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Biocompatibility of silk-tropoelastin protein polymers

Hongjuan Liu [a,h], Steven G. Wise [b,c], Jelena Rnjak-Kovacina [d,e], David L. Kaplan [d], Marcela M.M. Bilek [f], Anthony S. Weiss [c,g,h], Jian Fei [i,j,**], Shisan Bao [a,h,*]

[a] Discipline of Pathology and School of Medical Science, University of Sydney, NSW 2006, Australia
[b] The Heart Research Institute, Sydney, NSW 2042, Australia
[c] School of Molecular Bioscience, University of Sydney, NSW 2006, Australia
[d] Department of Biomedical Engineering, Tufts University, Medford, MA, USA
[e] Graduate School of Biomedical Engineering, University of New South Wales, NSW 2051, Australia
[f] School of Physics, University of Sydney, NSW 2006, Australia
[g] Charles Perkins Centre, University of Sydney, NSW 2006, Australia
[h] Bosch Institute, University of Sydney, NSW 2006, Australia
[i] School of Life Science and Technology, Shanghai Tongji University, China
[j] Research Centre for Model Organisms, Shanghai, China

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ABSTRACT

Blended polymers are used extensively in many critical medical conditions as components of permanently implanted devices. Hybrid protein polymers containing recombinant human tropoelastin and silk fibroin have favorable characteristics as implantable scaffolds in terms of mechanical and biological properties. A firefly luciferase transgenic mouse model was used to monitor real-time IL-1β production localized to the site of biomaterial implantation, to observe the acute immune response (up to 5 days) to these materials. Significantly reduced levels of IL-1β were observed in silk/tropoelastin implants compared to control silk only implants at 1, 2 and 3 days post-surgery. Subsequently, mice ($n = 9$) were euthanized at 10 days (10D) and 3 weeks (3W) post-surgery to assess inflammatory cell infiltration and collagen deposition, using histopathology and immunohistochemistry. Compared to control silk only implants, fewer total inflammatory cells were found in silk/tropoelastin (~29% at 10D and ~47% at 3W). Also fewer ingrowth cells (~42% at 10D and ~63% at 3W) were observed within the silk/tropoelastin implants compared to silk only. Lower IL-6 (~52%) and MMP-2 (~84%) (pro-inflammatory) were also detected for silk/tropoelastin at 10 days. After 3 weeks implantation, reduced neovascularization (vWF ~43%), fewer proliferating cells (Ki67 ~58% and PCNA ~41%), macrophages (F4/80 ~64%), lower IL-10 (~47%) and MMP-9 (~55%) were also observed in silk/tropoelastin materials compared to silk only. Together, these results suggest that incorporation of tropoelastin improves on the established biocompatibility of silk fibroin, uniquely measured here as a reduced foreign body inflammatory response.

1. Introduction

A variety of synthetic materials are used in diverse medical applications as replacements for damaged tissues, including vascular conduits, heart valves, dermal replacements and hip or knee joints [1,2]. Common to all these implants and locations is a necessity to modulate both the local and systemic host response of the material *in vivo* [2]. Natural polymers, mimicking the tissue they seek to replace are increasingly studied as an effective means of minimizing and modulating the biological responses [1,3].

Recombinant human tropoelastin is the soluble precursor of elastin and a promising biomaterial for elastic tissues, including arteries and skin [4]. Tropoelastin can be formulated and cross-linked into a variety of biomaterial formats, including scaffolds, electrospun mats and hydrogels, which have been shown to mimic native elastin's high elasticity and extensibility [5,6]. However, synthetic elastin alone has insufficient tensile strength for many tissue engineering applications, making the use of hybrid constructs useful for closely matching both biological and mechanical

* Corresponding author. Department of Pathology, University of Sydney, NSW 2006, Australia. Tel.: +61 2 93516156.
** Corresponding author. School of Life Science and Technology, Tongji University, China.
E-mail addresses: jfei@tongji.edu.cn (J. Fei), bob.bao@sydney.edu.auor (S. Bao).

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requirements. Tropoelastin hybrids have previously been shown to be valuable in engineering vascular conduits [7,8]. Electrospun tropoelastin/polycaprolactone (PCL) hybrid scaffolds demonstrated enhanced adhesion and proliferation of endothelial cells, increased elasticity and improved blood compatibility compared to PCL alone, while retaining a high strength and burst pressure [9].

Similarly, silk fibroin is a versatile natural polymer with remarkable mechanical properties [10]. Widely used as a suture material, purified silk is extremely well tolerated *in vivo*, demonstrating only a limited inflammatory response, superior to collagen and poly (lactic acid) constructs [11]. The biodegradability of silk can also be controlled during scaffold manufacture, making it a widely used biomaterial [12]. Hybrid scaffolds of silk and tropoelastin have demonstrated improved biological and mechanical properties compared to each of the components alone, including reduced host inflammatory response and combining strength with elasticity [13,14].

In this study, we examined both the acute and subacute immune response to silk/tropoelastin hybrid scaffolds, in comparison to silk alone. Given the established biocompatibility of silk, it provides a high benchmark for low immune response as a control. To observe any early differences in response to these materials, we employed an interleukin 1β (IL-1β) transgenic mouse model to monitor IL-1β levels at the site of biomaterial implantation, using bioluminescence to facilitate real-time monitoring in a live mouse [15,16]. The responses were contrasted with measures of the magnitude and duration of inflammatory and wound healing responses, foreign body reactions, and fibrous encapsulation following subcutaneous implantation [17].

2. Materials and methods

2.1. Reagents

Recombinant human tropoelastin corresponding to amino acid residues 27–724 of GenBank entry AAC98394 (gi 182020) was expressed and purified as previously described [18]. Cocoons of *Bombyx mori* silkworm were supplied by Tajima ShojiCo. (Yokohama, Japan). *B. mori* silk fibroin was prepared as previously described [19]. Human dermal fibroblasts (HDF; line GM3348) were obtained from Coriell Research Institute (Camden, NJ, USA) and used up to passage 14. All other reagents were purchased from Sigma–Aldrich unless otherwise stated.

2.2. Electrospinning

Different proportions of lyophilized tropoelastin and silk fibroin were combined in a 10% (w/v) protein solution in 1,1,1,3,3,3-hexafluoro-2-propanol (HFP). These included 100% silk: 0% tropoelastin (100S), 75% silk: 25% tropoelastin (75S25T), 50% silk: 50% tropoelastin (50S50T), 25% silk: 75% tropoelastin (25S75T). In addition, a 20% (w/v) tropoelastin solution was prepared (100T). Silk: tropoelastin solutions were loaded into a syringe equipped with a blunt 18 gauge needle and a flow rate of 3 mL/h was modulated using a syringe pump. The needle was connected to a 20 kV positive power supply (Gamma High Voltage Research, Inc.) and directed at a grounded, 30 mm diameter circular, brass collector at a collector distance of 20 cm. Scaffolds were then placed in an open stage desiccator and cross-linked by vapor from a separate 25% (v/v) aqueous glutaraldehyde (GA) solution. Unreacted GA in the scaffolds was quenched by immersion into 0.2 M glycine solution overnight. Scaffolds were then washed repeatedly in PBS.

2.3. Scanning electron microscopy/fiber diameter quantification

Samples for SEM analysis were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB), post-fixed with 1% (v/v) osmium tetraoxide in 0.1 M PB and dehydrated in ascending grades of ethanol before drying with hexamethyldisilasane (HMDS). The samples were sputter coated with 20 nm gold and imaged with a Philips XL 30 CP scanning electron microscope. For quantitation, the width of 10 fibers within an image for a total of 10 images per sample ($n = 3$ per treatment) was quantified using ImageJ (National Institutes of Health) [4].

2.4. Human dermal fibroblast (HDF) proliferation

HDF ($5 \times 10^3/cm^2$) were seeded onto a thin layer of electrospun fibers on glass coverslips in a 24-well cell culture plate. Cells were quantified at 3 and 5 days post-seeding using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay according to manufacturer's instructions. Dimethyl sulfoxide (DMSO) was used to dissolve insoluble formazan crystals, and the absorbance at 540 nm was measured using a spectrophotometer (Biorad).

2.5. Swelling

Pre-weighed scaffolds were submerged in Milli-Q water (Millipore, USA) for 24 h at 37°C. Excess water was drained and the scaffolds were weighed. The tared weight yielded the amount of water absorbed per gram of scaffold.

2.6. Mouse subcutaneous implantation

Study approval was obtained from the University of Sydney Animal Ethics Committee (protocol number K20/12-2011/3/5634). Experiments were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purpose. Control silk or silk/tropoelastin hybrid discs (4 mm diameter) were sterilized in 70% ethanol and stored in PBS at room temperature before subcutaneous implantation. For subcutaneous implantation, 18 male IL-1β mice, age 7 weeks and weighing 22 ± 2.5 g were obtained from the Shanghai Research Centre for Model Organisms [15,16]. Each mouse was anesthetized by intra-peritoneal injection of sodium pentobarbital (0.1 mg/g of body weight). The dorsal surface of each mouse were shaved and sterilized with betadine solution and then sterile saline. Four 1.5 cm incisions (two rows side by side) were then cut through the skin to create four subcutaneous pockets and an implant was inserted into each incision. Each animal received two silk fibroin and two silk/tropoelastin hybrids (25S75T). Tropoelastin only samples did not have sufficient mechanical strength to be implanted and retrieved from this model. The wounds were closed with 6-0 silk sutures. After the implantation, each mouse was caged individually with water and food. The *in vivo* luciferase signal, representing IL-1β expression in each mouse was imaged daily for 5 days post-surgery as described previously [16]. After periods of 10 days and 3 weeks, mice ($n = 9$) were euthanized by cervical dislocation and skin biopsies (1.5 × 1.5 cm) together with implants were collected for histological analysis.

2.7. IL-1β luciferase signal detection

IL-1β luciferase mice were generated by incorporating the firefly luciferase gene driven by a 4.5-kb fragment of human IL-1β gene promoter (cHS4l-hIL-1β). The subsequent bioluminescence represents the total IL-1β expressed and was monitored in real-time in a live mouse [16,20]. Mice were anesthetized with 4% isoflurane then placed into a MAG Biosystems Lumazone imaging system. Anesthesia was maintained with 1.5% isoflurane and the luciferase macroscopic images for the dorsal region of each mouse were captured.

2.8. Histological analysis

Skin biopsies were immediately fixed in 70% ethanol for 24 h, dehydrated overnight in a tissue processor, then embedded in paraffin. Tissue samples were cut into 5 μm thick transverse sections using a rotary microtome, deparaffinized and stained using standard hematoxylin-eosin staining (H&E) and Milligan's trichrome stains to assess total cell infiltration and collagen deposition, as previously described [21].

2.9. Immunohistochemistry

Tissue sections (5 μm) were deparaffinized and stained with antibodies including anti-Von Willebrand factor (vWF) (Dako, Sydney Australia) for neovascularization, anti-F4/80 (Abcam, Sydney Australia) for monocyte/macrophages, anti-PCNA (Abcam, Sydney Australia) and anti-Ki67 (Abcam, Sydney Australia) for proliferating cells, anti-MMP-2 (Abcam, Sydney Australia), anti-MMP-9 (Abcam, Sydney Australia), anti-IL-10 (Abcam, Sydney Australia) and anti-IL-6 (Abcam, Sydney Australia) for cytokine expression.

2.10. Quantitative and statistical analysis

Quantification of luciferase activity (photo/s) at the implant sites was performed using LivingImage software [15,16]. Histological and immunohistochemical slides were imaged using a Nikon Eclipses microscope, captured with DP Controller 2.2, and DP Manager Version 2.2, Olympus Corporation, Japan. Image analysis was used to perform quantitation for both immunohistochemical and histopathological analyses, as previously described [22]. Briefly, 20 fields per sample were captured from each animal. The images were analyzed using *ImagePro Plus 7* (Media Cybernetics, USA). For macrophage, $Ki67^+$ or $PCNA^+$ identification, positive staining areas were expressed relative to the total area of the tissue of interest within the image, and described as the number of cells per mm^2, as previously published [22]. For IL-10, IL-6, MMP-2, MMP-9 or $F4/80^+$, the percentage of positive staining relative to the total area of interest was calculated. The cytokine/chemokine expression results were expressed as positive pixel per area of interest, i.e. image units, as previously described [23]. All data are expressed as mean ± standard error of the mean (SEM) and were analyzed using statistical software in GraphPad Prism Version 5.0 (GraphPad Software, CA, USA). Student *t*-tests and two-way ANOVA with a Bonferroni post-test were used