RGD-Functionalized Bioengineered Spider Dragline Silk Biomaterial

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Spider silk fibers have remarkable mechanical properties that suggest the component proteins could be useful biopolymers for fabricating biomaterial scaffolds for tissue formation. Two bioengineered protein variants from the consensus sequence of the major component of dragline silk from *Nephila clavipes* were cloned and expressed to include RGD cell-binding domains. The engineered silks were characterized by CD and FTIR and showed structural transitions from random coil to insoluble β-sheet upon treatment with methanol. The recombinant proteins were processed into films and fibers and successfully used as biomaterial matrixes to culture human bone marrow stromal cells induced to differentiate into bone-like tissue upon addition of osteogenic stimulants. The recombinant spider silk and the recombinant spider silk with RGD encoded into the protein both supported enhanced the differentiation of human bone marrow derived mesenchymal stem cells (hMSCs) to osteogenic outcomes when compared to tissue culture plastic. The recombinant spider silk protein without the RGD displayed enhanced bone related outcomes, measured by calcium deposition, when compared to the same protein with RGD. Based on comparisons to our prior studies with silkworm silks and RGD modifications, the current results illustrate the potential to bioengineer spider silk proteins into new biomaterial matrixes, while also highlighting the importance of subtle differences in silk sources and modes of presentation of RGD to cells in terms of tissue-specific outcomes.

Introduction

Silk proteins are produced by specialized glands of insects and spiders. Although their sequences and properties vary depending on the producing species and function, silk proteins have some common characteristics: they tend to be high molecular weight proteins with a primary structure consisting of multiple repeats of specific motifs and they contain a high percentage of the amino acids alanine and glycine.[1−3] Silk proteins also form fibers of exceptional strength, toughness, and resistance to mechanical compression.[4−6] Silks are used by the larvae of insects mostly for cocoon fabrication and by spiders for various functions ranging from orb web construction to prey wrapping, egg protection, or dragline filament production.[4] Different spider silk proteins are used for the fabrication of each of these structures.

A spider orb-web consists of various silk components: the most extensively studied spider silk proteins are those produced by flagelliform and major ampullate glands and derive their names from the respective glands (Flag, MaSp). Specifically, two proteins MaSp1 and MaSp2 are employed for the formation of the radii in a typical orb-web as well as for the dragline thread, whereas the Flag protein is a major component of the capture spiral of the web. The MaSp proteins assemble into fibers possessing high tensile strength (1.3 GPa), and toughness of 16×10^4 J/Kg.[6] Due to their differing functions, the flagelliform silk combines high strength (0.5 GPa) and high elasticity.[7] The impressive mechanical characteristics of dragline protein MaSp1 are thought to arise partly due to the presence of repeats in the protein consisting of 6−10 alanine residues that alternate with glycine rich regions[8] and generate beta sheet secondary structures to form physical cross-links in the assembled and spun protein fibers. The remarkable mechanical properties of dragline spider silk have sparked interest in a broad range of industrial and biomedical applications.

Although silkworm silk cocoon fibers (fibroin protein) have been used successfully in the biomedical field as sutures for decades, this is not the case for spider silks. Silkworm silk also meets many criteria for biomaterials with outstanding mechanical properties, biodegradability, and biocompatibility.[9] Reprocessed silkworm fibroin has been used successfully to tissue engineer bone and cartilage,[10−13] and silkworm fibers have been used in ligament tissue engineering.[9,14,15] Unlike the silk above from the domesticated silkworm, *Bombyx mori*, that lends itself to large scale production via sericulture, there is no similar system to date to mass produce spider silk in order to study biomaterial applications. Therefore, heterologous expression of spider silk protein in a suitable host is the mode employed to accomplish this goal.[16−24]

An important advantage to the use of bioengineered fibrous proteins such as spider silks is the ability to design genetic variants with enhanced features within the otherwise native consensus sequence of the protein. In this paper, we report the design of a spider dragline-like sequence, based on the consensus repeat derived from the MaSp1 of *Nephila clavipes* but with the variation of an RGD cell binding sequence to enhance cell interactions. Since the native consensus sequence of the protein does not contain this cell-binding domain, the hypothesis was that improved cell interactions with the protein would result. This objective is important as silks are generally highly

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hydrophobic, and we have previously shown improvements in cell interactions by chemically coupling RGD peptides to the surfaces of silkworm films and fibers.[10,15] The bioengineered spider silk sequences were cloned and expressed in *E. coli*, and the new spider silk variant was cast into films and formed into fiber matrixes suitable for cell cultivation. Human bone marrow stromal cells (hMSCs) were cultured and differentiated into bone-like tissue on the bioengineered protein. The results obtained did not confirm the prior findings with silkworm silk where the RGD was chemically attached to the fibroin. In this case, when the RGD was part of the primary sequence of a spider silk, the enhanced impact on cell differentiation was not observed.

Materials and Methods

Design and Cloning of Spider Silk Sequences. The spider silk repeat unit was selected based on a consensus (SGRGGLGGQ-GAGAAAAAGGAGQGGY GGLGSQGT) derived from the native sequence of the spidroin 1 sequence of *Nephila clavipes* (Accession P19837). Multimers encoding this repeat were developed through the transfer of cloned inserts between two shuttle vectors based on pUC19 and pCR-Script, with different antibiotic resistance (ampicillin and chloramphenicol, respectively) according to our previously published procedures.[16,25,26] A linker was inserted into the *Bam*HI site of pUC18 (New England Biolabs, Beverly, MA) and pCR-Script (Stratagene, La Jolla, CA) plasmids, to introduce the restriction sites *Nhe*I and *Spe*I, which were needed for cloning and development of multimers. The two complementary oligonucleotides encoding the linker were: CTAGA*GGATCC*ATG*GCTAGC*GGTGACCTGAATAAC*ACTAGTG-GATCC*T and CTAGA*GGATCCACTAGT*GTTATTCAGGTCACC-*GCTAGC*CATGGATCCT. The restriction sites for *Bam*HI, *Nhe*I, and *Spe*I are italicized below. The sequences of the synthetic oligonucleotides encoding a single monomer (99 nt) were as follows:

SPI-a: 5′ *CTAGC*GGTCGTGGCGGTCTGGGTGGCCAGGGTG-CAGGTGCTGCTGCGGCAGCAGGC 3′

SPI-b: 5′ TGCCGCAGCAGCACCTGCACCCTGGCCACCCAG-ACCGCCACGACCG 3′,

SPI-c: 5′ GGTGCTGGCCAAGGTGGCTACGGTGGCCTGGGT-TCTCAGGGTA 3′

SPI-d: 5′ *CTAGT*ACCCTGAGAACCCAGGCCACCGTAGCCAC-CTTGGCCAGCACCGCCTGC 3′

SPI-a and SPI-b are complementary oligonucleotides and were annealed to form double stranded DNA. SPI-c and SPI-d are complementary oligonucleotides and were annealed to form another double stranded piece of DNA. The newly formed double stranded DNAs were then ligated to form the monomer before undergoing multimerization. At the end of the multimerization step, the construct pCR-15, carrying 15 repeats of the above monomer, was obtained.

The expression vector pET-30a (Novagen, Madison, WI) was modified with a linker carrying the *Spe*I site flanked by sequences encoding the amino acids CRGD to obtain pET30-link. The two complementary oligonucleotide sequences for the linker were *GGATC-C*TGTCGCGGTGAC*ACTAGT*CGCGGTGACTGTG and *GGATCC*A-CAGTCACCGCG*ACTAGT*GTCACCGCGACAG. The restriction sites of *Bam*HI and *Spe*I are underlined. The 15mer sequence obtained by multimerization was inserted into pET30-link to generate pET30-CRGD15mer. For the production of a spider silk protein without CRGD, the construct pET30-15mer was obtained by subcloning the *Nco*I-*Not*I fragment of pCR-15 into pET-30a vector.

Protein Expression and Purification. The constructs pET30-15mer and pET30-CRGD-15mer were used to transform the *E. coli* strains BLR (DE3) or RY-3041, a mutant strain defective in the expression of SlyD protein.[25,27] Cells were cultivated in LB broth at 37°C. Protein expression was induced by the addition of 1 mM IPTG (Fisher Scientific, Hampton, NH) when the OD_{600} was between 0.8 and 1.0. After approximately 2 h of protein expression, the cells were harvested by centrifugation at 9500 rpm. For large scale expression, *E.* coli was grown in a fermentor (Bioflo 3000, New Brunswick Scientific Co., Edison, NJ) in minimal medium[28] supplemented with 1% yeast extract. Ammonia was used as the base to maintain the pH at 6.8. When the pH exceeded 6.88, as a result of glucose exhaustion in the culture, a feed solution (50% glucose, 10% yeast extract, 2% $MgSO_4 \cdot 7H_2O$) was added. Pure O_2 was also provided to the culture to sustain the level of dissolved oxygen above 40%. All culture media contained kanamycin (30 μg/mL). For the fermentor grown cells, expression was induced when the absorbance was between 25 and 30 at OD_{600}. The cell pellets were resuspended by adding denaturing buffer (100 mM NaH_2PO_4, 10 mM Tris HCl, 8 M urea, pH 8.0) containing 10 mM imidazole. The cells were lysed by stirring for 30 min and were then centrifuged at 9500 rpm at 4°C for 30 min. His-tag purification of the proteins was performed by addition of Ni−NTA agarose resin (Qiagen, Valencia, CA) to the supernatant (batch purification) under denaturing conditions. After washing the column with denaturing buffer at pH 6.3, the proteins were eluted with denaturing buffer at pH 4.5 (without imidazole).

SDS-polyacrylamide gel electrophoresis (PAGE) was performed using 4−12% precast NuPage Bis-Tris gels (Invitrogen, Carlsbad, CA). Electrophoresis was performed for 50 min at 200 V. Western blots of crude extracts were performed using the S-Tag Western Blot kit (Novagen, Madison, WI), that is based on the specific binding of the S-protein alkaline phosphatase conjugate to S-tag. Purified samples were extensively dialyzed against several changes of H_2O. For dialysis, Snake Skin membranes or Slide-A-Lyzer Cassettes (Pierce, Rockford, IL) with MWCO of 7000 or lower were used. The dialyzed samples were lyophilized using a LabConco lyophilizer. For determination of the amino acid composition, the samples were submitted to the W. M. Keck Foundation Biotechnology Resource Laboratory (Yale University, New Haven, CT). The samples were analyzed from bands of interest cut out from the gel or lyophilized powder after purification and dialysis. Determination of protein concentration was performed by BCA assay (Pierce, Rockford, IL) or the molar absorptivity at 280 nm (19 440 and 19 200 M^{-1} cm^{-1} for CRGD-15mer and 15mer, respectively). Determination of recombinant protein yields before and after purification was perfomed using the S−Tag Rapid Assay Kit (Novagen, Madison, WI). Reducing treatment was performed by incubating protein samples in 10 mM DTT for 30 min at 55°C. Theoretical amino acid composition, molecular weight, isoelectric point and molar extinction coefficients were calculated by using the PeptideSort program of SeqLab (Accelrys, San Diego, CA), or ProtParam (ExPASy).

Protein Characterization and Materials Preparation. *Circular Dichroism.* To determine secondary structure, CD spectra of the recombinant proteins were obtained on a Jasco J-710 spectropolarimeter (Easton, MD) with a 1 mm path length cell, at a scanning rate of 10 nm/min, resolution of 0.1 nm, and 5 scans accumulation. Concentration of the proteins ranged between 0.18 and 0.72 mg/mL in water.

Preparation and Characterization of Films. Films were prepared as previously described[10] with minor modifications by dissolving the recombinant protein in hexafluoroisopropanol (HFIP) to a final concentration of 1.25%. For FTIR microscopy, 10−20 μL of the HFIP solution was pipetted directly onto a ZnS crystal and allowed to air-dry. For cell cultures, 150 μL of the solution was dispensed in 12-well plates to completely cover the bottom of the well and allowed to air-dry. Films treated with methanol were individually incubated for 10 min in methanol at different concentrations (70−100%). The methanol solution was then removed, and the films were allowed to air-dry. FTIR analysis was performed on a Bruker Equinox 55 infrared spectrophotometer (Billerica, MA) provided with a FTIR attachment.[29] Spectral features characteristic of silk II structure, such as the bands at 1625 (amide I), 1528 (amide II), and 1260 cm^{-1} (amide III) were monitored.

Preparation and Characterization of Fibers. Fibers of the recombinant spider silk protein were electrospun directly onto silica chips placed on the receiving plate. Electrospinning was performed using a 2% solution of the recombinant spider silk in HFIP using the apparatus

A CRGD-15mer

MHHHHHHSSG LVPRGSGMKE TAAAKFERQH MDSPDLGTDD DDKAMADIGS **CRGD**TSGRGG 60
LGGQGAGAAA AAGGAGQGGY GGLGSQGTSG RGGLGGQGAG AAAAAGGAGQ GGYGGLGSQG 120
TSGRGGLGGQ GAGAAAAAGG AGQGGYGGLG SQGTSGRGGL GGQGAGAAAA AGGAGQGGYG 180
GLGSQGTSGR GGLGGQGAGA AAAAGGAGQG GYGGLGSQGT SGRGGLGGQG AGAAAAAGGA 240
GQGGYGGLGS QGTSGRGGLG GQGAGAAAAA GGAGQGGYGG LGSQGTSGRG GLGGQGAGAA 300
AAAGGAGQGG YGGLGSQGTS GRGGLGGQGA GAAAAAGGAG QGGYGGLGSQ GTSGRGGLGG 360
QGAGAAAAAG GAGQGGYGGL GSQGTSGRGG LGGQGAGAAA AAGGAGQGGY GGLGSQGTSG 420
RGGLGGQGAG AAAAAGGAGQ GGYGGLGSQG TSGRGGLGGQ GAGAAAAAGG AGQGGYGGLG 480
SQGTSGRGGL GGQGAGAAAA AGGAGQGGYG GLGSQGTSGR GGLGGQGAGA AAAAGGAGQG 540
GYGGLGSQGT S**RGDC***GSEFE LRRQACGRTR APPPPPLRSG C*

B 15mer

*MHHHHHHSSG LVPRGSGMKE TAAAKFERQH MDSPDLGTDD DDKAMA*SGRG GLGGQGAGAA 60
AAAGGAGQGG YGGLGSQGTS GRGGLGGQGA GAAAAAGGAG QGGYGGLGSQ GTSGRGGLGG 120
QGAGAAAAAG GAGQGGYGGL GSQGTSGRGG LGGQGAGAAA AAGGAGQGGY GGLGSQGTSG 180
RGGLGGQGAG AAAAAGGAGQ GGYGGLGSQG TSGRGGLGGQ GAGAAAAAGG AGQGGYGGLG 240
SQGTSGRGGL GGQGAGAAAA AGGAGQGGYG GLGSQGTSGR GGLGGQGAGA AAAAGGAGQG 300
GYGGLGSQGT SGRGGLGGQG AGAAAAAGGA GQGGYGGLGS QGTSGRGGLG GQGAGAAAAA 360
GGAGQGGYGG LGSQGTSGRG GLGGQGAGAA AAAGGAGQGG YGGLGSQGTS GRGGLGGQGA 420
GAAAAAGGAG QGGYGGLGSQ GTSGRGGLGG QGAGAAAAAG GAGQGGYGGL GSQGTSGRGG 480
LGGQGAGAAA AAGGAGQGGY GGLGSQGTSG RGGLGGQGAG AAAAAGGAGQ GGYGGLGSQG 540
T*SGSARARAA ALEHHHHHH*

Figure 1. Amino acid sequences of the engineered spider silk proteins (A) with added CRGD sequences and (B) control protein without CRGD. CRGD-15mer start at position 51 and 15mer at position 47. Underline: His-tag. Dashed underline: S-tag. Double underline: representative monomeric unit. Italic: extra amino acids from vector sequence. Bold: CRGD sequence.

and procedures we have previously described.[30] The distance between plates was set at 12 cm, with an electric field of 0.57 kV/cm. The concentrated silk solution was infused at the rate of 0.02 mL/min. The fibers were treated with methanol to induce β-sheet formation by incubation for 10 min with 70% methanol in water, and then allowed to air-dry. Scanning Electron Microscopy (SEM) characterization of the electrospun fibers, with and without methanol treatment, was performed using a Leo 982 Field Emission Scanning Electron Microscope (LEO Electron Microscopy, Inc., Thornwood, NY).

Cell Responses. hMSCs were isolated as we have previously described,[14,15] and seeded onto the CRGD-15mer films air-dried in 12 well dishes at a concentration of 5000 cells/cm^2, while the engineered 15mer spider silk protein and commercially available treated and sterile polystyrene of the 12-well cell culture plates (BD Falcon, NJ) were used as controls.[10] The medium consisted of 10% fetal bovine serum, Pen-Strep and Fungizone, supplemented with 50 μg/mL ascorbic acid-2-phosphate, 10 nm dexamethasone, and 7 mM β-glycerolphosphate. Calcium content was assessed after 1, 2, 3, and 4 weeks by 5% trichloroacetic acid (TCA) extraction of the cells for 30 min. This procedure was performed twice. Calcium content in the two combined TCA washes was measured by a colorimetric assay employing *o*-cresolpthalein complexone (Sigma, St. Louis, MO). Assessment of residual endotoxin was performed on CRGD-15mer resuspended in fetal bovine serum (FBS) to a final concentration of approximately 0.9 mg/mL, and on serum exposed for one week to the CRGD-15mer film substrate. The serum was assayed by the Timed Gel Formation Endotoxin Kit (Sigma, St. Louis, MO) following the directions of the manufacturer.

Statistics. For statistical significance, two independent experiments were conducted with a minimum of *N=3* for each data point. The results of one independent experiment are displayed for each analysis. Data were analyzed using the Student-Newman-Keuls Multiple Comparisons Test. Differences were considered significant when equal or less than $p = 0.05$.

Results

Expression, Purification, and Characterization of Recombinant Spider Silk Proteins. The DNA sequences of the two spider dragline silk encoding genetic constructs, with and without CRGD motifs, were deposited in GenBank (Accession numbers AY555584 and AY555585). The corresponding amino acid sequences are shown in Figure 1. The two clones were initially expressed in *E. coli* BLR (DE3). A contaminant protein was co-purified with the silk protein, and the contaminant was identified as SlyD on the basis of its amino acid composition. This protein is expressed by *E. coli* and has been previously reported to co-purify with target proteins during purifications by Ni−NTA affinity chromatography, due to its high histidine content.[25,27] Pure silk protein was obtained by purifying extracts from the mutant expression host *E. coli* RY-3041 that does not produce SlyD[25] (Figure 2, lanes a−c). The amino acid composition of the two synthetic spider silks agreed well with the expected composition (Table 1).

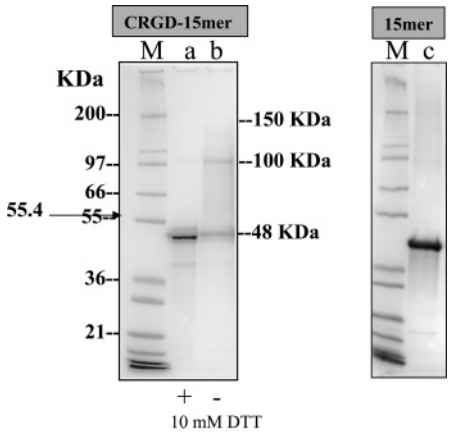

Figure 2. SDS−PAGE of purified recombinant spider silks: (a) CRGD-15mer, after 10 mM DTT treatment, (b) CRGD-15mer, no DTT treatment, (c) purified 15mer. M = molecular weight markers.

Yields of the two recombinant proteins in the crude extract were comparable and approximated 50 μg at OD_{600}, as determined by S-tag assay. This value, in a 1-L culture, grown in a fermentor to a final OD_{600} of 30, corresponds to a yield of about 1500 mg/L. However, after purification, the final yields were approximately 150 mg/L for CRGD-15mer and approximately 2.5 mg/L for the 15mer variant without CRGD. A

Table 1. Amino Acid Composition of Recombinant Spider Silks, Compared to the Predicted Amino Acid Composition of CRGD-15mer[a]

	CRGD-15mer		15mer	
	determined	determined	determined	expected
comp. Aa	mol %	mol % (100 KDa)	mol %	mol %
Asp+Asn	1.9	2.5	1.4	1.5
Thr	3.2	3.6	2.8	3.3
Ser	5.2	6.4	5.6	6.5
Glu+Gln	9.4	10.4	9.4	8.8
Pro	1.2	1.2	*b*	1.2
Gly	**45.4**	**42.3**	**44.4**	**43.0**
Ala	**20.0**	**21.0**	**20.6**	**19.3**
Val	0.2	0.6	0.1	0.2
Met	0.3	0.6	0.5	0.7
Ile	0.2	0.5	*b*	0.2
Leu	6.1	6.8	6.1	5.9
Tyr	2.6	2.7	2.6	2.6
Phe	0.4	0.5	0.2	0.3
Lys	0.7	0.9	0.6	0.5
Arg	3.4	*b*	2.9	4.1

[a] Key amino acids are in bold. [b] Composition was not determined.

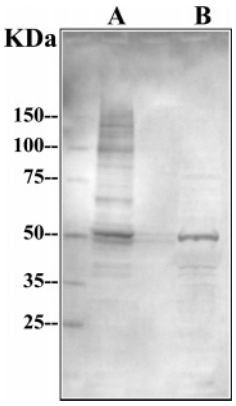

Figure 3. Western blot of crude extracts from cells expressing recombinant spider silk (A) CRGD-15mer and (B) control 15 mer. The blot was treated with S-protein alkaline phosphatase conjugate, which binds specifically to S-tag.

substantial loss of 15mer protein occurred during the dialysis step, attributed to adhesion to the dialysis membrane. Pure protein was analyzed by SDS−PAGE stained with Coomassie blue dye. The CRGD-15mer had an apparent molecular weight of approximately 50 kDa, which was in good agreement with the expected molecular weight of 48.55 kDa. The silk protein without CRGD also showed an apparent molecular weight of approximately 50 kDa (Figure 2, lane c). For the recombinant CRGD-15mer, two bands with apparent molecular weights of about 100 and 150 kDa were also present. The two bands are likely dimers and trimers of CRGD-15mer, due to the formation of disulfide bonds between Cys residues. This interpretation was confirmed upon treatment with 10 mM DTT (Figure 2, lane b), wherein the higher bands disappeared on the gels. Also, the 15mer does not contain Cys residues and did not show the additional high molecular weight bands (Figure 2, lane c). The amino acid composition of the 100 kDa band closely matched the composition of the CRGD-15mer, and both were consistent with the predicted composition based on the translation of the coding sequence (Table 1). Western blots also confirmed the presence of the desired engineered proteins (Figure 3)

Solubility of the Recombinant Proteins. The theoretical p*I*'s of CRGD-15mer and 15mer are 10.87 and 10.26, respectively.

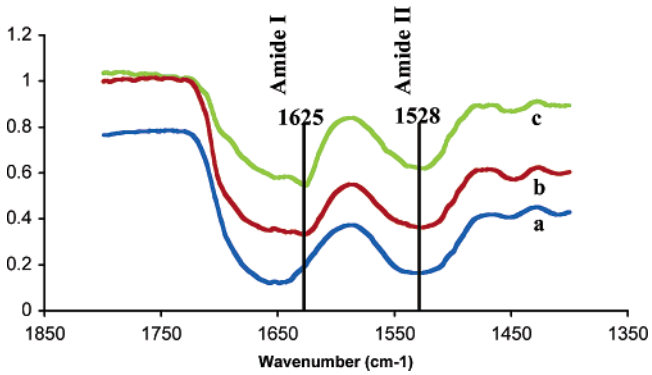

Figure 4. FTIR analysis of recombinant spider silk films. Films were cast from HFIP, allowed to dry and were (a) untreated, (b) treated with 100% methanol, or (c) treated with 70% methanol.

Therefore the two proteins are expected to be positively charged at neutral pH and at least partially soluble in water. Also, natural dragline silk is soluble at high concentration in the major ampullate gland.[31] To determine solubility, saturated solutions of CRGD-15mer and 15mer were prepared in distilled (Milli Q) water and UV measurements were taken at 280 nm. The protein concentrations, deduced from the A_{280} and the extinction coefficient of the recombinant silks, were approximately 0.8 mg/mL and 0.05 mg/mL for CRGD-15mer and 15mer, respectively. Therefore, despite their similarity in sequence with the exception of the CRGD, the two proteins showed large differences in solubility, and in both cases, they were only slightly soluble in water. In a previous report,[32] a recombinant silk protein based on a similar dragline silk consensus was characterized for solubility. The peptide sequence SGRGGLGGQGAG**M**AAAAAA**M**GGAGQGGYGGLGSQG carried two methionines that were oxidized and reduced, changing the solubility of the peptides substantially from 0.48 to 0.02 mg/mL, respectively.[32] Therefore, comparing CRGD-15mer and the methionine carrying peptide (oxidized form), it appears that the addition of two methionines, before and after the polyalanine motif, slightly decreased the solubility of the amino acid sequence. The C-terminal end of the 15mer is less hydrophilic than CRGD-15mer (Figure 1, panels a and b), and both ends lack the charged CRGD group, a difference which can explain the lowered solubility of 15mer with respect to CRGD-15mer.

FTIR Analysis. To establish if the recombinant material was capable of undergoing structural changes analogous to native silk, Fourier Transform Infra Red analysis was performed on two-dimensional films. All IR spectra showed minima in the amide II and I regions, at 1529 cm^{-1}, characteristic of the β-sheet structure and at 1640−1660 cm^{-1} due to random coil and turns, respectively (Figure 4). Therefore, all films, independent of methanol treatment, contained a mixture of these structures. After methanol treatment, a change in structure was observed with an increase of the proportion of β-sheet. A negative peak at 1627 cm^{-1} appeared in both treated specimens; this feature is typical of β-structure and was particularly marked in the film treated with 70% methanol (Figure 4). These transitions are similar to those observed for spider and silkworm silk. The same transitions were observed for the 15mer control protein films (data not shown).

CD Analysis. To determine the effect of temperature on the secondary structure of the protein in solution, samples of CRGD-15mer were characterized at 20, 70, and 98 °C. The protein concentration was approximately 0.18 mg/mL in distilled water. CD spectra of CRGD-15mer at 20°C showed a typical random coil curve with a minimum at 196 nm (Figure 5). The standard

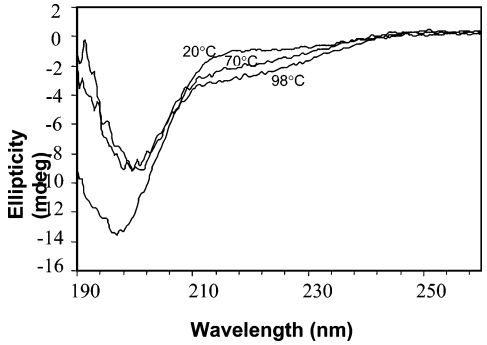

Figure 5. CD spectra of CRGD-15mer taken at 20, 70, and 98 °C. All samples were run at 0.18 mg/mL in water.

CD spectrum of the random coil secondary structure of proteins and peptides is positive between 212 and 240 nm,[33] this region is negative for the CRGD-15mer and flattening out toward 0 at longer wavelengths, suggesting the presence of additional structures, α-helix, β-sheet, or both. When the temperature increased to 70 and 98 °C, the minimum shifted to 200 nm with a diminution of intensity, and the region between 208 and 240 nm became more negative compared to the spectrum at 20°C. Therefore, the temperature increase caused conformational changes consistent with a decrease in random coil and an increase in α-helix, β-sheet, and possibly β-turn.

The CD spectra of the control protein 15mer without CRGD were taken but due to the low solubility of the protein in distilled water, the signal was very low and indistinguishable from noise. Therefore, CD analysis for 15mer protein yielded no significant data. Previously, a similar protein was genetically engineered by our group, and CD experiments showed a high content of β-sheets and negligible amounts of any other secondary structures.[32] However, it should be noted that the protein was only about half the size and also contained two methionines before and after the polyalanine motif which most likely contributed in higher solubility of the protein and higher CD signals.[32] Comparisons could be made between the 15mer protein and the similarly engineered octamer that suggest the difference in the lengths of the proteins play a significant role in solubility, likely reflecting a higher beta sheet content for the 15mer and thus lower solubility. The quantitative contribution of each structure was not evaluated because deconvolution of CD spectra is based on data derived from globular proteins. An accurate deconvolution will only be possible when a database of fibrous reference proteins is available.

Characterization of Fibers. To obtain a concentrated solution of recombinant silk, the protein was initially dissolved in HFIP to a final concentration of 4%. However, the solution was very viscous compared to a silkworm silk fibroin solution of the same concentration and would dry when exposed at the end of the capillary tip at the flow rate used for spinning. Therefore, an optimal concentration of 2% for electrospinning was determined empirically. Morphology and thickness of the electrospun fibers were analyzed by SEM. Fibers ranged from 50 to 250 nm, with an average diameter of 100 nm. The fibers appeared flat when compared to the more cylindrical shaped fibers obtained with regenerated fibroin[30,33] and some of the fibers appeared to contain two or three adjacent fibers (Figure 6a). After methanol treatment, the fibers appeared more flattened and partially fused at the intersection between multiple fibers (Figure 6b).

Cell Response to Recombinant Spider Silk. As a measure of mature bone-related outcomes from the hMSCs, calcification was determined both biochemically and by fluorescence microscopy after growth and differentiation of hMSCs on films formed from these silk proteins. The calcium content was used as a measure of cell response on the matrixes used as it is routinely considered a more mature indicator of cellular response toward osteogenic features. After 3 and 4 weeks, both the genetically engineered 15mer (control without RGD) and CRGD-15mer spider silk films showed more calcification than the treated polystyrene of tissue culture plastic. The calcium content on the CRGD-15mer silk film remained relatively constant for one week after week 3 while a slight decrease in calcium content was observed on the 15mer silk film. However, the 15mer film induced almost twice as much calcium deposition as CRGD-15mer film after 3 weeks (Figure 7). As a result, we can conclude that spider silk films from genetically engineered proteins showed increases in calcium deposition in comparison to tissue culture plastic, but the enhanced function of RGD on cell outcomes was not observed.

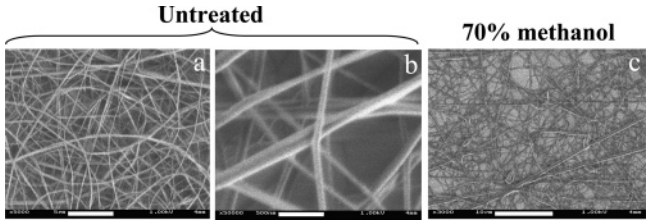

Figure 6. SEM images of electrospun recombinant spider silk (CRGD-15mer, 2% in HFIP) (a) untreated at magnification 5000 (scale bar = 5 μm), (b) untreated at magnification 50 000 (scale bar = 500 nm), and (c) treated with 70% methanol at magnification 3000 (scale bar = 10 μm).

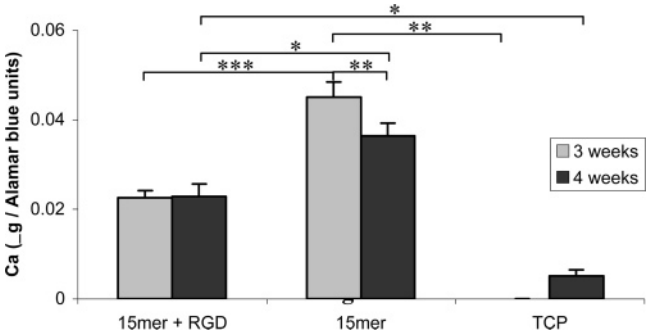

Figure 7. Comparison of relative amounts of Ca deposition on CRGD15mer and 15mer silk films, and tissue culture plastic (TCP); as a measure of cell response and hMSC growth on recombinant spider silk. (*), (**), and (***) represent statistically significant differences with $p \leq 0.5$, 0.01, and 0.001.

There is a possibility that residual endotoxin resulting from the expression in *E. coli* could lead to calcification as a consequence of an inflammatory response. The concentration of endotoxin in the medium was found to be 0.5−1.0 ng/mL. This level of lipopolysaccharide (LPS) enhances the production of interleukins in hBMSCs[35] but does not induce osteoclast differentiation in these cells.[36] Also, the significantly high calcium content on the recombinant spider silk corresponded to a higher cell density determined by DNA content quantitation (data not shown).

Discussion

Two dragline silk-like recombinant variants, CRGD-15mer and 15mer, were cloned, expressed, and purified from *E. coli*. The level of expression was high compared to previously expressed silk-like proteins or fragments of native silks.[24] A combination of codon optimization, use of the strong T7 viral

promoter, and expression in a host incapable of genetic recombination may help explain the higher yield. It should be noted that expression levels of many synthetic genes based on spider dragline silks have been low despite codon optimization and minimization of sequence repetitiveness in prior studies: 2−15 mg/L,[16] 2−10 mg/L cells,[37] 1.2−5.2 mg/L.[38] These reported yields were comparable to those obtained expressing native dragline silks in *E. coli*: 4 mg/L.[20] We previously reported the synthesis of dragline-like proteins almost identical to 15mer in primary sequence, and the level of expression was in the range of 27−41 mg/L (tetramer) and 25−95 mg/L (octamer) before purification.[32] A third sequence was expressed in *E.* coli with a final yield of 16 mg/L.[26] These constructs, like the CRDG-15mer and 15mer, were expressed in pET expression vectors (pET29a(+) or pET30a(+)) with the bacterial host *E.coli* BLR (DE3). CRGD-15mer and 15mer were eventually expressed in *E. coli* mutant host strain RY3041,[27] where the level of expression did not differ from *E. coli* host strain BLR (DE3) but yielded more and purer proteins during His-Tag purification since the histidine rich contaminant SlyD protein was no longer expressed in the mutant host strain. The difference in final yield between CRGD-15mer and 15mer was mostly due to loss of protein during dialysis. The low solubility of 15mer caused the precipitation or deposition of the protein on the surface of the dialysis membrane.

The recombinant spider silk proteins were successfully processed into films and electrospun fibers in the present work. These results, along with the structural characterization, suggest versatility in biomaterials formats from these novel proteins, as well as some recapitulation of native structural transitions during the processing and postprocessing treatments. The electrospun fiber mats offer an alternative to the native fibers for cell culture with higher flexibility and surface area, thus potentially offering useful features for cell interactions as we have found recently for reprocessed silkworm fibroin in similar formats.[39,40]

During natural spinning of spider and silkworm silks, the transition to β-sheet structure is accomplished by physical shear and results in the production of insoluble silk fibers. In the laboratory, *B. mori* silk fibroin undergoes a transition from amorphous to silk II (crystalline β-sheet conformation) after exposure to a methanol/water mixture. This change in conformation is a desirable property because it abolishes the solubility of silk in aqueous solutions. The genetically engineered spider silk protein was expected to behave in a similar way and this was found during materials processing. This ability to control structural changes is important for the processing of the material. Therefore, for the preparation of films or fiber mats that have applications in an aqueous environment, the scaffolds were treated with methanol to reproduce the amorphous to β-sheet transition. Films formed from the recombinant spider silk were made insoluble in water after the methanol treatment and were therefore suitable as substrates for cell growth.

Many studies have been conducted with *B. mori* silkworm silk fibroin as a biomaterial.[10,15] Recently, we reported the covalent decoration of *B. mori* silk films with the same integrin recognition sequence, RGD, as in the present study, as well as other factors including parathyroid hormone (PTH) and a modified PTH.[10] The adhesion of osteoblast-like cells (Saos-2) to these modified silk films containing these integrin recognition sequences was significantly increased as a result of these chemical modifications, reflecting both a change in hydrophobicity as well as cell binding domains. Stimulation of osteoblast-based mineralization in vitro was also found under these conditions. We have also shown the ability of RGD-coupled silkworm silk fiber matrixes to support the adhesion of hMSCs, along with spreading and proliferation and collagen type I formation.[15] These results demonstrate that silkworm silk is a suitable biodegradable protein matrix in film and fiber form that can be used to form scaffolds and that the functional properties in terms of interactions with cells improved by the introduction of the RGD via surface chemical decoration.

Although silkworm silk has been established as a promising biomaterial, spider dragline silk, whose mechanical properties exceed those of silkworm silk fibers, has not been explored as a potential candidate biomaterial for scaffolds in tissue engineering. The main limitations to the use of this source of silk proteins is due to the difficulties in harvesting sufficient amounts of these silks, as well as their purification, when compared to silkworm fibroin. However, through genetic engineering based on using multimers of the consensus repeat from the major ampullate protein of *N. clavipes* dragline protein, sufficient protein could be generated for biomaterial studies.[20,26,32] The ability to construct bioengineered spider silk proteins with designer features, such as the introduction of RGD motifs, suggests a wide range of new opportunities to match native features of the families of spider silk protein variants (e.g., dragline, cocoon, and adhesion) with biomaterial features, along with enhanced functions due to the addition of domains such as RGD in the present study. The additional inclusion of cysteine in the sequence leaves options for facile decoration of the recombinant proteins in biomaterials formats through disulfide interactions with growth factors or other useful components.

RGD motifs were included within the sequence of the recombinant silk to help stimulate cell adhesion on silk film surfaces.[41] However, a higher calcium content was observed on the recombinant 15mer substrate without RGD, leading to the conclusion that the recombinant spider silk primary sequence chemistry and secondary structure may be sufficient to increase cell response such that the RGD motif plays an insignificant role under these conditions. Furthermore, the extra amino acids present at the C and N-termini of the genetically engineered proteins might contribute in the formation of secondary structure that could result in the adjacent RGDs being unavailable for binding. This conclusion is significant in that prior studies with silkworm fibroin suggested the opposite outcomes, RGD chemically coupled via carbodiimide to silkworm silk fibroin films and fibers demonstrated enhanced osteogenic outcomes or other cellular functions.[10,15,42,43] These differences in cell interactions between the source of silk (spider dragline vs silkworm fibroin) and mode of decoration with RGD (part of primary sequence vs chemically appended) highlight the importance of subtle differences in biomaterial features such as hydrophobicity and surface chemistry in controlling cell interactions.

We also suggest that, during the process of forming the films on which the cells were grown, the recombinant proteins may assemble into secondary and tertiary structures, including β-sheets, such that few of the incorporated RGD motifs are exposed to the external medium and cells. This could partly explain the differences observed between the RGD-containing and the RGD-lacking spider silk films and why higher calcium content on the CRGD-15mer spider silk films was not found. Furthermore, the RGD peptide sequence needs to be accessible for binding to integrins; thus, the spacing between the RGD sequence and the anchoring moiety, in this case the spider silk consensus sequence, can be important for cell attachment.[44,45] Here the anchor is the 15mer spider silk protein, and the RGD sequence is added adjacent to the 15mer as part of the primary

sequence. In the design, it was assumed that the silk ends would be flexible enough for RGD access. However, spacers may be needed between the RGD sequences and the silk protein to increase binding to integrins on the cell surface.

Conclusions

The improved response of cells to the recombinant spider silk films when compared to tissue culture plastic provide support that recombinant spider silk is a potentially useful biomaterial for cell adhesion, growth, and differentiation. The strategy employed in the present work to bioengineer spider silk consensus repeats to enhance selective cell interactions provides a template for future options. For example, we have recently reported the chemical modification of silkworm silk with the bone inducing morphogen, BMP-2, with improvements in bone tissue outcomes as a result with seeded with hMSCs.[13] Bioengineered spider silks in which protein fusions, such as between the silk consensus repeat and morphogens, cytokines, or other matrix interacting function, would provide a route to the design and control of more complex biomaterial matrix systems to direct cell and tissue outcomes. Furthermore, these approaches would obviate the need for complex chemical coupling steps.

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References and Notes

(1) Zhou, C. Z.; Confalonieri, F.; Jacquet, M.; Perasso, R.; Li, Z. G.; Janin, J. *Proteins* **2001**, *44* (2), 119−22.
(2) Zhou, Y.; Morais-Cabral, J. H.; Kaufman, A.; MacKinnon, R. *Nature* **2001**, *414* (6859), 43−8.
(3) Gatesy, J.; Hayashi, C.; Motriuk, D.; Woods, J.; Lewis, R. *Science* **2001**, *291* (5513), 2603−5.
(4) Hinman, M. B.; Jones, J. A.; Lewis, R. V. *Trends Biotechnol.* **2000**, *18* (9), 374−9.
(5) Vollrath, F.; Knight, D. P. *Nature* **2001**, *410* (6828), 541−8.
(6) Vollrath, F.; Madsen, B.; Shao, Z. *Proc. R Soc. London B Biol. Sci.* **2001**, *268* (1483), 2339−46.
(7) Gosline, J. M.; Guerette, P. A.; Ortlepp, C. S.; Savage, K. N. *J. Exp. Biol.* **1999**, *202* (Pt 23), 3295−303.
(8) Xu, M.; Lewis, R. V. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87* (18), 7120−4.
(9) Altman, G. H.; Diaz, F.; Jakuba, C.; Calabro, T.; Horan, R. L.; Chen, J.; Lu, H.; Richmond, J.; Kaplan, D. L. *Biomaterials* **2003**, *24* (3), 401−16.
(10) Sofia, S.; McCarthy, M. B.; Gronowicz, G.; Kaplan, D. L. *J. Biomed. Mater. Res.* **2001**, *54* (1), 139−48.
(11) Meinel, L.; Hofmann, S.; Karageorgiou, V.; Zichner, L.; Langer, R.; Kaplan, D.; Vunjak-Novakovic, G. *Biotechnol. Bioeng.* **2004**, *88* (3), 379−91.
(12) Meinel, L.; Karageorgiou, V.; Fajardo, R.; Snyder, B.; Shinde-Patil, V.; Zichner, L.; Kaplan, D.; Langer, R.; Vunjak-Novakovic, G. *Ann. Biomed. Eng.* **2004**, *32* (1), 112−22.
(13) Meinel, L.; Karageorgiou, V.; Hofmann, S.; Fajardo, R.; Snyder, B.; Li, C.; Zichner, L.; Langer, R.; Vunjak-Novakovic, G.; Kaplan, D. L. *J. Biomed. Mater. Res. A* **2004**, *71* (1), 25−34.
(14) Altman, G. H.; Horan, R. L.; Martin, I.; Farhadi, J.; Stark, P. R.; Volloch, V.; Richmond, J. C.; Vunjak-Novakovic, G.; Kaplan, D. L. *FASEB J.* **2002**, *16* (2), 270−2.
(15) Chen, J.; Altman, G. H.; Karageorgiou, V.; Horan, R.; Collette, A.; Volloch, V.; Colabro, T.; Kaplan, D. L. *J. Biomed. Mater. Res. A* **2003**, *67* (2), 559−70.
(16) Prince, J. T.; McGrath, K. P.; DiGirolamo, C. M.; Kaplan, D. L. *Biochemistry* **1995**, *34* (34), 10879−85.
(17) Fahnestock, S. R.; Bedzyk, L. A. *Appl. Microbiol. Biotechnol.* **1997**, *47* (1), 33−9.
(18) Fahnestock, S. R.; Irwin, S. L. *Appl. Microbiol. Biotechnol.* **1997**, *47* (1), 23−32.
(19) Fahnestock, S. R.; Yao, Z.; Bedzyk, L. A. *J. Biotechnol.* **2000**, *74* (2), 105−19.
(20) Arcidiacono, S.; Mello, C.; Kaplan, D.; Cheley, S.; Bayley, H. *Appl. Microbiol. Biotechnol.* **1998**, *49* (1), 31−8.
(21) Lazaris, A.; Arcidiacono, S.; Huang, Y.; Zhou, J. F.; Duguay, F.; Chretien, N.; Welsh, E. A.; Soares, J. W.; Karatzas, C. N. *Science* **2002**, *295* (5554), 472−6.
(22) Pandey, A. *Trends Genet.* **2001**, *17* (8), 442.
(23) Scheller, J.; Guhrs, K. H.; Grosse, F.; Conrad, U. *Nat. Biotechnol.* **2001**, *19* (6), 573−7.
(24) Wong Po Foo, C.; Kaplan, D. L. *Adv. Drug Delivery Rev.* **2002**, *54* (8), 1131−43.
(25) Huang, J.; Valluzzi, R.; Bini, E.; Vernaglia, B.; Kaplan, D. L. *J. Biol. Chem.* **2003**, *278* (46), 46117−23.
(26) Winkler, S.; Wilson, D.; Kaplan, D. L. *Biochemistry* **2000**, *39* (41), 12739−46.
(27) Yan, S. Z.; Beeler, J. A.; Chen, Y.; Shelton, R. K.; Tang, W. J. *J. Biol. Chem.* **2001**, *276* (11), 8500−6.
(28) Panitch, A.; Matsuki, K.; Cantor, E. J.; Cooper, S. J.; Atkins, E. D. T.; Fournier, M. J.; Mason, T. L.; Tirrell, D. A. *Macromolecules* **1997**, *30* (1), 42−49.
(29) Winkler, S.; Wilson, D.; Kaplan, D. L. *Biochemistry* **2000**, *39* (45), 14002.
(30) Jin, H. J.; Fridrikh, S. V.; Rutledge, G. C.; Kaplan, D. L. *Biomacromolecules* **2002**, *3* (6), 1233−9.
(31) Kenney, J. M.; Knight, D.; Wise, M. J.; Vollrath, F. *Eur. J. Biochem.* **2002**, *269* (16), 4159−63.
(32) Szela, S.; Avtges, P.; Valluzzi, R.; Winkler, S.; Wilson, D.; Kirschner, D.; Kaplan, D. L. *Biomacromolecules* **2000**, *1* (4), 534−42.
(33) Greenfield, N.; Fasman, G. D. *Biochemistry* **1969**, *8* (10), 4108−16.
(34) Sukigara, S.; Gandhi, M.; Ayutsede, J.; Micklus, M.; Ko, F. *Polymer* **2003**, *44* (19), 5721−5727.
(35) Rougier, F.; Cornu, E.; Praloran, V.; Denizot, Y. *Cytokine* **1998**, *10* (2), 93−7.
(36) Zou, W.; Bar-Shavit, Z. *J. Bone Miner. Res.* **2002**, *17* (7), 1211−8.
(37) Lewis, R. V.; Hinman, M.; Kothakota, S.; Fournier, M. J. *Protein Expr. Purif.* **1996**, *7* (4), 400−6.
(38) Fukushima, Y. *Biopolymers* **1998**, *45* (4), 269−79.
(39) Jin, H. J.; Chen, J.; Karageorgiou, V.; Altman, G. H.; Kaplan, D. L. *Biomaterials* **2004**, *25* (6), 1039−47.
(40) Mao, C.; Qiu, Y.; Sang, H.; Mei, H.; Zhu, A.; Shen, J.; Lin, S. *Adv. Colloid Interface Sci.* **2004**, *110* (1−2), 5−17.
(41) Hersel, U.; Dahmen, C.; Kessler, H. *Biomaterials* **2003**, *24* (24), 4385−415.
(42) Asakura, T.; Tanaka, C.; Yang, M.; Yao, J.; Kurokawa, M. *Biomaterials* **2004**, *25* (4), 617−24.
(43) Higuchi, A.; Takanashi, Y.; Tsuzuki, N.; Asakura, T.; Cho, C. S.; Akaike, T.; Hara, M. *J. Biomed. Mater. Res. A* **2003**, *65* (3), 369−78.
(44) Pierschbacher, M. D.; Ruoslahti, E. *Nature* **1984**, *309* (5963), 30−3.
(45) Beer, J. H.; Springer, K. T.; Coller, B. S. *Blood* **1992**, *79* (1), 117−28.

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