Colloids and Surfaces B: Biointerfaces 130 (2015) 222–228

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Effect of negatively charged cellulose nanofibers on the dispersion of hydroxyapatite nanoparticles for scaffolds in bone tissue engineering

Minsung Park[a], Dajung Lee[a], Sungchul Shin[a], Jinho Hyun[a,b,c,*]

[a] Department of Biosystems and Biomaterials Science and Engineering, Seoul National University, Seoul 151-921, Republic of Korea
[b] Research Institute of Agriculture and Life Sciences, Seoul National University, Seoul 151-921, Republic of Korea
[c] Center for Food and Bioconvergence, Seoul National University, Seoul 151-921, Republic of Korea

ARTICLE INFO

Article history:
Received 19 January 2015
Received in revised form 6 April 2015
Accepted 7 April 2015
Available online 15 April 2015

Keywords:
Colloidal stability
Dispersant
Hydroxyapatite
Bacterial cellulose
Scaffold

ABSTRACT

Nanofibrous 2,2,6,6-tetramethylpiperidine-1-oxyl(TEMPO)-oxidized bacterial cellulose (TOBC) was used as a dispersant of hydroxyapatite (HA) nanoparticles in aqueous solution. The surfaces of TOBC nanofibers were negatively charged after the reaction with the TEMPO/NaBr/NaClO system at pH 10 and room temperature. HA nanoparticles were simply adsorbed on the TOBC nanofibers (HA–TOBC) and dispersed well in DI water. The well-dispersed HA–TOBC colloidal solution formed a hydrogel after the addition of gelatin, followed by crosslinking with glutaraldehyde (HA–TOBC–Gel). The chemical modification of the fiber surfaces and the colloidal stability of the dispersion solution confirmed TOBC as a promising HA dispersant. Both the Young's modulus and maximum tensile stress increased as the amount of gelatin increased due to the increased crosslinking of gelatin. In addition, the well-dispersed HA produced a denser scaffold structure resulting in the increase of the Young's modulus and maximum tensile stress. The well-developed porous structures of the HA–TOBC–Gel composites were incubated with Calvarial osteoblasts. The HA–TOBC–Gel significantly improved cell proliferation as well as cell differentiation confirming the material as a potential candidate for use in bone tissue engineering scaffolds.

1. Introduction

Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)$, HA) is the natural inorganic component of hard tissues and has been used extensively for bone repair, fillers, and substitution due to its excellent biocompatibility and bioactivity. In spite of the similarity of HA to the components of real bone, HA nanoparticles are not easily dispersed; instead, they easily aggregate to form precipitates due to the inherent unstable colloidal nature of HA that makes it difficult to form a controlled structure [1–3]. Because a solution process is usually required to fabricate bone tissue scaffolds, the colloidal stability of HA nanoparticles in solution is critical to obtain a uniform structure and desirable bulk properties [4–6]. In addition, HA-based composites crack under stress because of the brittle nature of HA [7]. To reinforce HA-based composites, stronger and more flexible constituents, such as natural nanofibers, are needed.

Bacterial cellulose (BC) nanofibers were chosen as a material to overcome the shortcomings of HA-based scaffolds due to their biocompatibility, potential to be used as a scaffold [8–11], and fibrous structure, with fiber diameters of several tens of nanometers, which is similar to that of collagen fibers [12,13]. 2,6,6-Tetramethylpiperidine-1-oxyl (TEMPO) radical oxidation is an easy method to obtain well-dispersed cellulose fibers without significant aggregation through the electrostatic repulsion of nanofibers [14], and oxidized cellulose nanofibers can effectively disperse nanomaterials [15,16].

In this study, we attempted to design a scaffold for bone tissue using nanofibrous TEMPO-oxidized BC (TOBC) as a structural framework and natural dispersant of HA nanoparticles. The colloidal stability of HA with TOBC and the mechanical properties of the scaffold were investigated. In addition, the unique properties of the composites, including physicochemical effects, viability, and differentiation of bone generating cells, were described.

2. Experimental

2.1. Materials

Gluconacetobacter xylinus (KCCM 40216) was obtained from the Korean Culture Center of Microorganisms. Calvarial osteoblasts isolated from the calvaria of neonatal (less than 1 day

* Corresponding author at: Department of Biosystems and Biomaterials Science and Engineering, Seoul National University, Seoul 151-921, Republic of Korea. Tel.: +82 28804624.
E-mail address: jhyun@snu.ac.kr (J. Hyun).

http://dx.doi.org/10.1016/j.colsurfb.2015.04.014
0927-7765/

old) Sprague–Dawley rats (SLC, Tokyo, Japan) via a digestive enzymatic process were provided from the lab of Prof. Byung-Soo Kim (Seoul National University, Seoul, Korea). Sodium hydroxide (≥98%) and D-mannitol (99%) were obtained from Samchun Chemicals (Korea). Yeast extract and bacto-peptone were purchased from BD (USA). 2,2,6,6-Tetramethylpiperidine-1-oxyl (TEMPO), NaBr, and NaClO were purchased from Sigma–Aldrich (USA). Hydroxyapatite (diameter >200 nm) nanoparticles, gelatin, and glutaraldehyde were purchased from Sigma–Aldrich. Phosphate buffered saline (PBS), Dulbecco's modified Eagle medium (DMEM), and fetal bovine serum (FBS) were purchased from Gibco (USA). Methylthiazolyldiphenyl tetrazolium bromide (MTT), ascorbic acid, β-glycerol phosphate, dexamethasone, Alizarin Red S, and cetylpyridinium chloride were purchased from Sigma–Aldrich. An ALP assay kit was purchased from Takara Bio (Japan).

2.2. Biosynthesis and purification of BC

The bacteria were cultured on mannitol media composed of 2.5% (w/w) mannitol, 0.5% (w/w) yeast extract, and 0.3% (w/w) bacto-peptone. Bacteria were introduced to Petri dishes containing culture medium at 28 °C for 5 days. After incubation, the BC membrane was harvested and purified with 1 wt% NaOH followed by washing with distilled water (DI water). The membrane was stored in DI water prior to use.

2.3. TEMPO-oxidation of BC

To obtain TEMPO-oxidized BC, hydrogels (20 g wetting weight) were cut into small pieces and then suspended in 500 ml of distilled water containing 20 mg of TEMPO and 0.5 g of NaBr. Subsequently, 15 ml of NaClO solution was added to the BC suspension to initiate oxidation while maintaining the system at pH 10 with NaOH. The mixture was vigorously agitated using a magnetic stirrer for 2 days at room temperature. The oxidation was quenched by adding ethanol to the suspension at the end of the reaction. The products were washed with DI water, collected by centrifugation (3×), and dialyzed with running DI water.

2.4. Preparation of HA–TOBC dispersions in an aqueous solution

HA nanoparticles (Sigma–Aldrich, diameter >200 nm) were dispersed in DI water using TOBC as a dispersant. HA nanoparticles (2.5, 5, and 10 mg) were dispersed in 10 ml of a TOBC (dry weight: 5 mg) suspension in DI water. The weight ratios of HA nanoparticles to TOBC were 1:2, 1:1, and 2:1. As a control, 5 mg of HA nanoparticles were dispersed in 10 ml of DI water without TOBC. All of the samples were contained in glass vials and were sonicated for 1 min and then stored at room temperature for 1 day.

2.5. Preparation of HA–TOBC-based scaffolds incorporating gelatin

Sixty milligrams of HA nanoparticles were dispersed in 12 ml of DI water containing 60 mg of TOBC under sonication. Subsequently, 30 or 60 mg of gelatin was added to the HA–TOBC suspension solution and stirred at 50 °C for 1 h. Next, 3 ml of the gelatin and HA–TOBC mixture was dropped into Petri dishes and cooled at 4 °C overnight. The gel form of the mixture was transferred to a freezer at −25 °C to solidify. After the cross-linking of the solidified gel mixture using 2.5% glutaraldehyde, the sample was freeze-dried for 48 h. A control sample without HA nanoparticles was prepared using the same method. Solidified HA–TOBC without gelatin was referred to as HA–TOBC–0Gel, samples containing a gelatin-to-TOBC of 0.5:1 (by weight) were referred to as TOBC–0.5Gel or HA–TOBC–0.5Gel, and samples containing a 1:1 ratio were referred to as TOBC–1Gel or HA–TOBC–1Gel.

2.6. Characterization of the composites

The chemical structures of BC and TOBC were characterized by Fourier transform infrared spectroscopy (FT-IR spectroscopy; Nicolet iS5, Thermo Scientific, USA) using 32 scans at a resolution of 8 cm^{-1} over the wavenumber range of 4000–600 cm^{-1}.

The morphologies of the samples were observed using field emission scanning electron microscopy (FE-SEM; SUPRA 55VP, Carl Zeiss, Germany) at an acceleration voltage of 2 kV. Transmission electron microscopy (TEM) images of HA and HA–TOBC dispersions were obtained using energy-filtering transmission electron microscopy (EF-TEM, LIBRA 120, Carl Zeiss, Germany) at an acceleration voltage of 200 kV.

The zeta potentials of HA, TOBC, and HA–TOBC dispersions were measured by dynamic light scattering (Zetasizer Nano, Malvern, UK). Samples were dispersed in DI water, and measurements were carried out at 20 °C with folded capillary cells. Three runs were performed and averaged for each sample.

The mechanical properties (tensile strength) of the samples were investigated using a universal testing machine (UTM; GB/LRX Plus, Lloyd, UK) fitted with a 500-N load cell at room temperature. The test specimens were rectangular, with a gauge length of 10 mm, a width of 5 mm, and a thickness of 0.5 mm. The Young's modulus was recorded at the maximum load.

The swelling ratio was measured by soaking the sample in a PBS solution at room temperature for 24 h. The samples were initially weighed (W_0). After soaking, excess water was removed from the sample surface with filter paper and the samples were reweighed (W). The swelling ratio (S) was calculated from the following equation: $S = (W - W_0)/W_0$.

2.7. Cell seeding on scaffolds

Calvarial osteoblasts that were isolated from the calvaria of neonatal (less than 1 day old) Sprague-Dawley rats (SLC, Tokyo, Japan) via a digestive enzymatic process were provided by the lab of Prof. Byung-Soo Kim (Seoul National University, Seoul, Korea). Cells were cultured in DMEM (high glucose) supplemented with 10% (V/V) FBS, 100 mM ascorbic acid, 10 mM β-glycerol phosphate, 100 nM dexamethasone, and 100 U/ml penicillin in a humidified 5% CO_2 incubator at 37 °C. To seed the cells on scaffolds, each scaffold was cut into circular discs (~15 mm in diameter), which were then sterilized in 70% ethanol for 30 min. The specimens were then placed in a 96-well plate and washed several times with DI water. Subsequently, the specimens were immersed in the incubation medium for 1 h. Osteoblasts from the cultures were trypsinized, and 1×10^4 cells were seeded on the sterilized scaffold specimens in 96-well plates. The medium was changed every other day, and the cultures were maintained at 37 °C.

2.8. Evaluation of cellular viability and proliferation

Cellular viability and proliferation were assayed using MTT. The medium was removed from the cell-culturing well plates, which were then washed with PBS. Scaffold samples or wells with cells were loaded with 20 μl of MTT (5 mg/ml in PBS) and 100 μl of medium for 2 min at 37 °C under 5% CO_2. The produced formazan crystals were dissolved in 100 μl of DMSO, and the absorbance of the suspension was measured at 570 nm using a plate reader. The empty wells of a tissue culture polystyrene (TCPS) plate were used as a positive control.