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Agarose particle-templated porous bacterial cellulose and its application in cartilage growth in vitro

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ABSTRACT

Bacterial cellulose (BC) is a biocompatible hydrogel with a three-dimensional (3-D) structure formed by a dense network of cellulose nanofibers. A limitation of using BC for applications in tissue engineering is that the pore size of the material (~0.05–10 μm) is smaller than the dimensions of mammalian cells and prevents cells from penetrating into the material and growing into 3-D structures that mimic tissues. This paper describes a new route to porous bacterial cellulose (pBC) scaffolds by cultivating *Acetobacter xylinum* in the presence of agarose microparticles deposited on the surface of a growing BC pellicle. Monodisperse agarose microparticles with a diameter of 300–500 μm were created using a microfluidic technique, layered on growing BC pellicles and incorporated into the polymer as *A. xylinum* cells moved upward through the growing pellicle. Removing the agarose by autoclaving produced BC gels containing a continuous, interconnected network of pores with diameters ranging from 300 to 500 μm. Human P1 chondrocytes seeded on the scaffolds, replicated, invaded the 3-D porous network and distributed evenly throughout the substrate. Chondrocytes grown on pBC substrates displayed a higher viability compared to growth on the surface of unmodified BC substrates. The approach described in this paper introduces a new method for creating pBC substrates with user-defined control over the physical dimensions of the pore network, and demonstrates the application of these materials for tissue engineering.

1. Introduction

Cartilage is an essential connective tissue found throughout the human body, including the ears, nose, and joints located between bones [1]. The inability of mature cartilage to heal effectively after damage can lead to a loss of joint function [2]. Several mechanisms, based largely on transplantation, are used to replace and repair damaged cartilage following injury or disease. The most common mechanism for cartilage replacement consists of receiving allogenous grafts derived from human donors or xenografts from animals. This mechanism is favorable due to the availability of these materials; however, risks of pathogen transmission and graft rejection can complicate cartilage grafts from donors. The best clinical outcomes of cartilage replacement therapies are from autografts derived from the patients who are being treated. Limitations in the quantity, shape and size of donor cartilage place restrictions on using autografts for repairing cartilage defects [3,4]. As the estimated annual number of cartilage grafts exceeds one million [5] and outstrips available resources, several mechanisms for creating new tissues to replace damaged cartilage have been explored.

The emergence of a field centered upon cartilage tissue engineering has provided cartilage for clinical grafts that can maintain or restore tissue function. Fabricating regenerative cartilage substitutes requires three major components: scaffolds to support cell growth, cells and signaling molecules [6,7]. Scaffolds provide a substrate for mimicking the human extracellular matrix (ECM) upon which cells interact, and provide structural support for newly formed tissues. Scaffolds consist of a network of interconnected pores that provide mechanical support for cells in three dimensions, supply nutrients and growth factors to cells and promote cell invasion. Studies have demonstrated that biocompatible scaffolds with a pore size of 300–500 μm promote chondrocytes to attach to the surface, spread and proliferate [8].

Bacterial cellulose (BC) is an exopolysaccharide secreted by *Acetobacter xylinum* that forms a hydrogel and has characteristics that make it promising for biomaterials applications, including

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high tensile strength, high purity, formation of a three-dimensional (3-D) nanofibril network and biocompatibility [9,10]. To date, BC has been used as a scaffold for growing tissues involved in skin replacement, blood vessel grafts and meniscus substitutes [11–13]. An intrinsic limitation of BC for tissue engineering is the ~0.05 to 10 μm pore size of the fibril network, which is poorly matched to the physical dimensions of mammalian cells, and limits cell penetration and migration during cultivation. Several approaches have been used to address this limitation, including the incorporation of paraffin wax particles as a porogen into BC during the fermentation of *A. xylinum* [14–16]. Paraffin wax particles are introduced into the BC scaffold during growth and removed later. Some challenges associated with this technique include controlling the size of pores due to the diameter and polydispersity of available wax particles, removing wax from the BC matrix to reveal the porous scaffold and controlling the size and shape of BC layers.

A. xylinum is a strict aerobe and only produces cellulose in aerobic environments [17]. Previous studies have shown that during the formation of a cellulose pellicle in a liquid culture under static conditions, cells move upward through the BC pellicle to the surface of the polysaccharide. The mechanism that guides the upward motion of cells is currently unknown [18]; however, it seems reasonable that it may be due to cells chemotaxing up a gradient in oxygen.

In this paper, we describe the fabrication of porous bacterial cellulose (pBC) scaffolds for in vitro cell culture by cultivating *A. xylinum* in the presence of agarose microparticles deposited on the surface of a growing BC pellicle. The percolating, upward growth and movement of *A. xylinum* cells through the pellicle to the moist, nutrient-rich surface guides BC formation around the agarose porogen particles. By controlling the physical dimensions and monodispersity of the agarose microparticles using a microfluidic system, we demonstrate that the removal of cells followed by autoclaving to sterilize the polymer and melt the porogen reveals pBC layers that contain a uniform and interconnected pore structure which facilitates mammalian cell growth in three dimensions. To demonstrate the function of BC materials fabricated using this simple approach, we grew human P1 chondrocytes on pBC substrates and analyzed their viability, morphology, attachment and 3-D distribution in pBC scaffolds after 1, 7 and 14 days of growth.

2. Materials and methods

2.1. Preparation of agarose microparticles using microfluidics

Monodisperse agarose microparticles with a diameter ranging from 300 to 500 μm were prepared using a controlled emulsion technique [19]. As illustrated in Fig. 1A, mineral oil was dispensed from a 50 ml syringe connected to poly(vinyl chloride) (PVC) tubing (inner diameter (ID): 0.035 inches; outer diameter (OD): 0.103 inches). A 25 ml syringe containing a warm solution (80 °C) of agarose (2% w/v) was connected to a 30-gauge needle that was inserted through the wall of the PVC tubing. The tip of the needle was positioned at the center of the PVC tubing. The syringes were connected to Harvard syringe pumps (PHD 22/2000) and the flow rates of mineral oil and warm agarose were controlled by adjusting the speed of the pumps; typical rates of flow varied between 1 and 5 ml min^{-1}. As warm agarose exits through the needle into the flowing mineral oil, droplets are sheared off, flow through the PVC tubing and collect in a beaker immersed in an ice bath where the agarose droplets gel. Agarose microparticles were collected, rinsed with water to remove the mineral oil completely and lyophilized.

2.2. Characterization of the size and morphology of agarose microparticles using optical microscopy

To measure the size and morphology of agarose microparticles, we used optical microscopy. Lyophilized agarose microparticles were hydrated by suspending them in water for 3 h or 3 days, a

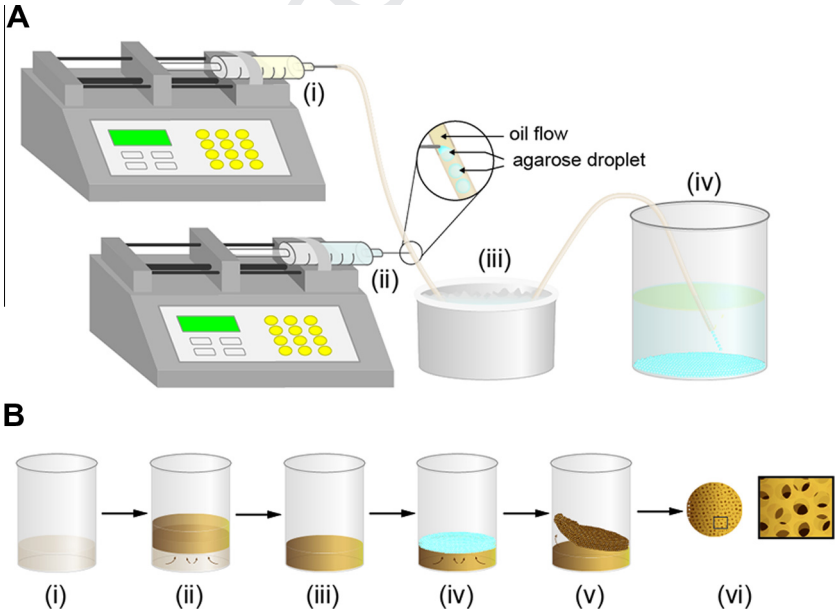

Fig. 1. Schematic illustration depicting the preparation of agarose microparticles and pBC scaffolds. (A) (i) Syringe pump with a 50 ml syringe connected to PVC tubing dispenses mineral oil. (ii) Syringe pump with a 25 ml syringe connected to a 30-gauge needle is inserted through the wall of the PVC tubing and injects a warm solution of agarose that is broken off into droplets suspended in the mineral oil. (iii) Ice bath for gelling agarose droplets into microparticles. (iv) 500 ml beaker containing 250 ml water collects agarose microparticles. (B) (i) A culture of *A. xylinum* incubated in 50 ml or 250 ml beakers containing H&S broth. (ii) BC pellicle forms at the air–liquid interface after 5 days of growth. (iii) Excess H&S liquid broth is removed. (iv) Agarose microparticles are spread on the surface of the BC pellicle. (v) After 2 days of incubation, the pBC layer is peeled off the BC pellicle. (vi) Removing the agarose poragens reveals the pBC scaffolds. Left image is a top-down view of a pBC substrate. Right image is a cartoon of a pBC substrate at high magnification.

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