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FUNCTIONALIZED SILK BIOMATERIALS FOR BONE REGENERATION

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INTRODUCTION

In this study, we extend our earlier strategies to functionalize silk-based biomaterials with mineralization domains, to new directions that exploit the addition of functional domains related to osteogenic repair needs and to infection control. To achieve this purpose a biotechnology was used to generate new chimeric silk proteins that can be combined into a single biomaterial system for application in bone defects, for improving interfaces with native tissue, for control of infections and for accelerating regeneration. Four new chimeric silk proteins were generated via recombinant DNA techniques, combining spider silk from the consensus repeat of *Nephila clavipes* major ampullate spidroin protein I with different functional peptides or proteins. In this way, a protein sequence consisting in six repeats of the major ampullate spidroin protein (MaSpI) consensus sequence named 6mer was combined with new function domains: integrin binding bone sialoprotein (BSP) hepcidin, human neutrophils defensins 2 (HNP-2) and 4 (HNP-4). Spider silk is a promising biopolymer with remarkable mechanical and biocompatibility properties, both necessary for bone engineering (Xu and Lewis 1990). BSP is a multi-domain protein involved in the deposition of calcium phosphates (CaP), cell adhesion, migration and differentiation (Fisher et al. 2001). The new fusion protein 6mer+BSP combines the remarkable mechanical performance and self-assembly capability of silk with the multi-domain BSP sequence for function mineralization and related properties. Additionally, HNP-2, HNP-4 and hepcidin were also selected based in their antimicrobial activities to be expressed together with the silk protein. During the last few years six different α-defensins in humans were described: HNP-1, HNP-2, HNP-3 (Selsted et al. 1985), HNP-4, which are expressed in neutrophils, and HD-5 and HD-6, expressed by the Paneth cells of the small intestine and by the epithelial cells of the female urogenital track (Jones and Bevins 1992). Different studies demonstrate that HNP-2 manifest bactericidal activity against gram+ and gram- bacteria (Pazgier and Lubkowski 2006). Alternatively, HNP-4 was considered to be more effective against the gram - *E. coli*. Hepcidin also exhibits bactericidal and fungicidal activity. Different studies demonstrate that this peptide has antibacterial activity against gram+ and gram- bacteria. The three new fusion proteins, 6mer+ HNP-2, 6mer+ HNP-4 and 6mer+hepcidin combine the spider silk self-assembly ability and mechanical properties with the antimicrobial features of hepcidin, HNP-2 and HNP-4. The goal of the present work was to generate a new family of functional biomaterials that combine the useful features of the silk protein component, such as self-assembly, mechanics and aqueous processing, with new features from the added peptides and proteins, such as to improved bone integration and infection control. Cloning and expression was accomplished in *Escherichia coli* and the purified proteins were characterized for purity and composition. Antimicrobial activity and stimulation of the deposition of calcium phosphate were assessed.

MATERIALS AND METHODS

Cloning

The assembly of the vector (pET30L) carrying the silk block with six repeats (6mer) was described previously (Rabotyagova et al. 2009). For purification purposes 6mer (control), 6mer+ HNP-2, 6mer+ HNP-4, 6mer+hepcidin and 6mer+BSP sequences were cloned with a histidine tag.

Protein Expression and purification

Expression of 6mer, 6mer+ HNP-2, 6mer+ HNP-4, 6mer+hepcidin and 6mer+BSP was induced with isopropyl β-D-thiogalactoside (IPTG, Invitrogen). Protein purification was performed in denaturing conditions using urea and Ni-NTA resin.

Film formation and characterization

Protein films were prepared by dissolving the proteins in in MQ water. After drying the films were treated with methanol to induce β-sheet formation. Attenuated-total reflectance fourier transform infrared spectroscopy (ATR-FTIR) was used for secondary structure analysis.

Mineralization and cell behaviour in 6mer+BSP films

In vitro mineralization was assessed by incubating the films in buffered accelerating mineralization solution (AMS) with Ca^{2+} and PO_4^{3-} for 1 and 6 hours. Human mesenchymal stem cells (hMSC) were used to assess *in vitro* cell responses in osteogenic media during 3, 7 and 14 days. Alamar blue was used to check cell viability. Quantitative real time reverse transcription-PCR (real time RT-PCR) was used to assess gene expression of: collagen type I (COL1A1), collagen type II (COL2), BSP, alkaline phosphatase (ALP). Scanning electron microscopy (SEM) and energy dispersive spectrometer (EDS) were used to characterize cell morphology and the obtained CaP coatings.

Antimicrobial assay for 6mer+ HNP-2, 6mer+ HNP-4 and 6mer+hepcidin proteins

Proteins were tested for antimicrobial activity against *E. coli* and *Staphylococcus aureus*, purchased from American-Type Culture Collection. Radial diffusion assay was used to test three concentrations of purified protein dissolved in phosphate buffer (pH 7.4): 10, 50 and 100µg/ml. 6mer protein solution was used as control. Filter paper discs were immersed in the different protein solutions, placed on the *E. coli* and *S. aureus* lawns. The zones of growth inhibition formed around the susceptibility disks were measure.

RESULTS AND DISCUSSION

The deconvolution of ATR-FTIR spectra allowed the calculation of the percentage of helix/random coil and antiparallel β-sheet and statistical analysis shows no significant difference between 6mer (control) and 6mer+BSP proteins. The introduction of the BSP, HNP-2, HNP-4 and hepcidin domains did not affect the self-assembly of the silk sequence. For 6mer+BSP films, after 1 hour in AMS, EDS analysis indicated Ca and P in the 6mer+BSP films (Ca/P=1.72). After 6 hours, the formation of mineral on the surface of the 6mer+BSP films was observed by SEM, while for the 6mer control mineral was not observed. Regarding the 6mer+BSP films after 14 days of cell culture the SEM shows a CaP coating with a globular cauliflower-like morphology. EDS analysis shows a Ca/P of 1.72, similar to hydroxyapatite (1.67). Furthermore, the expression of bone-related genes by hMSCs cultured on 6mer+BSP indicated significant ($p<0.05$) up-regulation of ALP, IBSP and COL1A1 during 14 days of cell culture. For the 6mer films a significant decrease in expression level was observed for ALP and COL1A1 from days 3 to day 14, and for IBSP gene expression was low at days 3 and 7, and increased at day 14. Finally, the three proteins 6mer+ HNP-2, 6mer+ HNP-4 and 6mer+hepcidin displayed obvious antimicrobial activity against *E. coli* with the formation of inhibition zones for all the three concentrations tested. In the case of *E. coli*, 6mr+HNP-4 and 6mr+hepcidin register the higher bactericidal values when compared with 6mr+HNP-2 peptide. When tested against the *S. aureus* there was a notorious decrease in the bactericidal activity for all the three peptides with a decrease in the diameter of the inhibition zone especially in the case of 6mr+ hepcidin and 6mr+HNP-2.

CONCLUSIONS

The present work describes the successful design of four new fusion proteins, 6mer+ HNP-2, 6mer+ HNP-4, 6mer+hepcidin and 6mer+BSP. The combination of the 6mer with BSP, HNP-2, HNP-4 and hepcidin domains did not interfere with the function of each domain and with the self-assemblage properties of silk. Furthermore, 6mer+BSP films sustained cell proliferation and viability, and also improve cell function towards the osteoblastic phenotype from hMSCs. These results suggest potential for this new fusion protein as a biomaterial for bone regeneration.

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