperature.

**Synthesis of Propargyl RGD SPAAC:** Propargyl p-nitrobenzyl carbonate (2.1 mg, 4.97 mmol, 1.26 equivalent) was dissolved in 1 mL dimethylformamide (DMF) and the resulting solution was used to dissolve cyclo(RGDfK) (5 mg, 8.3 mmol). Triethanolamine (TEA) was added dropwise until equal molar ratios of Cu(II) were reached. An equal volume of Cu(II) solution (2.5 mm sodium ascorbate, 0.5 mm copper sulphate and 2.5 mm Tris (3-hydroxypropyltriazolylmethyl) amine (THPTA)) was added, and allowed to react for 1 h at room temperature.
the solution turned clear yellow. It was left stirring for 18 h at room temperature. The reaction was followed by high resolution mass spectrometry (HRMS) to determine reaction completion. The desired product was purified using high performance liquid chromatography (HPLC), equipped with a semipreparative Agilent Eclipse XDB-C18 column, and displayed a retention time of 13.40 min (full method shown in the supporting information). The identity of the fraction collected at 13.40 min was confirmed by HRMS (ESI): C31H43N9O9 calculated [M+H]$: 686.3257; found [M+H]$: 686.3265. Collected fractions were freeze-dried yielding 0.9 mg (26%) of the target compound.

**Synthesis of RGD**

BCN carbonate (4.64 µg, 14.73 µmol, 1.77 equivalent) was dissolved in 1 mL DMF and the resulting solution was used to dissolve cyclodextrin (5 mg, 8.3 mmol). TEA was added dropwise until the solution turned clear yellow. It was left stirring for 18 h at room temperature. The reaction was followed by HRMS to determine reaction completion. The desired product was purified using HPLC, equipped with a semipreparative Agilent Eclipse XDB-C18 column, and displayed a retention time of 16.50 min. The identity of the fraction collected at 16.50 min was confirmed by HRMS (ESI): C31H43N9O9 calculated [M+H]: 686.3257; found [M+H]: 686.3265. Collected fractions were freeze-dried yielding 3.9 mg (60%) of the target compound.

**Expression and Purification of TRX**

A vial of commercially immortalized mesenchymal stem cells (CiMSCs) (Japanese Collection of Research Bioresources) were thawed and subsequently cultured in 175 mL flasks (Corning Costar). Cells were maintained in DMEM containing 10% (v/v) fetal bovine serum (Sigma Aldrich) and penicillin-streptomycin (100 units) (Sigma Aldrich) and incubated at 37 °C 5% CO2. Once confluent, cells were passaged by trypsin/EDTA (0.25%/0.02% v/v) (Sigma Aldrich), and their culture continued no further than 8–10 passages.

**Culture of CiMSCs on TRX**

Fibers: CiMSCs were harvested from a single T75 flask and counted using a Neubauer improved hemocytometer. A volume of media containing 25 000 CiMSCs was added to wells containing 1 mL pre-warmed media and either the positive control (tissue culture plastic—TCTP control) or unmodified TRX3Aha-4RepCT3Aha films, TRX-4RepCT-RGDSPAAC films, TRX-4RepCT-RGDSPAAC films, and the negative control (NTCTP—neg control). After seeding the well plates were returned to the 37 °C 5% CO2 incubator for 24 h.

**Culture of CiMSCs on Fiber-RGD**

Fibers: CiMSCs were harvested from a single T75 flask and counted using a Neubauer improved hemocytometer. A volume of media containing 25 000 CiMSCs was added to wells containing 1 mL pre-warmed media and either unfunctionalized 4RepCT3Aha fibers or fiber-RGD. Cells were seeded directly over the fibers to maximize chances of the cells landing on fibers.

**Visualization of CiMSCs Growing on Films**

Visualization of CiMSCs Growing on Films: After the 24-h incubation period the well plates were removed from the incubator and inspected using a phase contrast microscope (Leica DMRBE). Images were taken at 10x magnification using Velocity software (Improvision UK). Scale bars were added using ImageJ (Fiji).

**Fluorescent Cell Counting**

The following criteria were applied across fiber samples to ensure fair counting of fluorescent CiMSCs. Fibers were located on the bottom of each well and brought into the sharpest focus possible. This focal plane was applied across all samples. Only sharp defined green dots were counted. Blurred or out of focus fluorescent bodies were not counted since they were out of the fixed focal plane. Blobs or aggregates of cells were counted as one because it was not possible to resolve how many cells were in the aggregates.

**Water Contact Angle**

WCA measurements were performed on a CAM 200 optical contact angle meter (KSV Instruments Ltd., Finland) using the sessile drop method and CAM 200 image analysis software. Water droplets were placed on each surface and ten images were taken at 1 s intervals. All measurements were made at room temperature. WCAs were calculated using a Young-Laplace fitting function and the resulting right and left contact angles were averaged out. Five repeat measurements were made for each material in triplicate. Measurements are presented as average ± standard deviation.
Scanning Electron Microscopy: SEM was carried out on a Jeol 6060LV variable pressure scanning electron microscope (Jeol UK Ltd, UK). Before insertion into the chamber, samples were coated in gold using a Leica EM SCD005 Sputter Coater for 300 s. Images were acquired using a voltage of 12 kV at 25×, 100×, 250×, 500×, or 1000× magnification.

Energy Dispersive X-Ray Spectroscopy: EDX was conducted on a FEI QUANTA 650 scanning electron microscope in conjunction with an Oxford Instruments X-max 150 detector. Before analysis, sample films on microscope slides were coated with a fine layer of carbon using an Agar Turbo carbon coater. Images of the film edge, center, and part way between edge and center were acquired using a voltage of 20 kV. Within each image three areas were chosen for analysis by EDX, therefore totaling nine EDX analyses per film (see Figure S1, Supporting Information).

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

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