Mechanical Analysis of Biocomposite Materials from Bacterial Cellulose and Hydroxyapatite

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***Abstract*—Bacterial cellulose (BC), which is cultivated from *Gluconacetobacter xylinus*, is the finest natural cellulose with a diameter of 30 nm–50 nm. Because of its high levels of uniformity and purity, BC has unique physical and mechanical properties for applications in biomaterials. In this study, sample cellulose was prepared in advance from *Gluconacetobacter xylinus* under static cultivation. After the provision of hydroxyapatite was executed using dicalcium phosphate dehydrates and $CaCO_3$, BC was introduced in the alkaline solution at 55 ℃ for further reaction. Regarding mechanical strength and endotoxin evaluation, the biocomposite of BC and hydroxyapatite yielded stronger results after the addition of 5% BC. The compressive resistance of the trial product reached approximately 141.36 MPa, and the endotoxin level could be reduced to 0.3 EU/mL if the raw biocomposite was sintered using alginate as a hardener at 1200 ℃ for 1h. With its high level of compressive resistance and low level of endotoxins, the proposed biocomposite material has great potential for employment as a filling material.**

***Index Terms*—bacterial cellulose (BC), compressive resistance, endotoxin, hydroxyapatite (HA)**

I. Introduction

Hard tissues, such as bones and teeth, can be formed naturally through a biomineralization process, in which an inorganic phase is sequentially overlaid on an organic template. Before being implanted into a patient, the natural process of bone growth is partially mimicked in vitro [1]. Artificial bones are made from natural minerals composed of hydroxyapatite (HA) nanocrystals and collagen as a filling. HA-collagen nanocomposites have been widely investigated [2]-[6]. However, the applicability of these composites is limited because of high costs, concerns of cross-infection, and the poor definition of commercial sources [7]. Several techniques for preparing HA-based biocomposites have been investigated as bone substitutes. Compared to collagen, bacterial cellulose (BC, also known as microbial cellulose) eliminates cross-infections, and demonstrates superior strength and enhanced mechanical properties [8]. The BC produced by the Gram-negative bacteria, *Gluconacetobacter xylinus*, produces highly hydrated cellulose membranes (99% water by weight), with a higher molecular weight and crystallinity than plant cellulose [9], [10]. The cellulose membrane formed in static culture comprises a 3D structure consisting of an ultrafine network of nanofibers (3 nm–8 nm) and a large nanoporous surface area. In general, BC fibers have a high aspect ratio with a diameter of 35 nm–90 nm and a Young's modulus value of 78 ± 17 GPa, and the BC microfibrils have a density of 1600 kg m^{-3} [11], [12]. The BC possesses valuable qualities such as non-toxicity, biocompatibility, biodegradability, selective permeability, high levels of tensile strength, and high modulus values. The unique properties resulting from the nanometric structure yielded numerous medical applications, such as diagnostic biological probes and display devices, and it has been intensively examined for applications in wound dressings, artificial skin, artificial blood vessels, and specialty membranes [13]. Thus, combining BC and HA should yield a material with strong mechanical properties, a high level of porosity, controllable pore size, in situ shape mold ability, a 3D structure, excellent biocompatibility, adjustable biodegradation, and strong osteoconductivity and bone-bonding ability. Previously, BC has been used in critically and non-critically sized bone defects for guided bone regeneration (GBR, or guided tissue regeneration) and as a resorbable barrier membrane and fibrous connective tissue [14]. Svensson et al. suggested using BC as the scaffold in cartilage tissue engineering. [15]. Worldwide, the primary HA powder synthesis methods include the hot press sintering, hydrothermal synthesis, hydrolysis synthesis, calcium phosphate calcination, and wet granulation methods. The current study used a common low-temperature hydrolysis method to prepare HA powder; during the preparation, BC was added to enhance the molecular bonds and mechanical strength of the powder, and to determine the effects of BC on the HA. In addition, a hardener was added to the prepared HA powder to produce block-shaped artificial bones. The current study explored the optimal HA powder processing conditions, analyzed the mechanical strength and endotoxin levels of HA powder, and determined the feasibility of using BC for artificial bone material preparations in future products.

II. Materials and Methods

A. Static Fermentation

Gluconacetobacter xylinus were screened and collected from the Bioresource Collection & Research

Manuscript received May 10, 2013; revised July 21, 2013

doi: 10.12720/jomb.2.4.228-231

Center (Hsinchu, Taiwan) and the initial cultures were maintained on AC agar plates at 30 ± 2 ℃. After incubation for 48 h, the cultures were transferred into a yeast extract medium and grown at 30 ℃ for 7 d.

B. Pretreatment and Modification

After static fermentation, the metabolite of *Gluconacetobacter xylinus*, was washed using the medium in the film by using 1% NaOH solution, and then, sterilized at 121 ℃ for 15 min. The film was subsequently soaked in a water solution to perform structural modifications. Finally, various samples were obtained after vacuum filtration and hot-air drying.

C. Hydrolysis

The wet synthesis hydrolysis method was used to synthesize HA in an alkaline environment at a low temperature. Dicalcium phosphate dehydrates, or $CaHPO_4 \cdot 2H_2O$, was used as the initial material. Appropriate amounts of $CaCO_3$ were added based on the ratio of calcium and phosphorus, and various ratios of BC were added during synthesis. The compounds were processed in alkali sodium hydroxide solutions ($NaOH_{(aq)}$) and allowed to react under various temperatures and within varied time frames to synthesize the HA powder.

D. Preparation of Block-Shaped Specimens

For the control group, BC powders of various weight ratios were added to neutral (pH = 7) phosphate solutes. Sodium alginate solutions, at concentrations between 1.0% and 3.0%, were added in the experimental group. The powder to liquid blending ratio was 1 g:0.6 mL–1.0 mL. After this paste-like mixture was stirred for 1 min, it was poured into a stainless steel mold (12 mm high, 6 mm diameter). The mixture was then subjected to a 0.7 MPa holding pressure until the specimens hardened, and was stripped after 15 min.

E. Scanning Electron Microscopy

After the specimens were processed, they were fixed on carbon tapes and machined with gold plating to increase their electrical conductivity and avoid partial discharges. A Hitachi S-2500 field emission scanning electron microscope (SEM) was used to observe the surface morphology of the specimens.

F. Compressive Strength Test

Compressive strength tests were performed according to the ISO6873 dental gypsum product standards. The circular planes of the block specimens were staged facing upward on the microscope deck, and a universal testing machine (Shimadzu AGS-500D, Japan) was used to measure the compressive strengths by using a pressure speed of 0.5 mm/min. The average compressive strengths of five specimens were obtained, and the highest breaking load (Kgf) was recorded and converted into pressure units (MPa).

G. Antiwash-Out Test

The stripped test specimen block was placed in a 20-mL, 37 ℃ Hank's balanced salt solution (Sigma, USA) for 6 h to observe whether the specimen would dissolve or collapse, thereby causing the solution to become turbid.

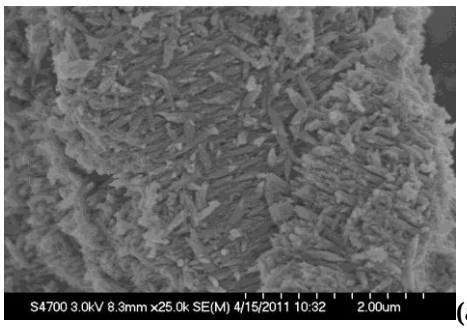
H. Pyrogen Assay (Limulus Amebocyte Lssate test)

A 100-μL diluted sample and the standard were added to 100 μL of LAL reagent and mixed slowly and gently in glass tube. The mixed sample was cultured at 37 ℃ for 1 h. The condition in the reversed glass tube was then observed to complete the LAL test. If the gel was agglutinated, then it tested positive for pyrogen, indicating an endotoxin in the sample.

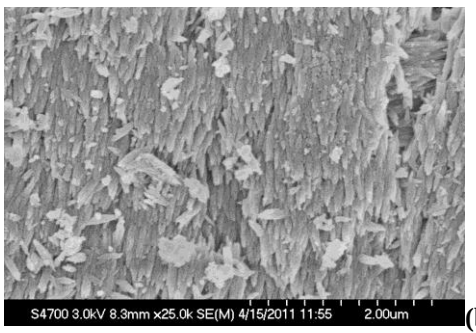
III. Results and Discussion

A. Effect of Adding Various Concentrations of BC on HA Powder Prepared using Hydrolysis

During SEM microstructure observation at 25 K magnification, the HA powders at various concentrations of BC underwent hydrolysis treatment in a 75 ℃ $NaOH_{(aq)}$ solution for 1 h. Fig. 1a and 1b show the observed acicular hydroxyapatite structures. After sintered at 1200 ℃ for 1 h, the crystal patterns transformed into columnar shapes with columnar hexagonal tips (Fig. 1c and 1d). When 0.5% BC was added, this arrangement was not affected; however, the lengths of the cylindrical precipitates were reduced to 100 nm–200 nm.



(a)



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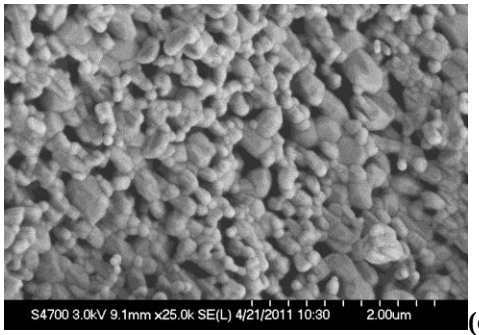
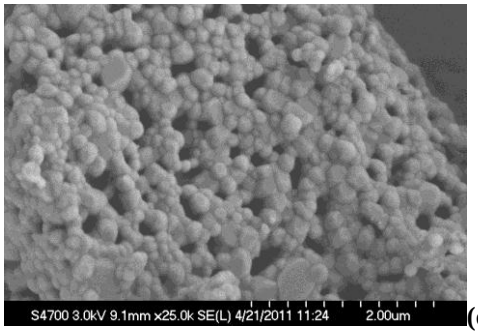


(c)



(d)

Figure 1. The morphology of the trial samples with SEM (a) original BC and (b) BC after sintering at 1200 ℃ for 1 h; the form of the BC-HA synthesized material under SEM (c) before sintering at a high temperature; and (d) after sintering at 1200 ℃ for 1 h.

B. Effect of Adding Various Concentrations of BC on the BC-HA Compressive Strength

The BC-HA block specimens were added to the HA based on various concentration ratios. The curing agent was alginate. Compressive strength tests were performed after sintering at 1200 ℃ for 1 h. Without adding BC, the calcium phosphate cement had a compressive strength of 96.72 MPa. When the BC concentration reached 5%, an optimal compressive strength of 141.36 MPa was achieved. If the BC concentration was increased beyond 5%, the compressive strength decreased (Fig. 2).

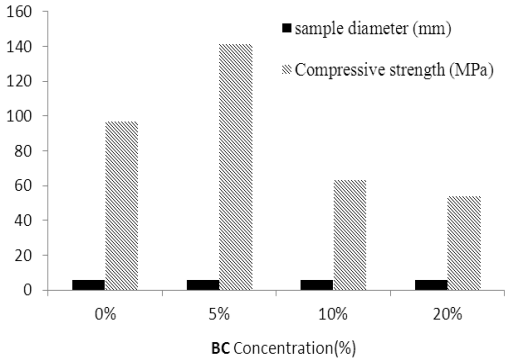


Figure 2. The effect of adding various concentrations of BC on the compressive strength of BC-HA

C. Effect of Various Concentrations of Curing Agents on the Anti-Erosion Properties of BC-HA

The BC-HA block specimens were placed in a Hank's balanced salt solution. The control calcium phosphate cement, which was prepared using an aqueous solution, was slightly soluble, resulting in a whitish, turbid consistency in the Hank's balanced salt solution. However, the BC-HA block specimens in the experimental group that were composed of 1.0% and 3.0% alginate concentrations were not soluble, and the Hank's balanced salt solution remained clear and transparent after static placement for 6 h.

D. Effect of Adding BC on the Endotoxin Content of the Sample

Based on the regulations of the U.S. Pharmacopeial Convention and U.S. Food and Drug Administration, the LAL gel endotoxin method was used to determine whether the endotoxin level of the product was excessive. A BC-HA composite at a 5% concentration was prepared and added to a commercially available hydroxyapatite (Sigma) to detect endotoxins. The results indicate that the level of endotoxin contained in the commercially available hydroxyapatite was less than 0.3 EU/mL. Prior to sintering, a 5% BC-HA composite had endotoxin levels of 0.3 EU/mL–3.0 EU/mL. After sintering at 1200 ℃, the endotoxin content was less than the heat source content of 0.3 EU/mL. Table I. lists the comparison of endotoxins.

TABLE I. EFFECT OF BC-HA ON ENDOTOXIN LEVELS.

	HA+5%BC	HA+5%BC 1200 ℃	HA (Sigma)	Control (BC untreated)
LAL test (EU/mg)	<3.0	<0.3	<0.3	>3.0

IV. CONCLUSION

The current study explored the potential advantages of BC, including its biocompatibility (non-toxic and non-residual heat sources), and mechanical strength (elasticity and ductility). The results indicated that adding BC to biological materials strengthens their resistance to erosion, enhancing their mechanical strength without affecting their stability during heat exposure. Thus, BC has a high potential as a filling material in moldable bones and in future applications such as artificial bones, vascular stents, and artificial heart valve stents.

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Pei-Ying Chen, who comes from Taiwan, is 33 years old. She was graduated from department of chemical engineering of National Yun-Lin University of Science & Technology in 2003, and received a master degree of bioengineering from Tatung University in 2005.
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Through several years on-the-job training, Miss Chen can run a research team work quite well whether academic or industry-oriented programs are given. That is why she can join so many outside learning activities and have been admired by the supervisors.

Jinn-Tsyy Lai, who was born in 1963 in Taiwan, has focused on the investigation of functional ingredients from microorganism over the past years. He was graduated from department of chemical engineering of Tamkang University in 1985, and got his master degree and PhD degree from institute of chemical engineering, National Taiwan University, in 1987 and 1997, respectively.
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