Proceedings of the 5th International Conference on Nanotechnology: Fundamentals and Applications
Prague, Czech Republic, August 11-13, 2014
Paper No. 149

Nanofiber Oriented Bacterial Cellulose Tubular Scaffolds: Mechanical Properties and Cellular Response

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Abstract - We have recently reported that native bacterial cellulose (BC), a randomly oriented nanofibril hydrogel, supports *in vitro* proliferation and osteogenic and chondrogenic differentiation of equine bone marrow-derived adult mesenchymal stem cells (EqMSCs). The objective of the present study was to generate and characterize the morphological, cellular response and mechanical properties of biodegradable and mineralized tubular-shaped bacterial cellulose scaffolds (BC-TS) of varying diameters. BC-TS and its mineralized composites were developed to mimic the highly aligned collagen nanofibers and hydroxyapatite crystal structures inherent in native bone tissue. The biocompatible gel-like BC-TS was synthesized using the bacterium *Gluconacetobacter sucrofermentans* under static culture in oxygen-permeable silicone tubes. The BC-TS scaffolds were modified using a periodate oxidation to yield biodegradable scaffolds. Additionally, calcium-deficient hydroxyapatite (CdHA) was deposited in the scaffolds to mimic native bone tissues. The mechanical and morphological properties of the resulting BC-TS and its composites were characterized in addition to their ability to support and maintain EqMSCs growth *in vitro*. The BC-TS and its mineralized composites exhibited aligned nanofibril structures. Calcein-AM and and propidium iodide fluorescent staining demonstrated viable cells on the engineered BC-TS and its composites. The biocompatible and biodegradable CdHA BC-TS composites have excellent mechanical properties and support the viability of EqMSCs cultured onto its surface in vitro, allowing for future potential use for tissue engineering therapies.

Keywords**:** Mechanical property, Nanofiber, Hydroxyapatite, Composite, Biomaterial, Tissue engineering, Stem cell.

1. Introduction

The ideal scaffold to treat damaged tissue resulting from traumatic injuries and diseases remains a challenge for tissue engineering of bone. Current clinical procedures to repair this tissue include the use of autographs and allographs (Lohmander, 2003). However, autographs may be unavailable for use and allographs may lack of immune compatibility (Marolt, Knezevic, and Novakovic, 2010). The current clinical treatment for bone tissue damages and diseases stresses the need for an alternative replacement therapy. Thus, developing advanced biomaterials that can mimic the chemical and structural nature of native bone tissue, permit stem cells to adhere and differentiate to regenerate the lost bone function is important in the development of stem cell therapies for bone defects.

Native bone tissue is a three-dimensional (3D) hierarchical tubular structure consisting of extracellular matrix, cells, collagen fibers and needle-shaped hydroxyapatite crystals (Cui et al., 2011). Hence, an accurate scaffold designed to replace bone tissue must mimic the mineralized 3D structural composite to properly replicate its function. Native bone tissue is formed in layers of lamellae fiber bundles which contain near parallel arrays of collagen type I, a 1.0 nm diameter nanofibril structure, which forms 100-200 nm triple helical collagen molecules in the tissue matrix (Bigg et al., 2007; Cui et al., 2011). In the present study, the near parallel arrays of collagen fibers in native bone are mimicked using bacterial cellulose (BC) scaffolds with oriented nanofibrils. Thus, the BC nanofibril scaffolds having diameter of approximately 32 nm, are very attractive materials for bone tissue engineering because they are chemically pure, biocompatible, moldable, cost-effective, possess appropriate high mechanical strength and large accessible surface area/volume ratio (Bielecki et al., 2001; Favi et al., 2013; Helenius et al., 2006; Hutchens et al., 2009). BC is derived from a natural source and can be easily synthesized from non-pathogenic bacterium such as *Gluconacetobacter sp* into non-degradable, nanoporous scaffolds in the form of hydrogels (Favi et al., 2013). Non-degradable BC have been used as a scaffold for treatment of second- or third-degree burns (Fontana et al., 1990), chronic ulcers (Kucharzewski, Slezak, and Franek, 2003), and for artificial blood vessels (Klemm et al., 2001). Non-degradable BC can be rendered degradable through a periodate ring-opening oxidation, which degrades by simple hydrolysis mechanism (Bielecki et al., 2001; Hutchens et al., 2009).

Recently, researchers have generated tubular BC (BC-TS) scaffolds with oriented fibers using oxygen permeable silicone tubes (Putra et al., 2008; Wang, Wan, and Huang, 2012). BC-TS is beneficial for bone tissue engineering because the oriented fibers may be used to imitate the inherent oriented collagen fibers in native bone. Furthermore, in bone, calcium-deficient hydroxyapatite (CdHA) minerals form along the oriented collagen fibers of the tissue (Cui et al., 2011). As demonstrated in previously studies, CdHA can be deposited in BC scaffolds to form BC-CdHA composites (Hutchens et al., 2009; Hutchens et al., 2006). Here, the structure and function of native bone tissue is mimicked, more specifically the collagen-CdHA composite in bone, by depositing osteoconductive and bioresorbable CdHA minerals in BC-TS with oriented fibers. Combined with osteocyte-forming mesenchymal stem cells, this biomimetic fibrous composite could be used for bone defect repair and reconstruction. We hypothesized that aligned mesenchymal stem cell morphology can be induced on the scaffolds due to the orientation of the fibers and as previously reported (Lyu et al., 2013).

The stiffness of engineered scaffolds have been shown to significantly influence the adhesion, self-renewal and differentiation of stem cells (Discher, Mooney, and Zandstra, 2009; Engler et al., 2006; Pelham and Wang, 1997), and subsequently the biomaterial's performance. Because cell fate and associated scaffold performance are influenced by the dynamic interaction of the cell with the scaffold stiffness, it is imperative to accurately determine the mechanical properties of the biomaterial (Tan et al., 2008).

Successful bone repair approaches may include an osteoconductive scaffold with mechanical properties that permits excellent cell adhesion and proliferation. In this study, biocompatible and biodegradable BC-TS-CdHA composite scaffolds with various diameters were prepared and their mechanical and morphological properties characterized. The ability of the scaffolds to support the viability of equine-derived bone marrow mesenchymal stem cells (EqMSCs) *in vitro* was also evaluated for potential tissue engineering use.

2. Materials and Methods

2. 1. Materials

All chemicals, cell culture supplements and disposable tissue culture supplies were purchased from Sigma-Aldrich (Saint Louis, MO) unless otherwise noted. During the bone marrow extraction to obtain equine-derived bone marrow mesenchymal stem cell, animal procedures were carried out according to Institutional Animal Care and Use Committee protocol no. 1953.

2. 2. Preparation of Tubular Bacterial Cellulose and its Composites

Silicone tubes (Fisher Scientific, Pittsburgh, PA), with inner diameters (ID) of 6.35 and 9.525 mm, wall thicknesses of 6.35 mm, and length of 100 mm, were used in the preparation of tubular BC (BC-TS) samples. The silicone tubes were washed, dried and sterilized in an autoclave (1 bar, 120 °C) for 30 min prior to use. Bacterial strain *Gluconacetobacter sucrofermentans* was commercially obtained from the American Type Culture Collection (Manassas, VA) (ATCC 700178). Pre-cultures of BC were made in a Schramm and Hestrin medium (Schramm and Hestrin, 1954) and the constituents are described by Favi et al. (Favi et al., 2013). For BC-TS production, pre-cultures of the bacteria were diluted 1:10 in fresh media and poured on the outside surface of the sterile silicone tubes or on the inside surface the sterile tubes. This method of synthesizing BC-TS is a modification of a method previously described by Putra et al. (Putra et al., 2008). The BC-TS were synthesized for 14 days and purified in distilled/deionized water using a method described by Favi et al. (Favi et al., 2013).

The oxidation of BC-TS (OBC-TS) was performed as previously described (Hutchens et al., 2009). Briefly, BC-TS samples were placed in a capped vessel covered with aluminum foil that contained 50 mM $NaIO_4$ (sodium metaperiodate) in 5% *n*-propanol, and placed on an orbital shaker for 24 h at 23 °C. The oxidation reaction was stopped and excess periodate consumed by placing the vessel in an ice bath and adding 0.5 mL of glycerol. The OBC-TS were then purified with numerous changes of deionized water.

Calcium-deficient hydroxyapatite (CdHA) minerals were deposited within the unmodified BC-TS (BC-TS-CdHA) and oxidized BC-TS (OBC-TS-CdHA) by performing an alternating incubation cycle with calcium and phosphate solutions (modified from Hutchens et al. (Hutchens et al., 2006)). Briefly, BC-TS and OBC-TS are suspended in 5.0 mM $CaCl_2$ under agitation in an ortibal shaker for 24 h (23 °C), rinsing the samples briefly in deionized water, and then transferring the pellicle to 3.0 mM Na_2HPO_4 under agitation for another 24 h (23 °C) to obtain CdHA.

2. 3. Characterization of Tubular Bacterial Cellulose and its Composites

The morphology of the lyophilized BC-TS and its composites were examined using a scanning electron microscope (SEM). For lyophilization, hydrated samples were placed in a -80 °C freezer for 24 h and then lyophilized in a FreeZone® 4.5 Freeze Dry System (Labconco, Kansas City, MO) for at least 24 h at -50 °C and 1.0×10^{-3} Pa. The lyophilized samples were mounted on carbon tape and sputtered with gold on a Spi Module Sputter Coater (Spi Supplies: Westchester, PA, USA) at 20 mA for 30 s. The samples were then analyzed on a LEO 1525 SEM (Zeiss: Oberkochen, Germany).

Mechanical properties including engineering elastic modulus, percent strain at break and ultimate tensile strength of the BC-TS and its composites were examined using the Instron ElectroPuls™ E1000 mechanical testing unit or the Instron 5943 mechanical testing unit, both equipped with Instron® Bluehill® Softwares. The mechanical integrity of the materials were determined following a previous describe method (Putra et al., 2008). Briefly, specimens were analyzed by lengthwise and breadthwise elongation. A minimum of three specimens were analyzed for each type of BC-TS sample. Specimen (~25 mm gage length with ~5 mm long gripping tabs) for lengthwise elongation was elongated at a rate of 0.5 mm/min until failure using a 1.0 kN or 100 N load cell. Specimen (~6 mm in length) for breadthwise elongation was elongated using 2 U-shaped wires inserted through the hollow opening of the sample. Analyses were performed at a rate of 0.5 mm/min until failure, using a 1.0 kN or 100 N load cell. Lengthwise and breadthwise sectioned specimens of the BC-TS hydrogels represent the longitudinal and transverse fiber direction of the samples in accordance to the potential use. The BC hydrogel samples with aligned fibers should exhibit anisotropic mechanical properties. i.e., mechanical differences in transverse and longitudinal directions.

2. 4. Cell Culture and Seeding

Previously isolated equine-derived bone marrow mesenchymal stem cell (EqMSCs) were obtained by centrifugation of bone marrow aspirates from the sternum of a healthy 11-years-old male horse, characterized and cryo-preserved in our laboratory as previously described (Dhar et al. (Dhar et al., 2012),

Favi et. (Favi et al., 2013)). The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (Hyclone, Logan, Utah) and 1% penicillin/streptomycin solution (P/S) (Invitrogen, Carlsbad, CA). Cell passages 2-6 were used for the experiments.

BC-TS and its composites were prepared as circular discs for the cell study using 6 mm Miltex Inc. disposable biopsy punches (Fisher Scientific) and a 17.46 mm round hole arch punch (McMaster Carr, Atlanta, GA). For cell culture studies, the BC-TS and its composites were sterilized by autoclave (1 bar, 120 °C) for 30 min, and were pre-soaked in phenol red-free growth media for at least 24 h prior to cell seeding.

2. 5. Cellular Adhesion and Cell Viability Staining Using Fluorescent Microscopy

EqMSCs were seeded on BC-TS and its composites at a density of 2.5×10^4 cells/well. All experiments were conducted in 96-well TCP. Cell adhesion and viability was assessed after 1, 2 and 3 days using calcein-AM (Invitrogen, Eugene, OR) and propidium iodide (PI) (Invitrogen, Carlsbad, CA). Cells were stained according to the manufacturer's protocols and subsequently visualized using a Zeiss Axiovert 40C microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY) equipped with a Nikon Digital Sight DS-Qi1Mc camera (Nikon Instruments Inc., Melville, NY).

2. 6. Statistical Analysis

Data are expressed as mean ± standard deviation (SD) of at least three independent samples. Statistical comparisons between groups were performed with a two-tailed Student's t-test, $p < 0.05$ was considered significant.

3. Results and Discussion

BC-TS samples were successfully generated in oxygen-permeable silicone tubes. Four different sizes of scaffolds were generated from the synthesis of *Gluconacetobacter sucrofermentans* in a Schramm and Hestrin medium. BC-TS scaffolds synthesized on the inner surface of the 6.35 mm ID silicone tubes produced water swollen scaffolds with 8.3 mm diameter and 1.6 mm thickness (Fig. 1A). BC-TS scaffolds synthesized on the outer surface of the 6.35 mm ID silicone tubes produced water swollen scaffolds with 20 mm diameter and 1.0 mm thickness (Fig. 1C). Therefore, the diameter of the scaffolds produced on the inner surface of the 6.35 mm ID silicone tubes were smaller (8.3 mm, Fig. 1A) than those produced on the outer surface of the equivalent size tube (20 mm diameter, Fig. 1C). Similar increase in scaffold diameter were also observed with the BC-TS scaffolds synthesized on the inner surface of the 9.525 mm ID silicone tubes (13.5 mm water swollen diameter, 1.1 mm thickness) (Fig. 1B) compared to the outer surface (24.2 mm water swollen diameter, 0.7 mm thickness) of the equivalent size tube (Fig. 1D).

Fig. 1. Photographs of BC-TS scaffolds synthesized on the inside surface (A) and on the outside surface (C) of 6.35 mm ID silicone tubes. Photographs of BC-TS scaffolds synthesized on the inside surface (B) and on the outside surface (D) of the 9.525 mm ID silicone tubes. Top view image (E) of the four sizes of BC-TS hydrogels synthesized using silicone tubes.

SEM images of the 8.3 mm BC-TS produced under various treatments demonstrated that aligned cellulose fibers were successfully generated in the scaffolds (Fig. 2). SEM images of the unmodified BC-TS (Fig. 2A) and after (Fig. 2B) periodate oxidation showed that the scaffolds maintained their morphological integrity of the nanofibers during the chemical reaction. CdHA ceramics were successfully deposited in the non-oxidized scaffold (Fig. 2C) and oxidized scaffold (Fig. 2D) as illustrated in the SEM images.

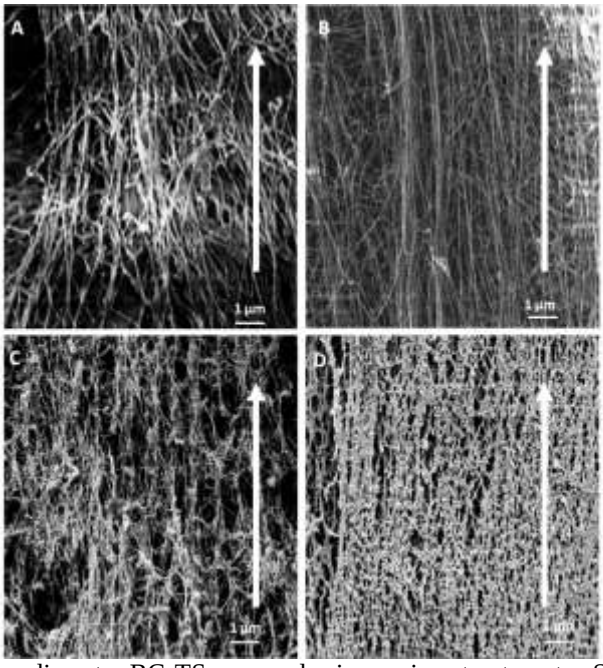

Fig. 2. SEM images of 8.3 mm diameter BC-TS prepared using various treatments. SEM images of BC-TS (A), OBC-TS (B), BC-TS-CdHA (C) and OBC-TS-CdHA. Arrows indicate the direction of the longitudinal axis of the silicone tube during scaffold synthesis.

The mechanical properties of the 8.3 mm diameter BC-TS (Fig. 1A) and its composites are summarized in Table 1. The ultimate tensile strength and elastic modulus of the scaffold composites decreased compared to the unmodified BC-TS scaffolds.

Table 1. Mechanical properties of 8.3 mm diameter BC-TS and its composites.

Sample	Form of Elongation	Ultimate Tensile Strength (KPa)	Strain at Break (%)	Elastic Modulus (MPa)
BC-TS	Lengthwise	243 ± 40	0.177 ± 0.032	1.90 ± 0.22
BC-TS	Breadthwise	596 ± 65	0.132 ± 0.062	13.12 ± 4.16
BC-TS-CdHA	Lengthwise	247 ± 123	0.170 ± 0.069	2.30 ± 2.61
BC-TS-CdHA	Breadthwise	471 ± 59	0.113 ± 0.014	9.63 ± 3.35
OBC-TS	Lengthwise	230 ± 66	0.188 ± 0.074	3.21 ± 0.08
OBC-TS	Breadthwise	135 ± 45	0.086 ± 0.013	2.16 ± 0.95
OBC-TS-CdHA	Lengthwise	29 ± 7	0.107 ± 0.019	0.66 ± 0.28
OBC-TS-CdHA	Breadthwise	50 ± 9	0.031 ± 0.004	0.67 ± 0.17

Calcein-AM and PI staining was performed to visualize the viability of the cells on the scaffolds. After 1 day in culture (Fig. 3), the cells on all the tubular BC scaffolds were viable. However, the cells on the oxidized scaffolds (Fig. 5B, 5D) showed distinct mesenchymal stem cell phenotype of fully spread-out morphology on the scaffolds. The cells on the non-oxidized scaffolds (Fig. 3A, 3C) showed less elongated shapes and their morphology was round. We had previously illustrated that up to day 7 in culture, EqMSCs seeded on non-oxidized BC are round in shape (Favi et al., 2013). Additionally, EqMSCs do proliferate over time and by day 7 and day 14 demonstrate the full spread-out morphology on non-oxidized BCs (Favi et al., 2013).

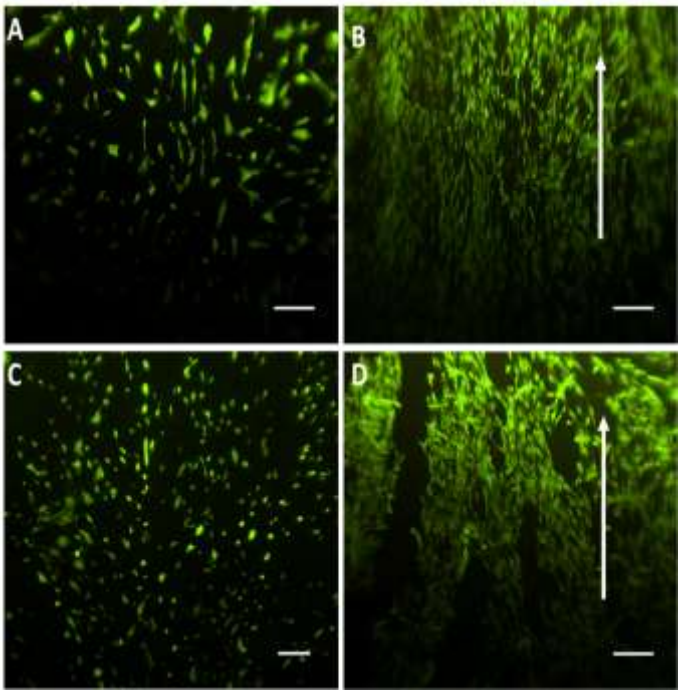

Fig. 3. Cellular adhesion and cell viability stained with calcein-AM and PI using fluorescent microscopy. Cell viability of EqMSCs seeded on BC-TS (A), OBC-TS (B), BC-TS-CdHA (C) and OBC-TS-CdHA (D) after 1 day in culture. Cells were analyzed by calcein-AM which exhibits green fluorescence and demonstrates live cells and PI which displays red fluorescence and demonstrates dead cells. Fluorescent micrographs showed that the cells were viable on the scaffolds. Arrows in image show the aligned direction in which the cells grew following the orientation of the cellulose fibers of tubular BC. Scale bar = 100 μm.

4. Conclusion

BC-TS is a natural nanofiber hydrogel scaffold that was successfully prepared, and the scaffold and its composites were characterized using SEM. The lyophilized BC-TS and its composites illustrated aligned nanofibrous morphology. It was demonstrated that the BC-TS scaffold and its composites were cytocompatible with EqMSCs *in vitro*. BC-TS scaffolds and its composites supported the adhesion, proliferation and osteogenic differentiation of EqMSCs. BC-TS scaffolds and its composites are promising alternative scaffolds for bone tissue engineering.

Acknowledgements

This study was supported by the College of Veterinary Medicine's Center of Excellence grant and the National Institute of Health Training Grant (NIH/ NIGMS-IMSD: R25 GM086761).

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