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Laser-structured bacterial nanocellulose hydrogels support ingrowth and differentiation of chondrocytes and show potential as cartilage implants

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ABSTRACT

The small size and heterogeneity of the pores in bacterial nanocellulose (BNC) hydrogels limit the ingrowth of cells and their use as tissue-engineered implant materials. The use of placeholders during BNC biosynthesis or post-processing steps such as (touch-free) laser perforation can overcome this limitation. Since three-dimensionally arranged channels may be required for homogeneous and functional seeding, three-dimensional (3-D) laser perforation of never-dried BNC hydrogels was performed. Never-dried BNC hydrogels were produced in different shapes by: (i) the cultivation of *Gluconacetobacter xylinus* (DSM 14666; synonym *Komagataeibacter xylinus*) in nutrient medium; (ii) the removal of bacterial residues/media components (0.1 M NaOH; 30 min; 100 °C) and repeated washing (deionized water; pH 5.8); (iii) the unidirectional or 3-D laser perforation and cutting (pulsed CO_2 Rofin SC ×10 laser; 220 μm channel diameter); and (iv) the final autoclaving (2 M NaOH; 121 °C; 20 min) and washing (pyrogen-free water). In comparison to unmodified BNC, unidirectionally perforated – and particularly 3-D-perforated – BNC allowed ingrowth into and movement of vital bovine/human chondrocytes throughout the BNC nanofiber network. Laser perforation caused limited structural modifications (i.e. fiber or globular aggregates), but no chemical modifications, as indicated by Fourier transform infrared spectroscopy, X-ray photoelectron scattering and viability tests. Pre-cultured human chondrocytes seeding the surface/channels of laser-perforated BNC expressed cartilage-specific matrix products, indicating chondrocyte differentiation. 3-D-perforated BNC showed compressive strength comparable to that of unmodified samples. Unidirectionally or 3-D-perforated BNC shows high biocompatibility and provides short diffusion distances for nutrients and extracellular matrix components. Also, the resulting channels support migration into the BNC, matrix production and phenotypic stabilization of chondrocytes. It may thus be suitable for in vivo application, e.g. as a cartilage replacement material.

1. Introduction

The biomaterial bacterial nanocellulose (BNC) is produced by cultivation of the bacterium *Gluconacetobacter xylinus* (synonym *Komagataeibacter xylinus*) in glucose-enriched medium under aerobic conditions. In the case of static cultivation, the bacteria biosynthesize an extracellular, highly crystalline cellulose hydrogel composed of a nanofiber network, forming flat-shaped hydrogels at the surface of the culture medium. Recently, BNC has become very attractive as a biomaterial for the regeneration of soft and hard tissue. Several in vitro and in vivo studies have indicated excellent biocompatibility and biofunctional properties of this material [1–4]. Therefore, the spectrum of potential applications ranges from wound dressings and guided tissue engineering scaffolds to cell-free grafts. The material structure and shaping of BNC during biosynthesis allows a structural imitation of host body tissue. Irregular alignment of the BNC nanofibers shows structures comparable to those of collagen matrices. Compared to smooth surfaces without nanostructures, the three-dimensional (3-D)

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biomimetic network structure of BNC is known to favor adhesion and prevent de-differentiation of connective tissue cells such as chondrocytes [5]. However, BNC hydrogels cultivated under static conditions on top of the culture medium display anisotropic, layered structures with pores of different sizes. The bulk of the BNC shows a relatively homogeneous network structure with interconnected pores (diameter between 2 and 10 μm, depending on the bacterial strain and the culture conditions). In contrast, the upper surface (in contact with air) is characterized by a dense architecture with much smaller pores (below 2 μm; own preliminary results) and the lower surface (in contact with the culture liquid) partially shows pores with a much larger diameter [1,6,7]. This heterogeneity of the pores limits the ingrowth of cells into the BNC hydrogels, especially the penetration of the top layer and the bulk material [1]. In this context, some studies suggest that the cells are able to push away the BNC fibers to migrate into the network [1], whereas others indicate that the network structure may be too dense for cell ingrowth [6,8,9].

Two main solutions have been suggested to overcome this limitation: the insertion of globular pores or (unidirectional) channels by using spacers/placeholders during BNC biosynthesis [10–13] and the generation of vertical pore arrays by post-processing steps such as laser patterning techniques [9]. However, in order to allow homogeneous and functional seeding of BNC with connective tissue cells, the generation of 3-D channels may be required. In the present study, the extension of the post-processing laser perforation of never-dried BNC hydrogels from the unidirectional to the 3-D level, as well as the effects on chemical surface structure, weight loss, mechanical stability and cell seeding, are described in detail.

2. Materials and methods

2.1. Biosynthesis of BNC hydrogels with different dimensions

For the cultivation of *Gluconacetobacter xylinus* (DSM 14666; synonym *Komagataeibacter xylinus*, Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany), a nutrient medium according to Schramm and Hestrin [14] was used, containing 20 g of D-glucose, 5 g of yeast extract, 5 g of Pepton, 3.4 g of disodium hydrogen phosphate dihydrate and 1.15 g of citric acid monohydrate per 1000 ml of water. The medium was inoculated with a pre-culture of the bacteria in a volume ratio of 20:1, and the BNC for unmodified samples (Fig. 1, left column; BNC native, Table 1) was cultivated in 96-well culture plates (200 μl of inoculated medium per well) for 14 days at 28 °C. In this case, the size of the BNC hydrogels generated on top of the culture medium was 8 mm in diameter and 1.5 to 2.5 mm in height (Table 1).

In contrast, BNC hydrogels for laser structuring were cultivated in 6-well plates in two different designs: (i) in order to generate hydrogels with a diameter of 35 mm and a height of 1.5–2.5 mm, cultures were performed with 3 ml of inoculated medium for 14 days at 28 °C (Fig. 1; center column; LS-BNC1-1D, LS-BNC2-1D, LS-BNC3-1D, LS-BNC4-1D, Table 1); (ii) for hydrogels with a diameter of 35 mm and a height of 5.5–6.5 mm, cultures were performed with either 7 ml (Experiment 1; Fig. 1, right column; final BNC sample LS-BNC8-3D, Table 1) or 9 ml of inoculated medium (Experiment 2; BNC scalpel-cut, BNC laser-cut, LS-BNC5, LS-BNC6-1D and LS-BNC7-3D, Table 1) for 14 days at 28 °C. BNC scalpel-cut samples were trimmed to their final dimensions using disposable surgical scalpels.

The volume–surface ratio (i.e. 6-well plates and 3 or 7 (9) ml of medium) was used instead of the culture time to guide the thickness of the BNC hydrogels, since after 14 days the culture reaches a stable, steady-state level, characterized by a glucose level close to 0 mg ml^{-1}, a constant number of bacteria and a constant dry weight of BNC with an optimized ratio between the middle layer vs. the surface and lower layers of the final BNC [3,15].

To remove bacterial residues and media components, the BNC hydrogels were treated in all cases with 0.1 M aqueous sodium hydroxide solution for 30 min at 100 °C, repeatedly rinsed with deionized water (pH 5.8) and stored at room temperature until further use.

2.2. Laser structuring of the BNC hydrogels

A pulsed CO_2 laser system (Rofin SCx10; Rofin Laser GmbH, Hamburg, Germany; max. 100 W, 100 kHz, pulse duration 170 μs, pulse energy max. 1 mJ, λ = 10,640 nm) equipped with a scanner (custom design; Laser Design GmbH, Essen, Germany) was used to perforate and cut out BNC hydrogels with a specified shape and channel array (Fig. 1; center and right column). For all laser modification steps, never-dried BNC hydrogels were mounted on a 2.5% (w/v) aqueous agar gel.

BNC laser-cut samples were trimmed to their final dimensions using BNC hydrogels with a height of 5.5–6.5 mm, a laser power of 90 W and a feed of the laser of 6 mm s^{-1} (in order to generate the highest laser impact in any of the samples employed in the present study; Table 1). Non-perforated LS-BNC5 samples for mechanical evaluation were cut using BNC hydrogels with a height of 5.5–6.5 mm, a laser power of 90 W and a feed of the laser of 8 mm s^{-1} (Table 1).

For unidirectional laser perforation of BNC hydrogels with a height of 1.5–2.5 mm, a laser power of 50 W and a feed of the laser of 10 mm s^{-1} were used (Fig. 1; center column; LS-BNC1-1D to LS-BNC4-1D, Table 1); the respective values for BNC hydrogels with a height of 5.5–6.5 mm were 90 W and 10 mm s^{-1} (LS-BNC6-1D, Table 1). For cutting of the final, round shapes of the BNC hydrogels with a height of 1.5–2.5 mm, a laser power of 30 W and a feed of the laser of 10 mm s^{-1} were used (see Table 1). For cutting of the final, squared shapes of the BNC hydrogels with a height of 5.5–6.5 mm, a laser power of 90 W and a feed of the laser of 8 mm s^{-1} were used (Table 1).

In the case of 3-D-perforated BNC (Fig. 1; right column; LS-BNC7-3D and LS-BNC8-3D, Table 1), unidirectionally perforated cuboids with an edge length of 8 mm and a height of 5.5–6.5 mm were first cut out from the larger hydrogels described in Section 2.1 using a laser power of 90 W and a feed of 8 mm s^{-1}. The final 3-D BNC hydrogels were then generated by perforating the other sides of the cuboids, using again a laser power of 90 W and a feed of the laser of 10 mm s^{-1}. Finally, the 3-D-perforated cuboids were either not further laser-cut (LS-BNC7-3D, Table 1) or, alternatively, cut into slices with a height of 2 mm using a laser power of 90 W and a feed of 8 mm s^{-1} in order to avoid further perforation of the rapidly collapsing, thin BNC slices. Exclusively the center pieces of this process (showing only laser-modified surfaces) were used for further analysis (Fig. 1; right column; LS-BNC8-3D; Table 1).

For all procedures, the focus diameter of the laser beam was ~300 μm (resulting in a channel diameter of 220 μm) and the laser beam was focused to one half of the height of the BNC hydrogel in order to achieve optimal distribution of laser intensity throughout the material. Each of the above process parameters, as well as the cutting and perforation outlines, were controlled by CAD-supporting scanner software (SamLight; SCAPS GmbH; Deisenhofen, Germany).

To remove bacterial residues and residues accompanied with laser processing, the samples were treated with 2 M aqueous sodium hydroxide solution in an autoclave at a temperature of 121 °C for 20 min. The samples were then repeatedly washed with pyrogen-free water (Roth, Karlsruhe, Germany) under aseptic conditions. To analyze the influence of aqueous sodium hydroxide solutions with different molarity, samples were autoclaved in 0 M, 1 M, 2 M and 4 M solutions (121 °C, 20 min).