Published in final edited form as:

Biomaterials. 2013 January ; 34(2): 402–412. doi:10.1016/j.biomaterials.2012.09.085.

The influence of specific binding of collagen-silk chimeras to silk biomaterials on hMSC behavior

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Abstract

Collagen-like proteins in the bacteria *Streptococcus pyogenes* adopt a triple-helix structure with a thermal stability similar to that of animal collagens, can be expressed in high yield in *E. coli* and can be easily modified through molecular biology techniques. However, potential applications for such recombinant collagens are limited by their lack of higher order structure to achieve the physical properties needed for most biomaterials. To overcome this problem, the *S. pyogenes* collagen domain was fused to a repetitive *Bombyx mori* silk consensus sequence, as a strategy to direct specific non-covalent binding onto solid silk materials whose superior stability, mechanical and material properties have been previously established. This approach resulted in the successful binding of these new collagen-silk chimeric proteins to silk films and porous scaffolds, and the binding affinity could be controlled by varying the number of repeats in the silk sequence. To explore the potential of collagen-silk chimera for regulating biological activity, integrin (Int) and fibronectin (Fn) binding sequences from mammalian collagens were introduced into the bacterial collagen domain. The attachment of bioactive collagen-silk chimeras to solid silk biomaterials promoted hMSC spreading and proliferation substantially in comparison to the controls. The ability to combine the biomaterial features of silk with the biological activities of collagen allowed more rapid cell interactions with silk-based biomaterials, improved regulation of stem cell growth and differentiation, as well as the formation of artificial extracellular matrices useful for tissue engineering applications.

Keywords

collagen; silk; stem cells; chimera

1. INTRODUCTION

Collagens, the major proteins in the extracellular matrix (ECM), are distinguished by their characteristic triple-helical molecular structure, which requires a (Gly-Xaa-Yaa)_n repeating amino acid sequence as well as a high content of Pro and the post-translationally modified hydroxyproline (Hyp) to promote stabilization [1]. The most abundant collagens form axially periodic fibrils in tendon, bone, cartilage and other tissues, and these collagens are widely used in tissue engineering and regenerative medicine because of their role in cell

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signalling and development, as well as their structural properties [2–5]. However, there remain concerns that collagens derived from animal sources could be contaminated with transmissible diseases, such as prion-related transmissible spongiform encephalopathies (TSEs) and procedures for extraction and purification of such collagens have proven difficult to standardize [6, 7].

Alternative approaches to obtain collagenous biomaterials have been developed to bypass these issues and to introduce the ability to modify the collagen sequences to customize these proteins for specific applications. Synthetic triple-helical peptides have been successfully used as models for collagen for many years [8–11] and recent progress has been made in inducing such peptides to form higher order structures such as fibers and hydrogels with the capacity to support cell growth [9, 12–14]. At this time, however, peptide synthesis is not economical for commercial level production. Approaches to express recombinant collagens in heterologous systems are also being widely pursued [15]. Human collagens can be successfully expressed in mammalian cell lines, but low yields with high costs make it not suitable for large scale production [16]. Production of collagen in well-developed prokaryote expression systems has proved challenging because of the lack of prolyl hydroxylase genes [6]. Human collagens have been successfully co-expressed with prolyl hydroxylase genes in yeast [17], and recently these genes were incorporated into *E. coli*, leading to partial hydroxylation of short collagen-like sequences [18, 19]. In these cases, optimization of expression conditions to balance protein yields and maximum hydroxylation level poses a challenge. Recently, another strategy to obtain high yield expression of collagenous proteins in *E. coli* has been pursued: expression of bacterial collagen-like proteins which form thermally stable triple-helices in the absence of hydroxyproline (Hyp) [7, 20].

Although collagen has long been considered a distinctive protein of multicellular animals, collagen-like proteins with typical repeating Gly-Xaa-Yaa sequences and triple-helical structures have been found in a number of bacteria [21, 22]. These bacterial collagen-like proteins lack the post-translational proline hydroxylation yet still form triple helical structures with a thermal stability of 35–40°C [23], similar to that found for mammalian collagens [24]. One well characterized bacterial collagen-like protein is Scl2, a membrane bound protein from *Streptococcus pyogenes* which includes an N-terminal trimerization (V) domain adjacent to a (Gly-Xaa-Yaa)₇₉ core collagen-like (CL) domain [21]. The CL collagen domain of Scl2 has a high content of charged residues (30%) which support electrostatic stabilization of the triple-helix (Tm=37°C) in the absence of Hyp [23]. Recombinant VCL can be readily expressed with high yields in *E. coli* and can be modified via genetic manipulation [25, 26]. Examination of the CL sequence suggests that this particular bacterial collagen-like protein lacks any known biologically active sites, while the introduction of a human collagen integrin binding sites [27] or MMP cleavage sequences [25] has resulted in expected binding or cleavage activities.

The potential to obtain collagenous proteins with controllable biological activity modules offers a promising system for biomaterials, but organization of the VCL protein into a stabilized and solid form is a prerequisite for such applications. VCL can be induced to form small fibril-like structures [28], and this protein has also been fabricated into bioactive hydrogels through poly(ethylene glycol) diacrylate photocrosslinking [20] or lyophilization after glutaraldehyde vapor crosslinking [7]. But such chemical modifications require multistep processes and can be challenging to characterize and can result in negative biological impacts if unreacted chemicals are not fully removed.

Here, a new approach is described to materialize VCL protein via specific non-covalent binding to solid silk biomaterials. Silk is well known for its excellent mechanical properties, its biocompatibility and biodegradability. Processed silk fibroin has been fabricated into

films [29, 30], porous scaffolds [31, 32], hydrogels [33, 34] and fibers [35, 36] for various tissue engineering and medical applications. In this study, recombinant collagen-silk chimeric proteins were designed to contain a short consensus sequence (GAGAGS)_n from *Bombyx mori* silk fused to the bacterial collagen sequence. Because of the capacity of silk for self-assembly, it was hypothesized that the introduction of the silk consensus sequence would give the collagen-silk chimera the ability to non-covalently bind to silk. The addition of the silk sequence promoted attachment of the collagen-silk chimeras to processed solid silk materials, with the binding affinity dependent on the length of the silk sequence. To examine the potential of collagen-silk chimera for regulating biological activity, integrin (Int) and fibronectin (Fn) binding sequences from mammalian collagens were introduced into the bacterial collagen domain, and their influence on the growth of hMSCs was investigated.

2. MATERIALS AND METHODS

2.1 Design and preparation of bacterial collagen silk chimeras

2.1.1 Gene cloning—The DNA sequence for the bacterial collagen VCL constructs (Figure 1A) was based on the Scl2 sequence from *S. pyogenes* [21], with DNA encoding an octa-histidine tag (His₈-tag) introduced at the N-terminus and DNA encoding the (GAGAGS)₆ silk sequence (ST₆) attached to the C-terminus of the VCL (Biomatik Corp. Wilmington, DE). An Int-binding site GFPGER was introduced by site-direct mutagenesis, changing the GLQ to GFP at triplet number 17 of the CL domain. A sequence encoding the Fn-binding site GLPGQRGER was inserted after triplet number 31 in the CL domain of Scl2 through restriction sites *ApaI* and *XmaI*. The final construct containing both the Int-binding site and the Fn-binding site, denoted VCL-Int-Fn-ST₆, was cloned into the pColdIII vector (Takara Bio Inc.) through *NdeI* and *XbaI* restriction sites. *BamHI* and *XbaI* were later used to replace the ST₆ silk sequence by ST₃ or ST₉. Clones with only Int- or Fn-binding site mutations were used for cell culture comparison studies. All enzymes for cloning were purchased from New England Biolabs (Ipswich, MA). DNA sequencing to confirm fidelity was carried out at the Tufts Core Facility.

2.1.2 Protein expression and purification—All VCL constructs in the pColdIII vector were expressed in *E. coli* BL21, grown in 20 ml LB medium with 100 µg/ml ampicillin overnight at 37°C. This starting culture was used to inoculate 500 ml of LB-ampicillin media in a shaking flask and grown at 37°C to an OD₆₀₀=0.8. To induce protein expression, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture and the temperature was lowered to 22°C. After 16 hrs induction, cells were harvested by centrifugation and resuspended in His-tag purification column binding buffer (20 mM sodium phosphate buffer pH 7.4, 500 mM NaCl, 10 mM imidazole) containing 0.25 mg/ml lysozyme and frozen at -80°C until purification.

Purification of the His-tagged collagen-silk chimeras was carried out by gravity driven immobilized metal ion affinity chromatography with nickel ion binding and imidazole gradient elution. Frozen cells were thawed and further lysed by sonication. Cellular debris was removed by centrifugation at 8,000 rpm at 4°C. Supernatant containing the soluble target protein was loaded onto equilibrated purification columns packed with Ni-NTA beads (Qiagen, Valencia, CA) and run through the column by gravity. The column was then washed sequentially with 2 bed volumes of binding buffer, binding buffer plus 60 mM imidazole and binding buffer plus 120 mM of imidazole. His-tagged protein on the column was eluted by elution buffer (binding buffer plus 400 mM imidazole). Protein purity was checked by SDS-PAGE and molecular weight was determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The

concentration of protein was determined using an extinction coefficient of $\epsilon_{280} = 9970 \text{ M}^{-1}\cdot\text{cm}^{-1}$ after dialysis into phosphate buffered saline (PBS, pH7.4). Chemicals used in all experiments are purchased from Sigma-Aldrich unless otherwise indicated.

2.2 Biophysical characterization

2.2.1 Circular Dichroism (CD) Spectrometry—CD spectra were obtained on an AVIV Model 420 CD spectrometer (AVIV Biomedical, Lakewood, NJ) using glass cuvettes with 1 mm path length. Protein solutions were equilibrated for at least 24 hrs at 4°C before measurement. Wavelength scans were collected from 190 to 260 nm in 0.5 nm steps with a 4 sec averaging time, 1.0 nm bandwidth and repeated three times. Temperature scans were monitored from 10°C to 60°C at 225 nm with a 10 sec averaging time and 1.5 nm bandwidth. Samples were equilibrated for 2 min at each temperature, and the temperature was increased at an average rate of 0.1°C/min.

2.2.2 Differential Scanning Calorimetry (DSC)—DSC was performed on a NANO DSC II Model 6100 (Calorimetry Sciences Corp, Lindon, UT). Each sample was re-dialyzed against PBS overnight before measurement to collect the dialyzed buffer as reference in the experiment. Sample solutions were loaded at 0°C into the cell and heated at a rate of 1°C/min till 80°C.

2.2.3 MALDI-TOF Mass Spectrometry—MALDI matrix was prepared by saturating α -Cyano-4-hydroxycinnamic acid (CHCA) in solution contains 50% (v/v) acetonitrile and 0.3% (v/v) trifluoroacetic acid. A 6 μl aliquot of 1 mg/ml sample was mixed with 24 μl of matrix, 1 μl solution was plated onto a 96 spot target plate and allowed to dry. MALDI-TOF mass spectra were acquired on a Microflex LT system (Bruker Corporation, Billerica, MA) with 50% laser intensity using standard LP (linear positive) 60 kDa method provided by the software.

2.2.4 Dynamic Light Scattering (DLS)—DLS measurements were performed using a DynaPro Titan instrument (Wyatt Technology Corp., Santa Barbara, CA) equipped with a temperature controller using Eppendorf UVette cuvettes with 1 cm path length. Samples were filtered through 0.2 μm Whatman Anotop filters before measurement. Samples were measured at 75% laser intensity. Twentily acquisitions were taken for every sample with each acquisition lasting 60 sec. To obtain the hydrodynamic radius (R_h), the intensity autocorrelation functions were analysed by Dynamic software.

2.2.5 Atomic Force Microscopy (AFM)—To observe surface profiles of silk films after treatment with the collagen-silk chimeras, AFM was performed on a VEECO Dimension 3100 Atomic Force Microscope (Veeco Instruments Inc., Plainview, NY) equipped with FESP AFM probe (Resonance frequency: 75kHz, spring constant: 3 N/m, Bruker Corporation, Billerica, MA) using methods previously reported [37]. For sample preparation, 2% silk HFIP solution was cast onto glass slides and dried to form a film. The films were subsequently treated with 90% methanol and washed by water twice before incubating with 1 μM collagen-silk chimeras or PBS control. After 2 hrs incubation, silk films were washed with excessive amounts of ultrapure water and dried before imaging. Surface height images of the silk films were acquired in tapping mode at a scan rate of 0.6–0.8 Hz and 3D plots were generated using the software provided by the equipment.

2.3 Silk film and scaffold preparation

B. mori silk fibroin solutions were prepared based on previously published procedures [38]. Silkworm cocoons were degummed by boiling 60 min in 20 mM Na_2CO_3 solution. Extracted silk fibroin was dissolved in 9.3 M LiBr solution at 60°C for 4 hrs for a final

concentration of 20 wt%. This solution was dialyzed against water using Slide-a-Lyzer dialysis cassettes (Pierce, MWCO 3,500) for 72 hrs. The aqueous silk solution was lyophilized to obtain dried silk and subsequently dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) to produce a 17 wt% silk dope. For silk film preparation, 30 μ l (96-well plate, for ELISA binding assay) or 150 μ l (24-well plates, for cell culture study) of 2 wt% silk-HFIP solution was added to each well in the testing plate and dried in a hood. The silk film at the bottom of the wells was then treated with 90% (v/v) methanol/water solution for 30 min, washed 3 times with ddH₂O and dried again. For silk scaffold preparation, 1 ml of silk HFIP solution (17 wt%) was added into 3.4 g of granular NaCl (particle size; 400–600 μ m) in a Teflon container. The container was covered and left overnight to provide sufficient time for homogeneous distribution of the solution over the salts. Subsequently, the solvent was evaporated at room temperature for 3 days. The scaffold in the container was then soaked in (90% v/v) methanol/water solution for 30 min, followed by immersion in water for 2 days to remove the NaCl. A 6 mm dermal punch was used to make cylindrical shaped silk scaffolds with 6 mm diameter and 10 mm height. The physical property and morphology of silk scaffold has been previously characterized.[32] For ELISA binding assay, scaffolds were prepared in smaller pieces to weigh 40–50 mg, with the final result normalized by the actual weight. For cell culture studies, 6 mm $\times$ 8 mm scaffolds were soaked in ddH₂O and autoclave for sterilization before seeding cells.

2.4 Silk binding assays

The binding of collagen-silk chimeras to silk films and porous scaffolds were carried out using an enzyme-linked immunosorbent assay (ELISA) previously described [39]. Serial dilutions of 1 mg/ml (~20 μ M) protein samples to 0.01 μ M, 0.05 μ M, 0.1 μ M, 0.5 μ M, 1 μ M, 5 μ M, 10 μ M solutions were prepared and the diluted protein solution in PBS was incubated with the silk films or porous scaffolds in 96-well plates at room temperature for 2 hrs with shaking. A 30 μ l solution was used for silk films and 100 μ l for the porous scaffolds in order to fully immerse the scaffolds. After incubation, silk films or scaffolds were washed 10 times with 200 μ l of PBST (PBS with 0.05% Tween-20, Pierce, Rockford, IL), then 50 μ l (for films) or 120 μ l (for porous scaffolds) of 1% bovine serum albumin (BSA) in PBS was added for blocking overnight on the shaker. Anti-polyHis antibody with conjugated HRP (Abcam, Cambridge, MA) was used to detect the His-tagged recombinant collagen-silk proteins with 1:20000 dilutions in 1% BSA-PBS solution for 1 hr. Silk films or porous scaffolds were subsequently washed 10 times with 200 μ l of PBST and colorimetric reactions were run by adding 100 μ l of 3,3',5,5'-tetramethylbenzidine solution (Pierce, Rockford, IL). Plates were incubated at room temperature for 15 min, and 50 μ l of solution from each sample was transferred to a new well with 1N HCl to stop the reaction. For silk films, an OD₄₅₀ was directly read from the 96-well plate using a SPECTRA MAX M2 plate reader (Molecular Devices, Sunnyvale, CA). For silk porous scaffolds, the solution was first diluted to half with PBS before the OD₄₅₀ measurement. Binding assay on spider silks followed the exact procedure mentioned above, the recombinant spider silk materials were generously provided in lyophilized form by the Lewis lab at Utah State University.

2.5 Cell culture study

2.5.1 hMSC cultures—2D hMSC cultures were carried out on protein-coated 24-well tissue culture plates (TCP). To coat the plates, collagen-silk chimeric proteins were diluted to 40 μ g/ml in ultrapure water, 500 μ l protein solutions were added to each well and allowed to dry in a cell culture hood. The plates were sprayed with 70% ethanol and allowed to dry in the cell culture hood for another 24 hrs. Coating efficiency was determined by Sirius Red staining with details provided in the Supplemental Material (Figure S1, S2). Passage 2 (P2) human mesenchymal stem cells (hMSC) (Lonza, Walkersville, MD) were seeded at a density of 5,000 cells per well (2,500 cells/cm²) and cultured at 37°C under a 5% CO₂

humidified atmosphere in hMSC growth media (high glucose Dulbecco's Modified Eagle Medium, 10% fetal bovine serum, 1% non-essential amino acids, 1% penicillin/streptomycin, 2 ng/ml basic fibroblast growth factor).

For 3D hMSC culture on silk porous scaffolds, sterilized samples were soaked in 1 μ M of filter sterilized VCL-Int-Fn-ST₆ PBS solution overnight and washed 3 times with excessive amount of Dulbecco's phosphate buffered saline (DPBS). P2 hMSCs were seeded at a density of 100,000 cells per scaffold. Scaffolds with freshly seeded cells sat in a cell culture incubator for 2 hrs to promote attachment before hMSC growth media was added.

All culture media were changed every three days and all experiments were conducted in triplicates. Chemicals used for cell culture studies were purchased from Invitrogen unless otherwise noted.

2.5.2 Evaluation of hMSC morphology—hMSC morphology in both 2D and 3D cultures were visualized by fluorescence F-actin staining. Prior to staining, plates or scaffolds with cells were washed two times with DPBS. Then, cells were fixed with 10% formalin PBS solution at room temperature for 15 min. After washing with DPBS three times, cells were permeabilized by adding Triton X-100 (0.1% in PBS) at room temperature for 5 mins. To avoid non-specific binding, samples were incubated with 3 wt% BSA in PBS at room temperature for 30 mins. Then, samples were incubated with 1:200 Alexa Fluor 488-phalloidin in the dark for 1 hr, with a subsequent DPBS wash three times, the 2 μ g/ml of Hoechst 33342 was added to stain the nucleus for 15 min before imaging on a Leica DM IL fluorescence microscopy equipped with a Leica DFC340 FX digital camera (Leica Microsystems CMS GmbH). Images are processed using Leica Application Suite 4.0 and ImageJ 1.45S.

2.5.3 Evaluation of hMSC proliferation—Proliferation of hMSCs in both 2D and 3D cultures was determined using alamarBlue assay at 1, 4, 7, 10, 13, 16 days of culture. At each time point, cell culture media containing 10 vol% alamarBlue was added to the samples. After 5 hrs incubation, the fluorescence signal (550 nm excitation 590 nm emission) of a medium aliquot (100 μ l) was measured using a plate reader. To estimate the cell number in 2D culture, a calibration curve was prepared by incubating a specific number of cells in the growth media containing 10% alamarBlue for 5 hrs. A no cell control with 10% alamarBlue in the media was used as a blank and subtracted from the sample. For 3D cultures, relative proliferation rate was determined by percentage reduction of alamarBlue at each time point. A 100% reduced form of alamarBlue was produced by autoclaving the culture medium with 10% alamarBlue at 121°C for 20 mins.

2.6. Statistical analysis

All quantitative analyses were performed at least in triplicate, and the mean values obtained. Results presented were based on the averages of data points and standard deviations as error bars. The significance level was determined by p-value using two sample paired student's t test between the means of two samples. Figures were plotted in Microcal Origin 6.0 or Microsoft Excel 2010.

3. RESULTS

Design, production and characterization of collagen-silk chimeras

A set of chimeric collagen-silk constructs was designed to be tested in terms of silk affinity and hMSC interactions. The constructs included the trimerization domain (V) and the collagen domain (CL) of the *S. pyogenes* Scl2 protein, together with 3-, 6- or 9-repeats of

the *B. mori* silk consensus sequence GAGAGS (or Silk Tag, denoted as ST₃, ST₆, and ST₉) at the C-terminus (Figure 1A). A linker sequence GAGAAGAGS was included for flexibility between the collagen and silk domains. Two sequences from human collagen with known biological activity were introduced into the CL domain (Figure 1A). The Int-binding sequence GFPGER, known to interact with $\alpha 2\beta 1$ integrin [12, 27], was generated by site-direct mutagenesis of GLQ to GFP at triplet number 17 in the CL domain. The sequence GLPGQRGER was reported to represent a minimum binding site for fibronectin type I domain [40], and a longer sequence: GLPGLAGQRGIVGLPGQRGER was inserted after triplet number 31 of CL domain by restriction digestion and splicing of the DNA sequence. An octa-histidine tag was included at the N-terminus of all constructs for protein purification.

All constructs were expressed in *E. coli* at 22°C, and the yields of purified proteins from the soluble fraction of *E. coli* lysate were approximately 100–120 mg/L. The similarity of the yields to those reported previously for bacterial collagen [7, 28] suggests the silk sequence, albeit its propensity of forming hydrophobic aggregates [41], did not significantly affect the yields of expressed soluble proteins. After elution from His-tag columns, all proteins were obtained with a high degree of purity, as indicated by SDS-PAGE (Figure 1B). The protein bands for the collagen-silk chimeras showed decreased migration with increasing length of the silk sequence, confirming the size differences expected for the silk length variation (Figure 1B). MADLI-TOF mass spectrometry confirmed the identity of each construct and again showed the expected increase in molecular weight with increasing *n* for (GAGAGS)_{*n*} (Figure 1C, Table 1).

The conformation and stability of these collagen-silk chimeric proteins were investigated by circular dichroism (CD) spectroscopy and differential scanning calorimetry (DSC). The CD spectra of VCL and the collagen-silk chimeras all showed typical triple helical CD features, with a maximum near 220 nm and a minimum near 200 nm (Figure 2A). The proteins containing the silk sequences (GAGAGS)_{*n*} have lower values of mean residue ellipticity at 220 nm than VCL, and the decrease in MRE_{220nm} is greater for constructs with larger number of silk repeats. This is consistent with an expected α -helix or random coil nature for the silk sequences in solution, prior to induction of β -sheet [42, 43], since these conformations would contribute a negative ellipticity at 220 nm. Monitoring of the 225 nm ellipticity as a function of temperature indicated a thermal stability T_m =36.8°C for VCL, and the thermal stability is slightly lower when the silk sequence (GAGAGS)₆ is added (T_m =36.1°C). A decrease of about 2°C in thermal stability was seen when both Int- and Fn-binding sites are introduced within CL (T_m =34.7–34.9°C) (Figure 2A; Table 1). The insertion of the Fn-binding site likely leads to this 2°C destabilizing effect on the triple-helix, since a similar decrease was observed when other sequences were introduced at this particular site (An, pers. comm.).

The DSC scan of the VCL control showed a sharp transition near 37.5°C, with a calorimetric enthalpy of 3,042 KJ/mol (Figure 2B). The addition of the silk sequence and introduction of the Int- and Fn-binding sequences again lowered the thermal transition; the DSC transition was higher than the CD T_m values because of the faster heating rate [44]. The calorimetric enthalpy decreased with the addition of the silk sequences, and the longer the silk sequence, the greater the decrease (Table 1). The collagen triple-helix is known to have an unusually high calorimetric enthalpy, due to hydrogen bonding involving its extensive hydration network [45], and as the molar fraction of triple-helix decreases, the ΔH_{cal} per mole appeared to decrease as well.

The aggregation state of the collagen-silk chimeras was investigated by dynamic light scattering (DLS), determining the hydrodynamic radii (R_h) of the soluble proteins in both

purification column elution buffer and PBS buffer (Table 1). In elution buffer, the VCL protein had an R_h value of approximately 10 nm, similar to the value reported previously for single VCL triple-helical molecules [23, 28]. The VCL proteins with silk sequences had slightly higher R_h values (1–2 nm increase), consistent with a small increase in length. These results suggest that the eluted proteins were predominantly in the non-aggregated form (mass percentage >97%). After dialysis of the control and all chimeric proteins into PBS, there was no longer any ~10 nm R_h value representing the single molecule form; rather, all molecules were in aggregated forms (Table 1). The predominant species in PBS appeared to be oligomers, with R_h values ranging from 17.6 nm (VCL-Int-Fn-ST₉) to 27.8 nm for the VCL control. Significantly higher molecular aggregates were also seen (R_h ranging from 69–99 nm), the fraction of these large aggregates increased for molecules with longer silk sequences.

Binding of collagen-silk chimeras to solid silk biomaterials

To determine whether the collagen-silk chimeras could bind to solid silk structures, an ELISA binding assay was performed on 2D silkworm silk films cast in the bottom of 96-well assay plates or fabricated 3D silk porous scaffolds. Three variations of collagen-silk chimeras with different lengths of silk sequence (VCL-Int-Fn-ST₃, VCL-Int-Fn-ST₆, VCL-Int-Fn-ST₉) were evaluated (Figure 3). The results showed that VCL without a silk sequence had only a minimal ELISA signal, even at high concentrations, while all three variants of collagen-silk chimeras showed noticeable ELISA signals, indicating significant binding to the silk films or porous scaffolds compare with control ($p < 0.01$). At the lower concentrations, the binding increased with increasing concentrations of the collagen-silk chimeras, and the ELISA signals appeared to reach a plateau after 0.5–1.0 μM . In addition to the binding to the regenerated films or porous scaffolds, binding was also observed to natural degummed silk fibroin, suggesting a general affinity to all silkworm silk based materials (Figure S3).

The copy number of silk sequence repeats influenced the binding of the collagen-silk protein to the silk materials. At all concentrations, the VCL-Int-Fn-ST₃ protein, with just 3 copies of ST, showed lower binding to silk films and the silk porous scaffold than VCL-Int-Fn-ST₆ and VCL-Int-Fn-ST₉. For binding to silk films, VCL-Int-Fn-ST₆ showed less binding than VCL-Int-Fn-ST₉ at lower concentrations (<0.5 μM) and more binding at higher concentrations. For binding to the silk porous scaffold, VCL-Int-Fn-ST₆ again showed less binding than VCL-Int-Fn-ST₉ at lower concentrations (<1 μM), but at higher concentrations their binding was comparable. The difference between the results on silk films and silk porous scaffolds may reflect the larger total surface area available for binding in the latter material, consistent with its stronger ELISA signal.

AFM was performed to confirm the binding of collagen-silk chimeras to silk and to illustrate the height and morphology of the surface deposition pattern on silk films (Figure 4). The surface roughness of the film increased after collagen-silk chimera solution treatment. VCL-Int-Fn-ST₆ or VCL-Int-Fn-ST₉ led to a rougher surface than VCL-Int-Fn-ST₃. This is consistent with more protein retained on the surface after washing, and corresponds with the ELISA results that VCL with longer silk sequence bound more strongly to the surface.

To investigate if the collagen-silk chimeras also showed binding affinity to another type of silk material with different sequences, the same ELISA assay was carried out on two types of recombinant spider silks: major ampullate spidroin 1 (MaSp1) and major ampullate spidroin 2 (MaSp2). MaSp1 carries GGX repeats and polyalanine regions [46]. MaSp2 contains GPGQQGPGGY repeats as well as polyalanine regions [47]. The binding affinity of VCL-Int-Fn-ST₆ at 1 μM and 10 μM was tested. Interestingly, we observed almost no

binding of VCL-Int-Fn-ST₆ to both types of spider silk films, while binding affinity to the silkworm silk film was again observed (Figure 3C).

hMSC responses on collagen-silk chimeras in 2D culture and 3D porous silk scaffolds

To select the best biologically active candidate to bind onto silk porous scaffolds and make a bioactive solid material, hMSC responses on VCL-Int-Fn-ST₆, VCL-Int-ST₆ and VCL-Fn-ST₆ were compared, along with rat tail collagen type I (BD Bioscience, San Jose, CA) as a positive control, and VCL, silk films and VCL-ST₆ as negative controls. The most effective recombinant chimera was VCL-Int-Fn-ST₆, which showed cell proliferation comparable to that on collagen type I (Figure 5). Cells proliferated more slowly on the negative control VCL, VCL-ST₆ or silk films. Proliferation on VCL-Int-ST₆ was slower than VCL-Fn-ST₆ and VCL-Int-Fn-ST₆, but better than on negative controls. hMSCs grown on proteins containing the Fn-binding motif showed a healthier morphology under fluorescence microscopy with more clustered cells and better extended F-actin, comparable to collagen type I (Figure 6).

Based on the above results and silk binding assays, VCL-Int-Fn-ST₆ was selected for study on 3D silk porous scaffolds because of its positive effect on hMSC growth and strong binding to solid silk biomaterials. hMSCs were seeded on silk scaffolds treated by VCL-Int-Fn-ST₆ solution while non-treated scaffolds were used as controls. 3D cell cultures were monitored by alamarBlue assay for cell proliferation (Figure 7) and fluorescence F-actin staining for cell morphology (Figure 8). On non-treated silk scaffolds, fewer cells were observed and were less extended, similar to what have been seen on 2D culture. On the other hand, hMSCs proliferated almost 3 times faster on VCL-Int-Fn-ST₆ treated silk, with better cell spreading and spindle- or stellate- shaped morphology.

DISCUSSION

The addition of a consensus *B. mori* silk sequence promoted the attachment of collagen-silk chimeras to solid silk biomaterials, providing an approach to form a biomaterial from this bacterial collagen-like protein. From another perspective, this protein design provides an approach to introduce selective biological activity into silk, one of the oldest and most versatile biomaterials [48, 49]. Despite its extensive study and applications, one aspect of silk biomaterials where improved biological features could enhance utility is in directed control of cell interactions. Silk proteins from *B. mori* lack integrin-selective binding sites or domains that bind to ECM elements to promote cell adhesion, proliferation or differentiation [38]. Functionalization of silk structures with defined sites for interaction and control of biological activity would be useful in many types of regenerative medicine studies to expand the versatility of silk biomaterials [50, 51]. A number of approaches have been used to introduce bioactivity to silk materials. Chemical modification of silk proteins has been used to covalently attach bioactive motifs, such as RGD sequences [52] or bone morphogenetic protein [53]. An alternate method is to blend silk protein with other materials or by multilayer fabrication, since silk is inherently an adsorbing material that could non-specifically interact with many substances. This approach has been used to incorporate collagen into silk materials [54, 55]. A third tactic employs fusion of the silk motif with another protein motif, such as silk-elastin-like proteins, to form block copolymers [56, 57] and the functionalization of spider silks with antimicrobial peptides and inorganic binding domains via chimeric designs [58, 59]. Similar approach is utilized here to form chimeras where the silk sequence is used as recognition motif to direct non-covalent binding of soluble collagen-like proteins to solid silk materials.

The GAGGS sequence was selected because it is the most commonly found repeating sequence in natural *B. mori* silk and is known to interact with similar sequences in the native

protein to generate β -sheets [49, 60]. The results demonstrated that inclusion of the (GAGAGS)_n motif adjacent to the collagen triple-helix directly resulted in binding of the chimeric protein to solid *B. mori* silk biomaterials, including degummed silk fibroin, regenerated silk films and porous scaffolds. It is known that the natural silk material contains β -sheet regions with GAGAGS repeating sequences, and the β -sheet structures of repeating (GAGAGS)_n polypeptides have been well characterized by x-ray diffraction and other methods [61]. Our results support the premise that an introduced ST_n silk sequence adjacent to the collagen domain can recognize and bind to similar sequences on the surface of solid silk materials. This binding is likely to occur through the formation of β -sheet structures with the silk fibroin.

The length of the (GAGAGS)_n repeat was varied to better characterize the interaction of the chimeras with solid silk and to optimize the binding affinity. Initially, it was expected that binding would be greater for longer ST_n sequences, and this was true for low concentrations of the chimeras. However, at higher concentrations, the binding efficiency of ST₆ was as good as or even better than ST₉. The greater binding of collagen-silk chimeras with ST₆ compared with ST₉ at high concentrations may be due to saturation of available binding sites in silk films. In *B. mori* silk fibroin, GAGAGS repeating sequences are interspersed throughout the polypeptide chain, together with sequences leading to amorphous domains. An analysis of the distribution of (GAGAGS)_n repeats in one putative silk fibroin heavy chain full sequence (Genbank accession number: NM_001113262) shows more n=6 than n=9 sequences (Figure S4), suggesting the number of n=9 domains could be a limiting factor for ST₉ binding. In addition, the increased length of the repeating silk sequence in the chimera could cause interchain self-association at high concentrations, making these sequences less available for binding to solid silk. This could be a factor since DLS studies indicated that in PBS, increasing length of ST_n regions led to an increased mass percentage of protein within soluble aggregates, although it did not affect aggregate size.

Our model for binding assumes a specific interaction between (GAGAGS)_n sequences and identical or similar sequences within the solid silk. To investigate this hypothesis, a study was carried out on the ability of the collagen-silk chimeras to bind to spider silks. The spider silks and the silkworm silk all have high glycine and alanine compositions and certain regions tend to form rigid β -sheet structures, but the spider silk has a very different primary sequence and consensus sequences, such as (GGX)_n and polyalanine. The chimeric protein with ST₆ showed no binding to films produced from two different types of recombinant spider silk protein, MaSp1 and MaSp2. This supports the requirement for an amino acid sequence in the solid silk material which is identical or very similar to that in the silk repeating motif within the soluble protein. This self-association may be analogous to binding of small (Gly-Pro-Hyp)_n synthetic collagen mimetic peptides (CMPs) to insoluble collagen fibers [11, 62] and related to the growth of amyloid fibers [63].

The construction of the collagen-silk chimeras which can bind to solid silk materials presents an opportunity to insert biologically active sequences within the collagen domain to improve the functional properties of the 3D biomaterials for tissue engineering and regenerative medicine applications (Figure 9). A proof of principle was illustrated when human collagen sequences for Int- and Fn-binding sites were inserted into the CL domain, and led to better hMSC proliferation and spreading than on the original silk material and comparable to that seen for extracted rat tail tendon type I collagen.

CONCLUSIONS

The attachment of a silk repeating sequence to a collagen domain provided a strategy to generate new functionalized silk-based biomaterials. The ability to non-covalently attach the

recombinant bacterial collagen to solid silk materials preserves the useful mechanical properties and stability of silk, while adding selective bioactive functions via the collagen chimeras. These protein chimeras, produced by genetic engineering enable direct modulation of protein sequence and function at a molecular level, and may provide a facile and versatile method for the design of artificial ECM and other useful biomaterials. This same strategy can be extrapolated to other functional domains for addition to silk biomaterials, such as antimicrobial peptides, inorganic binding domains and other cell membrane receptor binding domains.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work is supported by NIH (grant# EB011620 to BB and DK). The authors would like to thank Dr. Randolph Lewis from Utah State University for the spider silk proteins used in this study, and Dr. David Wilbur and the Tufts Chemistry Department for allowing us to use the MALDI-TOF MS equipment. Any opinions, findings, and conclusions or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the views of the NIH.

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Figure 1.

(A) Schematic of collagen-silk chimera constructs containing the bacterial collagen trimerization domain (V), collagen-like domain (CL) and silk repeating sequences ST_n. The integrin (Int) binding site and fibronectin (Fn) binding site in the CL domain are indicated. (B) SDS-PAGE of VCL (lane 2), VCL-Int-Fn-ST₃ (lane 3), VCL-Int-Fn-ST₆ (lane 4), VCL-Int-Fn-ST₉ (lane 5). An increase in protein mass was seen with the increased length of ST. Lane 1 is protein standard. Triple helical proteins migrate slower than normal in SDS-PAGE, thus the M_w prediction from protein standard is higher than actual M_w. (C) MALDI-TOF mass spectra showing molecular weight of the same four proteins in (B).

Figure 2.

(A) Circular dichroism temperature scans of collagen-silk chimeras: VCL-Int-Fn-ST₃ (blue); VCL-Int-Fn-ST₆ (red) and VCL-Int-Fn-ST₉ (green) with the control VCL (black); The CD wavelength spectra of these proteins are shown in the inset. (B) Differential scanning calorimetry of collagen-silk chimeras and the control VCL, showing heat capacity as a function of temperature. Areas under the curve indicate the calorimetric enthalpy. The color key is the same as in (A), with VCL-ST₆ also shown (purple).

Figure 3.

Binding of collagen-silk chimeras to (A) silk film and (B) silk porous scaffold, at a range of concentrations using ELISA assay. The recombinant proteins presented are VCL-Int-Fn-ST₃ (blue); VCL-Int-Fn-ST₆ (red); VCL-Int-Fn-ST₉ (green); and VCL (purple). Error bars are based on the standard deviation of three samples; larger error bars for the silk scaffold may be due to higher variation in surface area compare with films. The ELISA values for all collagen-silk chimeras are statistically different from the VCL control ($p < 0.01$). The statistical significance between all pairs (VCL-Int-Fn-ST₃ and VCL-Int-Fn-ST₆; VCL-Int-Fn-ST₃ and VCL-Int-Fn-ST₉; VCL-Int-Fn-ST₆ and VCL-Int-Fn-ST₉) were assessed at each concentration using a paired t-test, and an asterisk * is used to indicate pairs where the

means are statistically different ($p < 0.05$). (C) Binding affinity of VCL-Int-Fn-ST₆ at 1 μ M (blue) and 10 μ M (red) to recombinant spider silk films (MaSp1 and MaSp2) compared to silkworm silk film. VCL at 10 μ M (green) serves as negative control.

Figure 4. 3D surface height images of silk film acquired by AFM showing surface morphology changes after collagen-silk chimera attachment.

Figure 5.

Assessment of hMSC proliferation on collagen-silk chimeras with introduced integrin and fibronectin binding sites in 2D culture (up to 10 days) by alamarBlue assay: VCL-Int-Fn-ST₆ (blue); VCL-Int-ST₆ (red); VCL-Fn-ST₆ (green). Samples of VCL (purple), silk (black) and VCL-ST₆ (grey) with no Int- and Fn-binding sites serve as negative controls; rat tail tendon collagen type I (orange) serves as positive control. Error bars are based on standard deviations of three samples.

Figure 6. Fluorescence staining of F-actin (green) in hMSCs cultured for 3 days in 2D cell culture plates, showing cell morphology and the quality of cell spreading on each sample. Nucleus is stained blue. A scale bar = 100 μ m is shown in the first image, and all other images are on the same scale.

Figure 7. Assessment of hMSC proliferation on VCL-Int-Fn-ST₆ treated silk porous scaffold (square) in 3D culture (up to 16 days) compared with non-treated silk porous scaffolds (circle) by alamarBlue assay. Error bars based on standard deviation of three samples. Data of treated group is significantly different from non-treated group ($p < 0.01$) since Day 4.

Figure 8.

Fluorescence staining of F-actin (green) in hMSCs cultured for 3 days in 3D porous silk scaffolds either treated or not treated with VCL-Int-Fn-ST₆, showing cell morphology and the quality of cell spreading on silk porous scaffold. Background fluorescence signal in the images is high because silk scaffolds non-specifically take up the fluorescent stains, but cell shapes could still be seen clearly. Three pictures taken from different locations in the scaffolds are shown for each sample. Scale bar equals 100μm.

Figure 9.

Schematic of potential interactions of silk materials treated with collagen-silk chimeras in cell culture. The (GAGAGS)_n silk sequence on collagen-silk chimeras provides controllable specific binding to the crystalline GAGAGS repetitive domain of silk material. The collagen-like sequence with integrin and fibronectin binding sites interacts with cell surface integrin receptors or fibronectin to promote cell growth.

Table 1

Biophysical characterization of the collagen-silk chimeras, including molecular weight, CD spectral features, thermal stability from CD and from DSC, and the hydrodynamic radius from DLS in both the eluting buffer and PBS.

	Mw ¹ Actual (Calculated)	CD			DSC		DLS			
		MRE ₂₂₀ ²	MRE ₂₀₀ ²	Tm (°C)	Enthalpy ³	Tm (°C)	Rh _{elution buffer} (%Mass) ⁴		Rh _{pbs} (%Mass) ⁴	
							Peak 1	Peak 2	Peak 1	Peak 2
VCL	33.01 (32.85)	1500	-47500	36.8	3041.8	37.4	10 (97.1)	77 (2.9)	27.8 (94.3)	99 (5.7)
VCL-Int-Fn-ST ₃	38.07 (38.04)	1720	-43400	34.9	2783.7	35.5	11.5 (98.4)	54.7 (1.6)	19.5 (90.6)	58.3 (9.4)
VCL-Int-Fn-ST ₆	39.31 (39.24)	635	-35000	34.7	2447.0	35.0	11.6 (97.8)	73.4 (2.2)	17.7 (84.2)	76.2 (15.8)
VCL-Int-Fn-ST ₉	40.52 (40.44)	606	-30400	34.7	2353.0	35.5	12.4 (99.5)	62.6 (0.5)	17.6 (71.6)	69.1 (28.4)
VCL-ST ₆	(37.16)	631	-	36.1	2416.1	36.9	-	-	-	-
VCL-Int-ST ₆	(37.15)	645	-34100	-	-	-	-	-	-	-
VCL-Fn-ST ₆	(39.24)	632	-35200	-	-	-	-	-	-	-

¹Unit for molecular weight is kDa. Calculated Mw is based on amino acids sequence in the open reading frame of each construct. Actual Mw is acquired from MALDI-TOF results.

²Unit for MRE₂₂₀ and MRE₂₀₀ are deg·cm²/dmol.

³Unit for enthalpy is KJ/mol.

⁴Unit for Rh is nm. Percentage mass of each peak is presented in the parentheses.