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Osteoinductive recombinant silk fusion proteins for bone regeneration

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ABSTRACT

Protein polymers provide a unique opportunity for tunable designs of material systems due to the genetic basis of sequence control. To address the challenge of biomineralization interfaces with protein based materials, we genetically engineered spider silks to design organic-inorganic hybrid systems. The spider silk inspired domain (SGRGGLGGQG AGAAAAAGGA GQGGYGGLGSQGT)$_{15}$ served as an organic scaffold to control material stability and to allow multiple modes of processing, whereas the hydroxyapatite binding domain VTKHLNQISQSY (VTK), provided control over osteogenesis. The VTK domain was fused either to the N-, C- or both terminals of the spider silk domain to understand the effect of position on material properties and mineralization. The addition of the VTK domain to silk did not affect the physical properties of the silk recombinant constructs, but it had a critical role in the induction of biomineralization. When the VTK domain was placed on both the C- and N-termini the formation of crystalline hydroxyapatite was significantly increased. In addition, all of the recombinant proteins in film format supported the growth and proliferation of human mesenchymal stem cells (hMSCs). Importantly, the presence of the VTK domain enhanced osteoinductive properties up to 3-fold compared to the control (silk alone without VTK). Therefore, silk-VTK fusion proteins have been shown suitable for mineralization and functionalization for specific biomedical applications.

Statement of Significance

Organic-inorganic interfaces are integral to biomaterial functions in many areas of repair and regeneration. Several protein polymers have been investigated for this purpose. Despite their success the limited options to fine-tune their material properties, degradation patterns and functionalize them for each specific biomedical application limits their application. Various studies have shown that the biological performance of such proteins can be improved by genetic engineering. The present study provides data relating protein design parameters and functional outcome quantified by biomineralization and human mesenchymal stem cell differentiation. As such, it helps the design of osteoinductive recombinant biomaterials for bone regeneration.

1. Introduction

Recombinant biomaterials hold potential for the development of application-specific fine-tuned scaffolding for tissue regeneration and replacement, a major health challenge worldwide [1]. Organic-inorganic interfaces are integral to biomaterial functions in many areas of repair and regeneration, therefore the surface modification of implantable biomaterial surfaces with bioactive peptides is one approach to design materials for bone formation [2,3]. Several fibrous proteins have been investigated for this purpose [4,5]. Collagens are of particular interest as scaffolds for bone tissue engineering as they represent the major protein fraction of bone extracellular matrix [6]. However, collagen-based biomaterials generally lack mechanical stability and lose integrity over time [7]. Another unique family of fibrous proteins with impressive mechanical properties, biocompatibility and biodegradability are silks [8–12]. The mechanical properties of spider silk exceed other natural polymers and most synthetic materials, rivalling even high-performance fibers such as Kevlar, making silk a suitable candidate for bone-related biomaterials [13].

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Biomimetic inorganic-organic hybrid systems have been analysed as a route to stiffer and stronger materials [14–17]. To enhance osseointegration, silk has been specifically functionalized with different biological molecules [15–18]. Nevertheless, there is limited data on how these functional domains influence the material properties of silk [16]. Previously, silk-silica peptide designs were generated with studies focusing on understanding the role of the biomineralization domain position relative to the silk component in the silk-silica fusion proteins in terms of silica formation *in vitro* [16]. The silica-binding peptide R5 (SSKKSGSYSGSKGSKRRIL) taken from the silaffin gene of *Cerithiopsis fusiformis* was fused to the N- or C-terminus of the silk sequence (SGRGGLGGQGAGAAAAAGGAGQGGYGGLGSQGT)$_{15}$, derived from the consensus repeat of *Nephila clavipes* dragline silk protein [16]. These fusion proteins were around 43 kDa. Herein, we designed silk based biomaterials to induce hydroxyapatite formation and enhance *in vitro* bone regeneration. Hydroxyapatite was selected as the target inorganic material due to its importance in bone tissues [19].

Optimal peptide candidates for bone regeneration have been identified *via* phage display, with VTKHLNQISQSY (VTK) as a candidate with preferential interactions with both bone-like minerals and hydroxyapatite [20,21]. Since, the VTK peptide alone does not possess adequate mechanical properties for bone graft engineering, combination with appropriate scaffolding material is required.

In this study, we genetically modify the artificial silk polymer, 15mer ((SGRGGLGGQGAGAAAAAGGAGQGGYGGLGSQGT)$_{15}$, ~39 kDa), derived from the consensus repeat of *N. clavipes* dragline silk protein with the hydroxyapatite binding peptide VTK, with the aim to build a biomaterial with potential application in bone grafting, exploiting both the remarkable mechanical properties of silk and the biomineralization properties of the VTK peptide. To identify optimal protein design with regard to mechanical performance and osteoinductive properties, the VTK peptide was fused separately to the N-, C- or both termini of the spider silk. The influence of the designs on calcium phosphate deposition with respect to crystallinity was assessed *in vitro*, along with the impact on beta sheet content as a proxy for mechanical strength. These assessments provide insight into structure-function relationships and the effect of functional domains on silk secondary structure/folding and functionalization for bone regeneration.

2. Materials and methods

2.1. Construction of recombinant silk and silk-VTK chimeras

The following constructs were designed: 15mer-ch, nh-15mer, VTK-15mer-ch, nh-15mer-VTK and VTK-15mer-VTK (Fig. 1). The 15mer-ch and nh-15mer are spider silk constructs built of 15 repeating units (SGRGGLGGQG AGAAAAAGGA GQGGYGGLGSQGT)$_{15}$ that carry a Histidine tag (His$_6$ = h) on the C-terminal and N-terminal, respectively. VTK-15mer-ch is a 15mer-ch construct that has the VTK sequence (VTKHLNQISQSY) [20,21], at the N-terminus, whereas nh-15mer-VTK is a nh-15mer that has the VTK sequence at the C-terminus. Plasmids pET30ch and pET30nh were used as cloning vectors, where the His-tag was located at the C- or N-termini of the genetic constructs, respectively. Both pET-30ch and pET-30nh are pET-30a(+) (Novagen, San Diego, CA, USA) derivative vectors. The construction of the cloning vectors pET-30ch and pET-30nh was performed as described previously [16]. Next, a 1485 bp DNA *Nhe*I/*Spe*I fragment containing genetic sequence coding for the artificial silk protein, 15mer (SGRGGLGGQGAGAAAAAGGAGQGGYGGLGSQGT)$_{15}$, was inserted into pET30ch and pET30nh to yield pET30ch-15mer and pET30nh-15mer, respectively. To prepare the chimeras with the VTK sequence fused at the C-, N- or both termini of the 15mer, pET30ch-15mer and pET30nh-15mer were digested with *Spe*I and then treated with antarctic phosphatase (NEB, Ipswich, MA, USA) to prevent self-ligation. The nucleotide sequences of VTK were designed with restriction endonuclease sites *Nhe*I and *Spe*I flanked at the 5′ and 3′ termini, respectively. Codons were optimized for expression in *Escherichia coli* strain BL21(DE3) using the on-line tool OPTIMIZER and were synthesized commercially (Invitrogen, Grand Island, NY, USA). The synthesized nucleotides were annealed to generate double strands and then ligated to generate the constructs pET30ch-VTK-15mer, pET30nh-15mer-VTK and pET30-VTK-15mer-VTK. *E. coli* DH5α cells were transformed and positive clones were selected with Luria Bertani (LB) (Sigma-Aldrich, St. Louis, MO, USA) plates supplemented with kanamycin (50 μg/mL) (Sigma-Aldrich, St. Louis, MO, USA).

2.2. Expression and purification of recombinant silk and silk-VTK chimeras

The recombinant silk constructs were expressed in *E. coli* strain BL21 Star (DE3) (Invitrogen, Grand Island, NY, USA). A fermentor (Bioflo 3000, New Brunswick Scientific, Edison, NJ, USA) was used for the expression. Cells were cultivated at 37 °C in LB medium with 50 μg/mL kanamycin. Once the optical density OD_{600} reached 0.8, isopropyl β-d-1-thiogalactopyranoside, IPTG (Sigma-Aldrich, St. Louis, MO, USA) was added at a final concentration of 1 mM to induce expression. After 5 h cells were harvested by centrifugation for 20 min at 8000 rpm. Recombinant silk protein and chimeras were purified by Ni-NTA affinity chromatography as previously described [22], while the VTK-15mer-VTK protein that

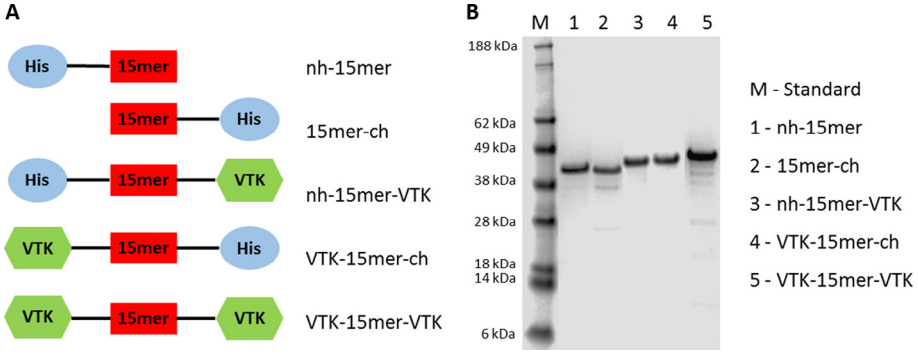

Fig. 1. Recombinant silk fusion protein design and production. (A) Schematic representation of fusion proteins design strategy; His-tag (blue circle) has been added to spider silk 15mer (red box) at N-terminal end of nh-15mer and nh-15mer-VTK constructs, and C-terminal end of 15mer-ch and VTK-15mer-ch constructs; VTK domain (green hexagon) has been added to the C-terminal of nh-15mer-VTK, N-terminal of VTK-15mer-ch and both N- and C-terminal of VTK-15mer-VTK. (B) SDS-page of purified nh-15mer (~40 kDa), 15mer-ch (~40 kDa), nh-15mer-VTK (~42 kDa), VTK-15mer-ch (~42 kDa) and VTK-15mer-VTK (~43 kDa), run on the 4%–12% Bis-Tris acrylamide gel and stained with Simple Blue dye. Marker (M) sizes are indicated on the left. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)