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Porous biocompatible three-dimensional scaffolds of cellulose microfiber/gelatin composites for cell culture

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ABSTRACT

Physiological tissues, including brain and other organs, have three-dimensional (3-D) aspects that need to be supported to model them in vitro. Here we report the use of cellulose microfibers combined with cross-linked gelatin to make biocompatible porous microscaffolds for the sustained growth of brain cell and human mesenchymal stem cells (hMSCs) in 3-D structure. Live imaging using confocal microscopy indicated that 3-D microscaffolds composed of gelatin or cellulose fiber/gelatin both supported brain cell adhesion and growth for 16 days in vitro. Cellulose microfiber/gelatin composites containing up to 75% cellulose fibers can withstand a higher mechanical load than gelatin alone, and composites also provided linear pathways along which brain cells could grow compared to more clumped cell growth in gelatin alone. Therefore, the bulk cellulose microfiber provides a novel skeleton in this new scaffold material. Cellulose fiber/gelatin scaffold supported hMSCs growth and extracellular matrix formation. hMSCs osteogenic and adipogenic assays indicated that hMSCs cultured in cellulose fiber/gelatin composite preserved the multilineage differentiation potential. As natural, biocompatible components, the combination of gelatin and cellulose microfibers, fabricated into 3-D matrices, may therefore provide optimal porosity and tensile strength for long-term maintenance and observation of cells.

1. Introduction

Cellulose fibers are natural material abundantly present in many plants and bacteria. Cellulose microfibers have been widely applied in many areas, like the paper industry and textile industry. Cellulose is also a very promising material for cell culture and biomedical applications. These positive properties include biocompatibility, reactive surfaces for protein binding, mechanical strength and resistance to breakdown in vivo. Specific examples of these positive aspects are given below.

First, the major composition of cellulose fiber has been proven to be biocompatible for both granulation tissue and bone formation [1,2]. Furthermore, the regenerated cellulose scaffold can promote cardiac cell growth and enhance cell connectivity and electrical functionality [3]. Calcium phosphate-coated cellulose fibers were employed to provide a favorable environment for the development of cartilaginous tissue [4]. Cellulose fiber has a high density of reactive hydroxyl groups on its surface which facilitates the immobilization of cell adhesive proteins such as fibronectin [5].

Secondly, the densely packed glucan chain structure in cellulose fibers gives them sufficient mechanical strength to support cell aggregate structures [6]. Regenerated cellulose was also proved to be very stable under dynamic stress [7].

Thirdly, cellulose does not have good degradability in vivo [3], though it is biodegradable by hydrolysis with cellulase, the final product being glucose. Therefore, the cellulose fibers could potentially be removed from a cell culture construct when no longer needed, as has been described for other systems [6].

However, there have been very few studies into the application of cellulose microfibers as a scaffold for cell culturing due to the absence of an intrinsic three-dimensional (3-D) architecture. Use of gelatin may provide additional 3-D architecture for cellulose-based cell scaffolds. Gelatin is a derivative of collagen and is biodegradable, inexpensive and nonimmunogenic. It has been shown that gelatin-based scaffolds have wide applications in different areas of tissue engineering [8–10]. Here we elaborate the use of 3-D composites based on cellulose microfibers connected by gelatin as bioglue for cell culture. The morphology and structural characteristics were observed by scanning electron microscopy. The biocompatibility of the scaffolds was tested by culturing brain tumor cells (BTCs) and human mesenchymal stem cells (hMSCs) in vitro. To our knowledge, this is the first report of cellulose–gelatin composites for three-dimensional cell scaffolds.

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2. Experimental

2.1. Materials

Beaten bleached Kraft hardwood fiber sheets, supplied by International Paper Company, (Bastrop, LA), were dispersed in water to obtain cellulose fibers. These fibers are of 1–2 mm in length and 10–30 μm in diameter. All other chemicals were bought from Sigma–Aldrich or Invitrogen and used without further purification.

2.2. Scaffold fabrication and characterization

The 3-D microscaffolds were prepared by solid–liquid phase separation and subsequent sublimation of the solvent [11]. Briefly, 1 wt.% gelatin solution was prepared by dissolving gelatin B (M_W 20,000–25,000; Sigma–Aldrich) powder in deionized H_2O heated at 50 °C. A controlled amount of 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) were added to crosslink the gelatin. The final concentration of EDC and NHS was 5 mM at a molar ratio of 1:1 [12]. Different amounts of dried cellulose fibers were mixed with the above gelatin solutions and gently rotated for even distribution. The mixture was put in ice bath to initiate gelation. After being kept in 4 °C refrigerator overnight, the resulting gel was placed at −20 °C to freeze. The frozen samples were then lyophilized in a freeze-dryer for at least 24 h. Samples were cut into thin slices, and a scanning electron microscope (AMRAY, Model 1830) was used to characterize the morphology of the scaffolds. Pore size and porosity was determined by Autopore II mercury intrusion porosimeter.

2.3. Mechanical testing

The mechanical property (tensile strength) of scaffolds was tested with an eXpert 2611 twin screw electromechanical materials testing machine (ADMET, 10KN). The specimens tested were rectangular, with a length of 7 mm and thickness of 1.5 mm. The gage length was set at 5 mm. Young's modulus, peak stress and break position were recorded at the maximum load.

2.4. Swelling ratio

The swelling ratio, or water sorption capacity, was measured by soaking the sample in 0.01 M phosphate-buffered saline (PBS) solution at room temperature for 24 h. The samples were weighed initially (W_0) and then, after swelling, were wiped with filter paper to remove excess water and weighed (W_{24}) again. The swelling ratio (S) was calculated from the following equation:

$$S = \frac{W_{24} - W_0}{W_0}$$

We did not directly measure the time needed for each scaffold to reach swelling equilibrium.

2.5. Protein adsorption characterization

Samples of 3-D cellulose fiber/gelatin scaffolds (with 75 wt.% fibers) and pure gelatin scaffolds were weighed and put into complete medium (Dulbecco's modified Eagle's medium from the American Type Culture Collection (ATCC)) containing 10% fetal bovine serum (FBS; Lonza Group Ltd., Switzerland) and 1% penicillin/streptomycin (Sigma) at 37 °C for a specific time period. The samples were taken out and washed repeatedly in PBS, then put into a freezer overnight and freeze-dried in the lyophilizer for 24 h. The samples were weighed again and the weight changes were calculated as average percentage increases of three samples. The medium was changed every 3 days.

2.6. Brain cancer cell seeding and culture

Brain cancer cells CRL-2020 were obtained from ATCC and grown in the recommended medium: RPMI 1640 plus 10% FBS. Both gelatin and cellulose fiber/gelatin microscaffolds (with 75 wt.% fibers) were cut into thin slices about 1.5 mm thick and sterilized using 70% ethanol followed by three washes with PBS. A 1 ml cell suspension with 2×10^6 cells was seeded onto the matrix soaked in 1 ml of culture medium in a well of 24-well tissue culture plate. After seeding, the medium was changed every other day and the cultures were maintained for 16 days at 37 °C under 5% CO_2.

2.7. Calcein staining

After 16 days in culture, the brain cancer cells were stained using a fluorescent dye (calcein AM). Briefly, 5 μl of pluronic acid at a concentration of 20% (w/v) in DMSO and 10 μl of a stock solution of calcein AM at a concentration of 1 mM were added into 5 ml of pre-warmed Locke's solution. The cells were incubated with the pre-warmed solution for 25 min and imaged using a LEICA DM IRE2 confocal laser scanning microscope or Nikon epifluorescence microscope, as indicated.

2.8. MTT assay

Cellular metabolism/viability was assayed by using methylthiazolyldiphenyl tetrazolium bromide (MTT), obtained from Sigma (Product # M5655). To assay cellular metabolism/viability levels, scaffold samples or wells with cells were loaded with 1 ml of MTT (1.25 mg ml^{-1} stock solution in Locke's) for 60 min at 37 °C under 5% CO_2, then formazan product crystals were dissolved in 500 μl of 91% isopropanol, and absorbance at 595 nm of 200 μl of dissolved formazan product was measured using a Thermo Scientific-Multiskan Spectrum plate reader. When the resulting color product approached absorbance of 2.5 or greater, samples were diluted with 91% isopropanol and readings taken to avoid going above threshold limits for readings from the instrument.

2.9. Human mesenchymal stem cell seeding and culture

hMSCs were obtained from the bone marrow of health donors in a method described previously [8]. The collected cells were expanded using α-minimum essential medium (α-MEM) with 20% FBS at 37 °C under 5% CO_2. The sixth passage cells were seeded onto each scaffold in a 24-well plate using 1 ml of cell suspension containing about 2.5×10^5 cells. The cell culture medium was changed every other day and maintained for up to 28 days at 37 °C under 5% CO_2. The cellulose microfiber/gelatin scaffold containing 75 wt.% fibers was used for all the hMSCs experiments.

2.10. Immunocytochemistry staining

Cytoskeleton protein F-actin and the extracellular matrix (ECM) proteins fibronectin and collagen IV were examined using immunocytochemistry staining. The hMSCs grown in a 3-D scaffold were fixed using 0.3% glutaraldehyde solution, permeated with 1.0% Triton X-100 and blocked with 10% FBS. The cells were then incubated with anti-fibronectin or collagen IV primary antibodies for 1 h at 37 °C, followed by a mixture of fluorescein isothiocyanate (FITC)-conjugated secondary antibody and phalloidin conjugated to Alexa Fluor 594 (for F-actin staining) for 1 h at 37 °C. Before imaging, the samples were mounted using Vectashield with 4′,6-diamidino-2-