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Research Paper

Gelatin-bacterial cellulose composite sponges thermally cross-linked with glucose for tissue engineering applications

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ABSTRACT

Freeze-drying and thermal cross-linking techniques were used to prepare gelatin-bacterial cellulose (GB) composite sponges for potential application as scaffolds in tissue engineering. To avoid the use of toxic and costly cross-linking agents, glucose was used to cross-link the gelatin via the Maillard reaction. The effects of the weight ratio of gelatin to bacterial cellulose (BC) and the cross-linking conditions (temperature and duration) on the GB sponges were examined. An open and highly interconnected porous structure was attained for the GB sponge with a gelatin:BC weight ratio of 25:75 that was cross-linked at 140 °C for 3 h. Its high porosity, good swelling properties, good structural stability in water, non-toxicity and good biocompatibility against Vero cell are promising for its application as a scaffold for tissue engineering.

1. Introduction

Gelatin has been widely used in many fields, especially for biomedical applications such as controlled drug-delivery vehicles (Ooi, Ahmad, & Amin, 2016) and scaffolds for bone (Sharma, Dinda, Potdar, Chou, & Mishra, 2016), skin (Ramana Ramya, Thanigai Arul, Sathiamurthi, Asokan, & Narayana Kalkura, 2016), and cartilage (Balakrishnan, Joshi, Jayakrishnan, & Banerjee, 2014). Gelatin mimics the extracellular matrix and is biodegradable, non-immunogenic, and inexpensive (Xing et al., 2010). However, it must be supplemented with other substances to eliminate its drawbacks or to add other functional properties. For example, gelatin is brittle and exhibits reduced flexibility in the dry state; however, its mechanical strength can be improved through reinforcement with different types of cellulose, such as cellulose microfiber (Xing et al., 2010) and dialdehyde carbonylmethyl cellulose (Guo et al., 2013; Li, Ye, Li, Li, & Mu, 2016). Recently, to promote wound healing and antibacterial properties, chitosan (Fan, Yang, Yang, Peng, & Hu, 2016), aloe vera, and curcumin (Tummalapalli et al., 2016) were loaded into gelatin matrices.

Meanwhile, bacterial cellulose (BC), a pure fine-fibril cellulose synthesized using *A. xylinum*, exhibits high mechanical strength, high water adsorption capacity (40–60 times (Phisalaphong & Jatupaiboon, 2008; Saibuatong & Phisalaphong, 2010)), non-toxicity, and biocompatibility (Sulaeva, Henniges, Rosenau, & Potthast, 2015). Nonetheless, BC is not biodegradable in the human body and exhibits low bioactivity (Wang, Wan, Luo, Gao, & Huang, 2012), which have limited its applications in tissue engineering. In addition, the fabrication of BC into desired shapes requires complex techniques or equipment. Homogenized BC is an alternative form of this cellulose that overcomes these restrictions. Chiaoprakobkij, Sanchavanakit, Subbalekha, Pavasant, and Phisalaphong (2011) successfully used the homogenized form of BC to fabricate a BC/alginate composite sponge using the lyophilization technique; the interior of the sponge consisted of a three-dimensional network of nanofibrils and interconnected porous structure.

To benefit from advantages of both gelatin and BC and overcome their disadvantages, gelatin-BC (GB) composites have recently been fabricated. Wang et al. (2011) were the first researchers to synthesize a GB composite film using the impregnation method. They observed that the amount of gelatin adsorbing in/on the BC pellicle was limited. In addition, Taokaew, Seetabhawang, Siripong, and Phisalaphong (2013) used a biosynthesis technique to synthesize a GB composite by supplementing the BC culture medium with gelatin solution. The GB composite was synthesized by bacteria; however, the fiber qualities were affected if a high concentration of gelatin (≥5% w/v) was added because of the increase of the viscosity of the medium, which led to a decrease of oxygen transfer into the medium.

One issue with the use of gelatin is that it is very sensitive to

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temperature change; it easily dissolves in warm water (temperature > 40 °C) and spontaneously forms a hydrogel at room temperature. The cross-linking technique has been used to enhance the structural stability of gelatin hydrogel. Glutaraldehyde, a chemical cross-linking agent, is commonly used to cross-link gelatin because it is inexpensive and easy to handle; however, it is toxic to cell culture (Bigi, Cojazzi, Panzavolta, Rubini, & Roveri, 2001). For gelatin applied in the biomedical field and in tissue engineering, natural extracts, such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and genipin, have been extensively used as cross-linking agents (Chang, Chen, Lin, & Chen, 2012; Fu, Sheu, Chen, Chen, & Yao, 2015; Li, Guo, Wei, MacDiarmid, & Lelkes, 2006); however, they are expensive. Glucose is an alternative to these toxic and expensive cross-linking agents for cross-linking gelatin via the Maillard reaction, which initiates with the condensation between the carbonyl group of the reducing sugar and the amine group in the amino acid of gelatin (Zhang, Ames, Smith, Baynes, & Metz, 2009).

In this study, gelatin-homogenized BC was lyophilized and thermally cross-linked with glucose to obtain a 3D interconnected porous structure with good mechanical strength and high biocompatibility. The effect of the gelatin to BC ratio on the chemical and physical properties was investigated. In addition, the optimal cross-linking conditions (temperature and duration) were also determined. Finally, the cytotoxicity and biocompatibility of the composite sponges were evaluated to determine their potential for application as scaffolds in tissue engineering.

2. Material and methods

2.1. Chemicals and BC

Gelatin (300 bloom type A) was purchased from Sigma-Aldish, USA. D-Glucose anhydrous was supplied from Merck Millipore, USA. BC pellicles (size 1 cm × 1 cm × 1 cm), which were synthesized by *A. xylinum* AGR 60, were kindly provided by Pramote Thammarad from the Institute of Research and Development of Food Product, Kasetsart University, Bangkok, Thailand. The BC pellicles were purified with 1 wt.% NaOH and washed with DI water until they were neutral.

2.2. Fabrication of GB composite sponges

A 15 wt.% gelatin solution was prepared by dissolving gelatin powder in deionized (DI) water and stirring at 60 °C. A 15 wt.% glucose solution was then added to the gelatin solution as a cross-linking agent and was continually stirred for 30 min. The purified BC pellicles were homogenized using a blender. Then, the homogenized BC was added into the gelatin-glucose solution in the weight ratios and was stirred for another 30 min. Afterwards, 50 g of the mixture was poured into a plastic box and was then rapidly frozen at −20 °C in a freezer (SF-PC697, Panasonic, Thailand) for at least 24 h. The frozen mixture was then lyophilized at −40 °C under vacuum condition (0.01 mbar) in a freeze dryer (Labconco, USA) for 48 h to obtain the GB composite sponge. The composite sponge was treated in an oven (UN 110, Memmert, Germany) at various cross-linking temperatures (100 °C, 120 °C, and 140 °C) and times (1.5 and 3 h) to initiate the condensation reaction between amino acids and glucose via the Maillard reaction. Each cross-linked composite sponge was labeled using the notation sponge code-temperature(time). For example, GB75-140(3) represents the composite sponge consisted of gelatin:BC weight ratio of 25:75 and cross-linked at 140 °C for 3 h.

2.3. Sponge characterization

2.3.1. Fourier transform infrared spectroscopy (FTIR)

FTIR spectra of GB sponges was recorded using a Nicolet 6700 analytical FTIR spectrometer (Thermo Scientific Inc., MA, USA). The sponge was characterized in the wavelength range of 4000–400 cm^{-1} at a resolution of 2 cm^{-1}.

2.3.2. Swelling ability

Sponge (1 × 1 cm^2) was examined its swelling ability or water adsorption capacity. The sponge was initially weighted (W_i) and then was submersed in DI water at room temperature (30 ± 1 °C) until it was equilibrated. After that, the swollen sample was then removed from the water and excess water at the surface of the samples was blotted out and it was weighted (W_w). The procedure was repeated until there was no further weight change. The swelling was calculated according to Eq. (1). The presented value was the average value determined from at least five specimens.

$$Swelling\ (time) = (W_w - W_i)/W_i \quad (1)$$

2.3.3. Porosity

The porosity of the sponge was determined by the liquid displacement method. Hexane (99.7%, J.T. Baker, USA) was used as displacement liquid because it could penetrate easily into the pores and did not induce shrinkage or swelling effect. Density of hexane (ρ_h) at 30 °C is 0.663 g/cm^3. Firstly, the sponge sample of 1 × 1 cm^2 was weighted (W_i) and its thickness was measured. Total volume (V_t) of the sponge was determined from its length, width, and thickness. Secondly, the sponge was immersed in hexane under vacuum condition to facilitate hexane transport into the pore of the sponge. After it was equilibrated, the sponge was weighted (W_h). The percentage of porosity of sponge was calculated according to Eq. (2).

$$Porosity\ (\%) = 100 \times (W_h - W_i)/(\rho_h \times V_t) \quad (2)$$

2.3.4. Weight loss

Sponge (1 × 1 cm^2) was weighted (W_i) before it was immersed in DI water for 7 days. After that, the immersed sponge was dried in the oven at 60 °C for 24 h. The dried sponge was re-weighted (W_f). Percent weight loss was calculated using Eq. (3). The weight loss was reported as the average value determined from at least five specimens.

$$Weight\ loss\ (\%) = 100 \times (W_i - W_f)/W_i \quad (3)$$

2.3.5. Color

Degree of cross-linking was examined by color measurement. Due to the Maillard reaction, a reddish-brownish substance, melanoidin, was produced from the reaction. The color of sponge was analyzed by Colorimeter (3nh, NR200, China), which could be defined by the Commission Internationale de l'Eclairage (CIE), L^* for lightness (black-white axis), a^* for redness (red-green spectrum) and b^* for yellowness (yellow-blue spectrum). In addition, hue angle ($h°$), an angular measurement, was also computed using Eq. (4). Hue angle is starting with red at 0°, yellow at 60°, green at 120°, cyan at 180°, blue at 240° and magenta at 300°.

$$h^\circ = \tan^{-1}(b^*/a^*) \quad (4)$$

2.3.6. Morphology

Morphological examination of the sponges was determined by Scanning Electron Microscope and Energy Dispersive X-ray Spectrometer (JEOL, JSM-6610LV, Tokyo, Japan). The sponge was sputtered with gold and then was photographed. The pore size of the sponge was direct measured from SEM image (at least 50 pores) using ImageJ software.

2.3.7. Mechanical test

Sponge was cut into 10 × 100 mm^2 (width × length). The mechanical properties (tensile strength and elongation at break) were tested

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