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Perichondrium directed cartilage formation in silk fibroin and chitosan blend scaffolds for tracheal transplantation

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ABSTRACT

The purpose of this study was to investigate the potential of silk fibroin and chitosan blend (SFCS) biological scaffolds for the purpose of cartilage tissue engineering with applications in tracheal tissue reconstruction. The capability of these scaffolds as cell carrier systems for chondrocytes was determined *in vitro* and cartilage generation *in vivo* on engineered chondrocyte–scaffold constructs with and without a perichondrium wrapping was tested in an *in vivo* nude mouse model. SFCS scaffolds supported chondrocyte adhesion, proliferation, and differentiation, determined as features of the cells based on the spherical cell morphology, increased accumulation of glycosaminoglycans, and increased collagen type II deposition with time within the scaffold framework. Perichondrium wrapping significantly ($P < 0.001$) improved chondrogenesis within the cell–scaffold constructs *in vivo*. *In vivo* implantation for 6 weeks did not generate cartilage structures resembling native trachea, although cartilage-like structures were present. The mechanical properties of the regenerated tissue increased due to the deposition of chondrogenic matrix within the SFCS scaffold structural framework of the trachea. The support of chondrogenesis by the SFCS tubular scaffold construct resulted in a mechanically sound structure and thus is a step towards an engineered trachea that could potentially support the growth of an epithelial lining resulting in a tracheal transplant with properties resembling those of the fully functional native trachea.

1. Introduction

Excessive tracheal resection may be required due to cancer influx into host tissues and/or benign diseases of the trachea and is treated by tracheal reconstruction [1]. While defects of up to 50% of the tracheal length in adults and 30% in infants may be reconstructed by primary closure, longer circumferential tracheal defects will require more elaborate surgical reconstructions with grafts and/or flaps [2]. Due to the unique rigid structure and function of the trachea and its air interface (airway) traditional grafting techniques, employing autografts, allografts, and/or prosthetic materials generally do not provide a stable airway, which results in failure [3,4]. Different strategies have been attempted to combine living cells and biocompatible scaffold materials to produce tracheal substitutes. Several *in vivo* studies have shown the importance of both stable cartilaginous support and a functional epithelium in the long-term success of tracheal reconstruction [5,6]. Therefore, the scaffold used for tracheal engineering will need to support regeneration of both tissue components, i.e. cartilage and epithelial lining, in a biological environment that supports the regenerative process, rather than inhibiting it.

Silk fibroin (SF) and chitosan (CS) are both naturally occurring biological materials that have been extensively used for cell and tissue applications due to their excellent biocompatibility, degradability, ease of processing, and uniquely engineered structures and mechanical properties [7–9]. We have developed a blended biomaterial to mimic the biochemical composition, architecture, and biomechanical properties of the extracellular matrix (ECM), particularly the structural protein and compressive glycosaminoglycan components [10]. Employing different mold geometries and varying the blending ratios we were able to obtain three-dimensional (3-D) matrices with optimal mechanical properties [10]. SFCS blend scaffolds serve as an effective carrier for stem cells, supporting cell adhesion and migration *in vitro* and *in vivo* [11,12]. It also facilitates host cell infiltration and vascular in-growth, and has been deployed successfully for abdominal wall reconstruction [13], skin wound healing [11], and bone regeneration [14]. Recently this hybrid matrix has been proven to support adipose tissue-derived stem cell differentiation into vascular and epithelial phenotypes [11]. Based on these findings we hypothesized that SFCS is a scaffold that

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would support the growth of cartilage for tracheal tissue engineering.

In this study we have examined the potential of SFCS in combination with precursor cells and perichondrium for the fabrication of neo-trachea using *in vitro* and *in vivo* methods. Rabbit auricular chondrocytes were seeded on SFCS scaffold, as they have been shown to be an efficient cell source for cartilage repair [15,16]. Perichondrium is a physiologically thin barrier covering cartilage and protects it from external influences and is capable of generating cartilage *in vivo* [17–19]. In an attempt to stabilize the immature *in vitro* engineered tissue (cells + scaffold) and also provide a source of cartilage precursor cells we wrapped the engineered SFCS constructs with perichondrium before *in vivo* implantation. The aim of this study was, first, to evaluate whether SFCS could serve as a supportive carrier for chondrocyte delivery and, second, to determine the feasibility of SFCS combined with chondrocytes and perichondrium to construct tracheal cartilage.

2. Materials and methods

2.1. Tubular SFCS scaffold fabrication

Raw silk (Sau Paulo, Brazil via the Korean Sericulture Institute) was graciously donated by Dr. Samuel M. Hudson (North Carolina State University, Raleigh, NC) and degummed according to previously described procedures [10]. In brief, 0.25% (w/v) sodium dodecylsulfate (SDS) (Sigma, St. Louis, MO) and 0.25% (w/v) sodium carbonate (Sigma) were dissolved as the temperature was raised to 100 °C. Raw silk was added at 1:100 w/v and heated for 1 h. The alkaline soap solution was then drained and the degummed silk was heated to 100 °C in distilled water for an additional 1 h. Finally, any remaining sericin and surfactants were removed completely by rinsing the silk in running distilled water. The washed silk was then air dried. The degummed silk was then dissolved by adding it to a calcium nitrate tetrahydrate–methanol solution (molar ratio 1:4:2 $Ca(NO_3)_2{\cdot}4H_2O$:MeOH at 65 °C). The silk fibroin was dissolved at 10% (w/v) concentration over a 3 h period with continuous stirring. The final protein concentration of silk fibroin was calculated to be 4.08% (w/v).

High molecular weight chitosan (82.7% deacetylation, Sigma) was dissolved in an aqueous solution of 2% acetic acid to create a solution of 4.08% (w/v), equal to that of the silk fibroin. Silk fibroin and chitosan were blended at 75:25 % (v/v) to prepare silk fibroin–chitosan, as this ratio showed superior mechanical properties compared with the other blends [10]. The mixture was then dialyzed (molecular weight cut-off 6–8 kDa) against deionized water for 4 days and strained through a 100 μm filter. The final aqueous solution was clear and homogeneous and was kept at room temperature.

A mold to resemble the shape of the trachea was created from two concentric tubes with 5 mm separation between the outer polystyrene tube and the inner silastic tube. Tubular sfcaffolds were prepared by adding 5 ml of SFCS solution between the polystyrene tube and the silastic tube, with their ends sealed with paraffin wax. The solution was frozen in the mold at −80 °C overnight, followed by lyophilization for 72 h. The dry scaffolds were then treated in a 50:50 % (v/v) methanol:sodium hydroxide (1 N) solution for 15 min to crystallize the SF content and neutralize the CS content. The scaffolds were rinsed in distilled water and phosphate-buffered saline (PBS) (Lonza, Walkersville, MD). Before cell seeding the tabular scaffolds were sterilized by immersion in 70% ethanol overnight and then washed in PBS three times on the day of cell seeding. The final dimensions of the SFCS tubular scaffolds prior to implantation were 30 mm in length, 7 mm in outer diameter, and 2 mm in thickness (Fig. 1A).

2.2. Chondrocyte isolation and culture

All animal care and surgical procedures were in compliance with the Institutional Animal Care and Use Committee (IACUC) of The University of Texas M.D. Anderson Cancer Center.

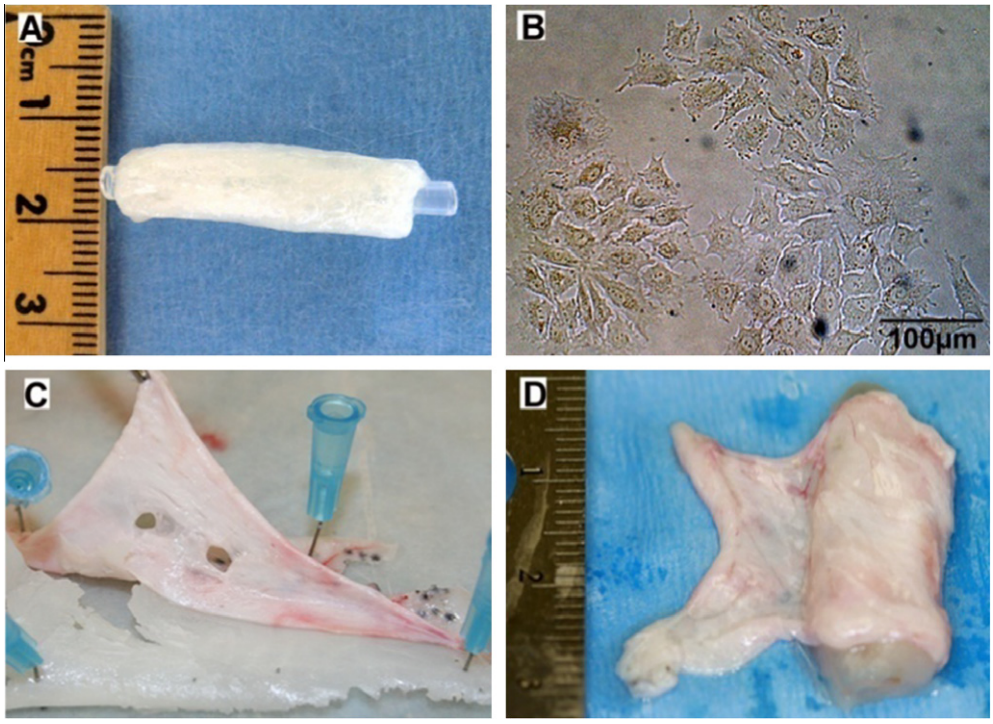

Fig. 1. Preparation of SFCS scaffold–cell–perichondrium constructs. (A) The tubular SFCS scaffold after aqueous crystallization shows an intact scaffold around a siloxane tube. (B) Phase contrast photomicrograph of passage 3 chondrocytes in monolayer culture after 24 h culture (magnification 200×). (C) At the time of implantation the perichondrium was elevated from the rabbit ear cartilage and straightened in place with a needle. (D) The cell–scaffold construct (CS) was wrapped with perichondrium (CSP) before being implanted.