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In vitro behaviors of rat mesenchymal stem cells on bacterial celluloses with different moduli

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ABSTRACT

Compressive moduli of bacteria-synthesized cellulose (BC) were altered by two drying techniques: ambient-air drying and freeze drying. While no significant differences in dry weight were found, their cross-sectional structures and thickness varied greatly. Freeze dried BCs had loose cross-sectional structures and a thickness of ~4.7 mm, whereas air dried BCs had more compacted cross-sectional structures and a thickness of ~0.1 mm. The compressive moduli of the rehydrated freeze dried and rehydrated air dried BCs were measured to be 21.06 ± 0.22 kPa and 90.09 ± 21.07 kPa, respectively. When rat mesenchymal stem cells (rMSCs) were seeded on these BCs, they maintained a round morphology in the first 3 days of cultivation. More spread-out morphology and considerable proliferation on freeze dried BCs were observed in 7 days, but not on air-dried BCs. The cells were further grown for 3 weeks in the absence and presence of differentiation agents. Without using any differentiation agents, no detectable differentiation was noticed for rMSCs further cultivated on both types of BC. With differentiation inducing agents, chondrogenic differentiation, visualized by histological staining, was observed in some area of the rehydrated freeze dried BCs; while osteogenic differentiation was noticed on the stiffer rehydrated air dried BCs.

1. Introduction

Bacterial cellulose (BC), a biopolymer extracellularly synthesized by several bacterial genera, particularly *Acetobacter* strains, holds a great promise as a biomedical material. BC has the analogous chemical structure as plant celluloses; however, it possesses pure cellulose without pectin, lignin, and hemicellulose [1,2]. BC has been used in medical fields such as artificial skin for burns [3], artificial blood vessel for microsurgery [4], scaffolds [5,6], and wound dressing [7] due to its fine-assembled nanofibrous structure and good physicochemical properties such as moldability, high tensile strength, crystallinity, and water uptake rate [2,7]. Mechanical properties of BC can also be varied using differently drying techniques for different applications.

Mechanical properties of extracellular environment or underlying substrate play a vital role for directing stem cell behaviors such as adhesion, proliferation, and differentiation [8,9]. Substrate stiffness is a factor affecting the interactions between cells and substrate, especially in in vitro studies. Substrate stiffness that mimics the natural extracellular matrix (ECM) appears to promote stem cell differentiation [10–16] into the right cell lineage. For instance, collagenousbone-like substrate with elastic modulus in kPa range has shown to promote differentiation into osteogenic lineage of mesenchymal stem cells (MSCs) [10].

MSCs are multipotential cells isolated from adult bone marrow compartment [17] or adipose tissue [18]. Depending on the source of cells [19], they have a potential to commit into different anchorage cell lineages such as osteoblasts, chondrocytes, adipocytes [17], myoblasts [18,20] or even neurons in the presence of specific induction factors and suitable matrix elasticity [10]. These multilineage cells are therefore attractive cell sources for tissue engineering, self-cell repair and gene-based therapy. BC could be a good candidate for studying the effect of its matrix elasticity on the regulation of stem cell functions.

In this study, BC samples were dried by two different methods, air drying under ambient condition and freeze drying, to produce BC layers having different compressive moduli. Matrix stiffness relating to compressive modulus was measured by contact mechanics. Rat MSCs (i.e. rMSCs) were cultured on BC samples for 1 week to 3 weeks in the presence and absence of a specific induction medium to follow the difference in chondrogenesis and osteogenesis on the two types of BCs.

2. Materials and methods

2.1. Materials

All chemicals and cell culture medium (DMEM-HG containing 10% of FBS and 1% of antibiotic) and supplements were purchased from

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Sigma-Aldrich, Inc. unless otherwise noted. The Sprague–Dawley (SD) rat mesenchymal stem cells (rMSCs) were purchased from Oricell™ Cyagen and used in this experiment. *Acetobacter xylinum* bacterial strain AGR60 was supplied by Pramote Thammarad, the Institute of Research and Development of Food Product, Kasetsart University, Bangkok, Thailand.

2.2. Preparation of BC samples

BC samples were produced using a coconut-water based medium supplemented with 5% w/v of sucrose (Ajax Finechem), 0.5% w/v of ammonium sulfate (Ajax Finechem), and 1 ml of 30.0% v/v acetic acid (QRec) to achieve acidic pH 4–5. The medium was sterilized at 110 °C for 5 min and cooled prior to use. The 5% v/v of pre-culture of *A. xylinum* was added to 75 ml of the culture medium in a 14.5 cm diameter of a sterile glass petri-dish. The incubation was carried out at 30 °C under ambient condition. After 7 days, all the BC gels were washed with running water for 30 min and were then treated with 1% w/v NaOH (Ranken) at room temperature for 24 h followed by rinsing with DI water until a pH of 7 was achieved. The purified films were stored in DI water at 4 °C until use. The BC gels were cut into 1 × 1 cm^2 and dried by two different methods, which were air drying under ambient condition on glass slide for 48 h or freeze drying for 24 h. For the freeze drying, the gels were frozen in liquid nitrogen for 2 h before transferring the frozen sample to a freeze drier (VirTis-SP Scientific Sentry 2.0), which had a condenser temperature of −80 °C and vacuum pressure of 14 mTorr.

2.3. Characterizations

Dry weight (using an analytical balance with an accuracy of 0.1 mg) of dried BC samples was measured. At least four different samples of each type of dried BC were used, and the values were averaged. The length, width and thickness samples were also measured (using Traceable® Digital Carbon Fiber Calipers from Fisher Scientific) and used to estimate the volume of the sample.

Water absorption capacity (WAC) of BC samples was assessed by immersing the dried samples in DI water under ambient condition for overnight. The rehydrated samples were then removed and gently wiped with a piece of Kimwipes® prior to weighing. The weight of the sample was measured and the water content absorbed into the sample was then calculated by using $\%\mathrm{WAC} = \frac{W_h - W_d}{W_d} \times 100$, where W_h and W_d denote the weights of the rehydrated and dried sample, respectively.

Porosity (ε) of BC samples was calculated using $\varepsilon = \left(1 - \frac{\rho_{sample}}{\rho_{cellulose}}\right) \times 100\%$, where ρ_{sample} and $\rho_{cellulose}$ were the density of the BC sample and the cellulose, respectively. The value of ρ_{sample} was estimated by dividing the dry mass of BC sample to the estimated volume of dried or rehydrated BC sample, and the value of $\rho_{cellulose}$ was 1.5 g/cm^3.

Surface and cross section morphologies of samples were examined by using Scanning Electron microscopy (SEM, Hitachi TM3000 and JOEL JSM-5410LV). The images were taken with 3000× magnification and an accelerating voltage of 5 kV. For higher resolution images, the dried samples were sputtered with a thin layer of gold in a Balzers-SCD 040 sputter coater (Balzers, Liechtenstein), and then imaged at 10,000× magnification and an accelerating voltage of 15 kV. The surface topography of freeze dried BC was also scanned using an atomic force microscope (AFM, Veeco multimode NanoScope IIId) at a scan rate of 1 Hz for a scan size of 1.64 × 1.64 μm^2. The pore size was manually estimated from the SEM and AFM images.

The compression moduli of rehydrated BC samples were performed using the contact-mechanics/indentation method. The linear equation (Eq. (1)) of Hertz theory was applicable to obtain moduli of the samples.

$$a^3 = \frac{RP}{K} \tag{1}$$

where a is the contact radius between a sample and an indenter, R is a radius of curvature of the indenter, P is an applied load, and K is a function of the Young's modulus (E) and the Poisson ratio (ν):

$$K = \frac{3}{4}\left(\frac{1-\nu_s^2}{E_s} + \frac{1-\nu_i^2}{E_i}\right)^{-1} \tag{2}$$

where ν_s and ν_i represent the Poisson ratios of the sample and the indenter, respectively, and E_s and E_i represent the Young's moduli of the sample and the indenter, respectively. The samples were immersed in deionized water (DI water) and autoclaved. The octadecyltrichlorosilane (OTS, Gelest) modified glass indenter with a radius of curvature of 1.17 mm was used. The modification was achieved by soaking the indenter in the OTS solution for 20 min. After modification, the indenter was thoroughly rinsed with hexane, blow-dried with a stream of nitrogen, and was then held using a translating stage equipped with a micro-manipulator. The micro-manipulator brought the indenter down slowly to make contact with a prepared sample, which was placed on a rigid glass support on the analytical balance. The applied load at the point of contact was recorded. Then, a series of applied load (P) and indenter displacement (δ) was recorded as the indenter traveled vertically from a zero load to a load of ~2 mN. The displacements were taken using a Navitar zoom 6.5× 6000 lens and with Win TV software (Version 7.0.30237, Hauppauge Computer Works). The images were analyzed to obtain the displacements using Image J (Version 1.43t, National Institute of Health). The contact radius was calculated from displacement–contact radius relationship as follows:

$$\delta = \frac{a^2}{R}. \tag{3}$$

The water evaporation rate was also measured and it was found to be very small (~2 mg/min over 3 min, the duration of each experiment) as compared to the load applied between −82 mg to 200 mg.

2.4. In vitro cell differentiation study

5×10^4 rMSCs in 20 μl of growth medium were seeded on a BC sample and tissue culture plastic to control the culture area to the size of the BC samples (~8 × 8 mm^2). The cells were allowed to attach for 30 min

Table 2
Summary of mechanical properties of rehydrated BC samples. Values are the means ± SD of three independent indentation experiments.

Sample	E (kPa)
Rehydrate air dried BC	90.1 ± 21.1
Rehydrate freeze dried BC	21.1 ± 0.2

Table 1
Physical properties of the ambient-air dried or freeze dried BC samples. Results are the means ± standard derivation (SD) of at least four specimens.

Sample	Dry weight (mg)	Dry thickness (mm)	Dry porosity (%)	Water uptake (%)	Rehydrated thickness (mm)	Rehydrate porosity (%)
Air dried BC	4.9 ± 0.3	0.12 ± 0.04	68.1 ± 15.6	472 ± 14	0.20 ± 0.03	77.7 ± 9.5
Freeze dried BC	4.1 ± 0.5	4.73 ± 0.25	98.3 ± 9.2	2984 ± 350	4.88 ± 0.14	99.0 ± 5.0