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Novel bilayer bacterial nanocellulose scaffold supports neocartilage formation *in vitro* and *in vivo*

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ABSTRACT

Tissue engineering provides a promising alternative therapy to the complex surgical reconstruction of auricular cartilage by using ear-shaped autologous costal cartilage. Bacterial nanocellulose (BNC) is proposed as a promising scaffold material for auricular cartilage reconstruction, as it exhibits excellent biocompatibility and secures tissue integration. Thus, this study evaluates a novel bilayer BNC scaffold for auricular cartilage tissue engineering. Bilayer BNC scaffolds, composed of a dense nanocellulose layer joined with a macroporous composite layer of nanocellulose and alginate, were seeded with human nasoseptal chondrocytes (NC) and cultured *in vitro* for up to 6 weeks. To scale up for clinical translation, bilayer BNC scaffolds were seeded with a low number of freshly isolated (uncultured) human NCs combined with freshly isolated human mononuclear cells (MNC) from bone marrow in alginate and subcutaneously implanted in nude mice for 8 weeks. 3D morphometric analysis showed that bilayer BNC scaffolds have a porosity of 75% and mean pore size of 50 ± 25 μm. Furthermore, endotoxin analysis and *in vitro* cytotoxicity testing revealed that the produced bilayer BNC scaffolds were non-pyrogenic (0.15 ± 0.09 EU/ml) and non-cytotoxic (cell viability: 97.8 ± 4.7%). This study demonstrates that bilayer BNC scaffolds offer a good mechanical stability and maintain a structural integrity while providing a porous architecture that supports cell ingrowth. Moreover, bilayer BNC scaffolds provide a suitable environment for culture-expanded NCs as well as a combination of freshly isolated NCs and MNCs to form cartilage *in vitro* and *in vivo* as demonstrated by immunohistochemistry, biochemical and biomechanical analyses.

1. Introduction

Serious auricular defects such as anotia and microtia, along with auricle damage caused by cancer and trauma, demand an effective treatment for auricular cartilage reconstruction. For such cases, the field of tissue engineering (TE) provides a promising potential alternative therapy to the conventional and complex surgical reconstruction of auricular cartilage by using ear-shaped autologous costal and nasoseptal cartilage [1–3]. Bacterial nanocellulose (BNC), a novel biomaterial with excellent biocompatibility and remarkable tissue integration capability [4–8], has been evaluated

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for several TE strategies and has shown to support adhesion, proliferation and differentiation of different cell types [9–15]. BNC is a natural biopolymer synthesized by various bacteria species, particularly *Gluconacetobacter xylinus* [16,17]. Its three-dimensional and interconnected network is composed of highly hydrated nanofibrils ranging from 70 to 140 nm in width, similar to collagen fibrils found in extracellular matrix (ECM) of several tissues, with high tensile strength [11,18]. BNC is considered a hydrogel since it is mostly composed of water in its native state (99%). All together, these outstanding properties make BNC an exceptional biomaterial for many biomedical applications [19–21], including auricular cartilage reconstruction [8,14,22].

Although several groups have attempted to engineer auricular cartilage [1], few successful outcomes have been reported [23–25]. Development of artificial auricular grafts with adequate mechanical properties has been identified as a key factor for successful auricular cartilage TE [26]. Most studies that have used biodegradable scaffold materials have resulted in poor structural integrity (i.e. shape and size stability) of the auricular scaffold after implantation; caused by the short-lived chemical and mechanical stability [27–31]. On the other hand, recent studies that have investigated the use of non-degradable biomaterials for auricular cartilage reconstruction have reported a better structural integrity of the implant [23,25,32] – likely caused by the chemical stability of the support biomaterial, which translates into long-lasting mechanical properties even after implantation. As opposed to the many biodegradable scaffolds previously evaluated for auricular cartilage TE, the long-term structural integrity of BNC scaffolds should not be compromised after implantation since humans do not produce enzymes capable of breaking down cellulose [33]. Besides being a chemically stable material, BNC with increased cellulose content of 17% (densified hydrogel) is a competitive scaffold material for repair, reconstruction or regeneration of auricular cartilage since it matches the elastic mechanical properties (e.g. equilibrium modulus) of human auricular cartilage [22], can be fabricated in patient-specific auricular shapes [34] and exhibits excellent biocompatibility *in vivo* – causing a minimal foreign body response [8].

When densified, BNC hydrogel is a mechanically and biologically appropriate biomaterial for use in auricular cartilage reconstruction [8,22]. However, its dense nanocellulose network prevents cells from penetrating the material. To circumvent this problem, several techniques have been developed to support cell ingrowth in BNC scaffolds by tuning pore size and pore interconnectivity during biosynthesis of BNC [35], via laser ablation [10] and freeze-dry processing [36,37]. Such macroporous BNC scaffolds have been shown to provide an adequate environment that supports ingrowth and differentiation of chondrocytes. For example, human primary articular, auricular and nasoseptal chondrocytes cultured in macroporous BNC scaffolds *in vitro* have been shown to adhere, migrate, proliferate and maintain their chondrogenic phenotype – as confirmed by the synthesis of cartilage-specific ECM [14,37,38].

Engineering stable and functional auricular cartilage tissue also depends on the cell source used. Pleumeekers et al. showed that human auricular and nasoseptal chondrocytes possess a high chondrogenic capacity *in vivo*, making them attractive cell sources for auricular cartilage repair [39]. The use of cells in cartilage repair is an attractive strategy as it may result in regeneration of the lost tissue. However, the clinical application of a cell-aided treatment does feature challenges – a limited supply of autologous chondrocytes with the proper phenotype being the most stringent one. To cancel out cell culture, including the concomitant laboratory logistics and the double surgery, autologous cells should be isolated within the operating room and applied directly. In addition, the combination of chondrocytes with a less limited source of autologous cells, such as bone marrow mononuclear cells (MNC), can overcome the challenge of having too few cells and may even increase the treatment's performance [40,41]. By resuspending the cells in alginate, also the factor of cell loss after scaffold seeding can be diminished whilst simultaneously providing the cells with a 3D environment to suppress dedifferentiation [42].

Several studies that have evaluated BNC as a scaffold material for auricular cartilage TE [8,14,22,37] have contributed to the design and development of BNC scaffolds with a two-layer (bilayer) architecture. This study investigates the *in vitro* and *in vivo* performance of bilayer BNC scaffolds, composed of a dense nanocellulose layer joined with a macroporous composite layer of nanocellulose and alginate, designed to be mechanically stable and maintain a long-term structural integrity while providing a porous architecture that supports cell ingrowth and neocartilage formation. Moreover, this study explores the application of a clinically relevant strategy by seeding a low number of freshly isolated (uncultured) human chondrocytes combined with freshly isolated human mononuclear cells, in order to test the translation of this auricular cartilage TE technology to the clinic.

2. Materials and methods

2.1. Fabrication and purification of bilayer BNC scaffolds

2.1.1. Production of dense and porous scaffold layers

BNC hydrogel disks with increased cellulose content (i.e. dense layer) were produced and purified as described elsewhere [8]. Briefly, a suspension of *G. xylinus* (ATCC® 700178, LGC Standards, Sweden) was inoculated in 250 ml conical flasks containing sterile culture medium (described by Matsuoka et al. [43]) and cultured at 30 °C for 18 days, until large BNC cylinders (Ø 48 mm × 20 mm) were biosynthesized. The BNC cylinders were purified in a built-in-house perfusion system and compressed to 1 mm in height to increase the cellulose content. The compressed BNC pellicles were frozen to −80 °C overnight and lyophilized (Heto PowerDry PL3000, Thermo Fisher Scientific, MA, USA) for 3 days. Dense BNC disks (Ø 8 mm × 1 mm) were then cut with a sterile biopsy punch (Miltex GmbH, Germany). The criterion for selecting the thickness of the dense BNC layer is based on morphometric analysis from MRI scans of human auricular cartilage, where Nimeskern et al. reported a cartilage thickness of 1.15 ± 0.10 mm [44].

BNC/alginate composite scaffolds (i.e. porous layer) were fabricated by a freeze-drying process. First, purified BNC pellicles were homogenized with a blender, until a pulp consistency was obtained, and then with a dispersing element (S25N-18G, IKA, Germany) at 25,000 rpm for 20 min. Afterwards, the homogenized BNC suspension was steam sterilized (100 kPa, 121 °C for 20 min) and the cellulose content was determined using a halogen moisture analyzer (HB43, Mettler-Toledo, OH, USA). The following steps were carried out in sterile conditions. The BNC suspension was mixed with 1.1% w/w clinical grade alginate dissolved in 0.9% NaCl (CellMed AG, Germany) to get a final composition of 90% dry weight BNC and 10% dry weight alginate compared to the total dry weight. The weight of alginate solution (W_{Alg}) added to a known weight of BNC suspension (W_{BNC}) was calculated by using the formula: $W_{Alg} = W_{BNC} \times (\%DW_{Alg} \div \%DW_{BNC}) \times (\%CC_i \div \%AC_i)$. Where $\%DW_{Alg}$ and $\%DW_{BNC}$ are the targeted percent dry weight of alginate (10%) and BNC (90%) compared to the total dry weight; and $\%CC_i$ and $\%AC_i$ are the initial cellulose and alginate concentrations. The BNC/alginate mixture was then dispersed at 25,000 rpm for 15 min, transferred to sterile containers (TP52, Gosselin, France) and degased in a vacuum desiccator. The containers were then placed inside Nalgene® cryo freezing containers (Thermo Fisher Scientific) and frozen to −80 °C overnight at a rate of 1 °C/min. The frozen BNC/alginate mixtures were lyophilized for 5 days to sublimate the ice crystals, creating a macroporous architecture. The dry BNC/alginate sponges were then sliced to 2 mm-thick slices and porous BNC/alginate composite scaffolds (Ø 8 mm × 2 mm) were cut with a sterile biopsy punch (Miltex GmbH).

2.1.2. Fabrication of bilayer BNC scaffolds

A novel cellulose solvent system (i.e. ionic liquid EMIMAc) was used to attach the dense and porous layers and achieve a strong interfacial molecular bonding between the layers. The following steps were carried out in sterile conditions. First, dry homogenized BNC was dissolved in ionic liquid EMIMAc (1-ethyl-3-methylimidazolium acetate; Sigma–Aldrich, MO, USA) at a concentration of 10 mg/ml. The cellulose solvent solution was preheated to 80 °C and then smeared on the dense BNC layers. Subsequently, the porous layers were aligned on top of the dense layers and the bilayer BNC scaffolds were placed on a heating plate at 80 °C for 2 min to accelerate the dissolution of nanocellulose at the interface. The bilayer BNC scaffolds were then stabilized in 100 mM $CaCl_2$ in ethanol to precipitate the dissolved cellulose between the layers (i.e. attach the layers), while simultaneously