Mechanical integrity

Bacterial nanocellulose (BNC) vs. auricular cartilage

Equilibrium modulus (MPa)

R = 0.79

Effective cellulose content $C_C$

Patient-specific biofabrication

MRI scan

BNC implant

1 cm
Bacterial nanocellulose (BNC) matches the mechanical moduli of human ear cartilage

Different relaxation kinetics between BNC and native cartilage are observed

Concepts for improved relaxation kinetics are proposed

BNC can be produced in complex shapes, such as patient-specific auricles
Mechanical evaluation of bacterial nanocellulose as an implant material for ear cartilage replacement

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Abstract

Bacterial nanocellulose (BNC) is a novel non-degradable biocompatible material that promotes chondrocyte adhesion and proliferation. In this work, its potential use in ear cartilage tissue engineering (TE) is investigated. Firstly, the mechanical properties of native ear cartilage are measured in order to set a preliminary benchmark for ear cartilage replacement materials. Secondly, the capacity of BNC to match these requirements is assessed. Finally, a biofabrication process to produce patient-specific BNC auricular implants is demonstrated.

BNC samples (n = 78) with varying cellulose content (2.5 - 15%) were compared using stress-relaxation indentation with human ear cartilage (n = 17, from 4 males, aged 49 – 93 years old). Additionally, an auricle from a volunteer was scanned using a 3T MRI with a spoiled gradient-echo sequence. A negative ear mold was produced from the MRI data in order to investigate if an ear-shaped BNC prototype could be produced from this mold.

The results show that the instantaneous modulus $E_{in}$, equilibrium modulus $E_{eq}$, and maximum stress $\sigma_{max}$ of the BNC samples are correlated to effective cellulose content. Despite significantly different relaxation kinetics, the $E_{in}$, $E_{eq}$ and $\sigma_{max}$ of BNC at 14% effective cellulose content reached values equivalent to ear cartilage (for $E_{eq}$, BNC: 2.4 ± 0.4 MPa and ear cartilage: 3.3 ± 1.3 MPa). Additionally, this work shows that BNC can be fabricated into patient-specific auricular shapes. In conclusion, BNC has the capability to reach mechanical properties of relevance for ear cartilage replacement, and can be produced in patient-specific ear shapes.

Keywords:

Stress relaxation, auricle, bacterial cellulose, microbial cellulose, tissue engineering.
1. Introduction

Bacterial nanocellulose (BNC) is a natural biopolymer produced by various species of bacteria, particularly *Gluconacetobacter xylinus* (formerly named *Acetobacter xylinus*) (Ross et al., 1991). It consists of an interconnected entangled network of cellulose fibrils similar in size to collagen fibers (approximately 30 nm wide) (Gatenholm and Klemm, 2010; Gelin et al., 2007) creating an extensive surface area holding a large amount of water (Czaja et al., 2007) while the whole structure is interconnected and stabilized by intra- and inter-fibrillar hydrogen bonds (O'Sullivan, 1997). This fibrillar structure provides high tensile mechanical characteristics to the material (Gea et al., 2011) and a hydrogel-like behavior as cellulose nanofibers bind water (Czaja et al., 2007). In contrast to plant cellulose, BNC is devoid of macromolecules such as lignin or hemicellulose (Petersen and Gatenholm, 2011). Additionally it is non-degradable in the human body, which lacks the enzymes to degrade cellulose, and it has been shown to be biocompatible (Backdahl et al., 2006; Helenius et al., 2006; Rosen et al., 2011); all factors which make BNC an exciting biomaterial for biomedical applications (Czaja et al., 2007; Petersen and Gatenholm, 2011).

Interconnected porosity is a key feature in a scaffold to allow the seeded chondrocytes to penetrate and migrate throughout the material. In its natural state, BNC is known to be impenetrable by cells, due to the small size of the pores (Backdahl et al., 2006; Klemm et al., 2005). However, this issue has been addressed by several investigators, who successfully synthesized BNC scaffolds with large pores, allowing the cells to penetrate the scaffold (Bäckdahl et al., 2008; Rambo et al., 2008). Therefore, in the scope of this work the mechanical
properties of BNC are the primary focus, including investigation of the tunability of the natural material.

Due to its good tensile mechanical properties, BNC has been used in previous work for blood vessel (Bodin et al., 2007a; Klemm et al., 2001; Malm et al., 2012), meniscus (Bodin et al., 2007b; Martinez et al., 2012), and articular cartilage tissue engineering (TE) strategies (Andersson et al., 2010; Svensson et al., 2005). It has been shown that BNC supports adhesion and proliferation of different cell types (Andersson et al., 2010; Backdahl et al., 2006; Bodin et al., 2010; Brackmann et al., 2011; Svensson et al., 2005), in particular chondrocytes (Andersson et al., 2010; Svensson et al., 2005). This ability to induce chondrocyte proliferation makes BNC a promising scaffold material for ear cartilage TE.

Although several groups have investigated engineering ear cartilage (Bichara et al., 2012), few successful outcomes have been reported (Yanaga et al., 2009). Many studies have investigated the potential of biodegradable scaffold materials (Cao et al., 1997; Haisch et al., 2002; Isogai et al., 2004; Kusuhara et al., 2009; Shieh et al., 2004); for example, results reported by Shieh et al. (Shieh et al., 2004) in an immuno-competent animal model showed poor shape stability due to an immune response presumably caused by degradation byproducts. As opposed to degradable biomaterials, non-degradable ones have the advantage of providing both durable mechanical properties and long-term chemical stability. Non-degradable biomaterials have been used for a wide range of biomedical applications (Chirila, 2001; Dziubla et al., 2001; Ruszymah et al., 2005; Xue and Greisler, 2003), but have only recently been investigated for ear cartilage engineering (Lee et al., 2011; Ruszymah et al., 2011; Zhou et al., 2011). Lee et al. (Lee et al., 2011) and
Ruszymah et al. (Ruszymah et al., 2011) investigated, in vivo, the use of commercial HDPE\(^1\) implants covered with cell-fibrin constructs, and Zhou et al (Zhou et al., 2011) studied in vivo the potential of type I collagen implants reinforced with coiled titanium wires. Good histological (Lee et al., 2011; Ruszymah et al., 2011; Zhou et al., 2011) and mechanical results were reported (Lee et al., 2011). Nevertheless, the use of non-viscoelastic and stiff biomaterials such as HDPE or metals leads to complications - in particular, extrusion – reported with high occurrence in clinical outcomes for regular HDPE alloplastic implants (Cenzi et al., 2005). The use of a non-biodegradable, more compliant material such as BNC may provide an alternative implant material, on condition that the mechanical properties can be tuned to match the native tissue. That said, since a non-biodegradable implant like BNC will remain implanted over the patient life-time, it is critical that its mechanical properties are consistent with the native tissue, i.e. in this work, ear cartilage. Among the three types of cartilage present in the human body - hyaline cartilage, fibrocartilage and elastic cartilage - ear cartilage is classified as elastic cartilage (Sayed et al., 2010). The mechanical characteristics of hyaline cartilage (e.g. articular cartilage, nasoseptal cartilage) (Mow and Guo, 2002; Rotter et al., 2002) and fibrocartilage (e.g. meniscus) (Joshi et al., 1995) are well documented, yet there is little data available for ear cartilage (Naumann et al., 2002). In comparison to hyaline and fibrocartilage, elastic cartilage contains significantly more elastin (Naumann et al., 2002), which is known to be a highly resilient protein, and which plays a mechanically functional role in tissues like heart valves (Lee et al., 2001) and skin (Oxlund et al., 1988). For this reason, a profile of the mechanical properties of ear cartilage is required, since using published mechanical properties of hyaline cartilage or fibrocartilage may not be an appropriate substitute.

\(^1\)high density polyethylene
Two distinct mechanisms are involved in the response of cartilage to loading: the intrinsic mechanical properties of the ECM\(^2\), and the resistance to interstitial fluid flow through the ECM (Mow and Guo, 2002), which is governed by the permeability and swelling pressure (Donnan osmotic pressure) of the ECM (Bartel DL et al., 2006). During loading, there is an instantaneous response involving a change in shape of the material without change in volume (instantaneous modulus); followed by a transient phase where the fibrillar stress relaxes and fluid flows out of the matrix (relaxation kinetic). Finally, the tissue reaches an equilibrium or steady-state response where fluid flow ceases; then the load is carried by the solid matrix, and is characterized by the elastic properties of only the solid component (equilibrium modulus) (Armstrong, 1986). Creep and stress relaxation tests are commonly performed to characterize the mechanical properties of cartilage to find these properties (Bartel DL et al., 2006).

Beside the control of the implant mechanics, it is key in the field of ear cartilage replacement, to control the external shape of the implant to obtain satisfying aesthetic results. This outcome is often used as a measure of success for a potential biomaterial (Lee et al., 2011; Shieh et al., 2004; Zhou et al., 2011). Most efforts in ear cartilage TE so far, have used standardized auricle shapes (Lee et al., 2011; Ruszymah et al., 2011; Shieh et al., 2004; Zhou et al., 2011), rather than patient-specific forms. Liu et al (Liu et al., 2010) describe a method to 3D print ear scaffolds using CT\(^3\) data from a patient scan. However, the use of CT is controversial since it uses a radiation source, and is not ideal for good quality soft tissue contrast; and hence the direct visualization of cartilage. Better soft tissue contrast would allow segmentation of the various soft tissues present, specifically ear cartilage, adipose and skin tissues, rather than the whole

\(^2\) extracellular matrix
\(^3\) computed-tomography
ear. Reconstruction of the individual components would then further improve the aesthetic result. MRI\(^4\) is a non-invasive 3D imaging technique, which provides good soft tissue contrast.

MRI is used clinically for evaluation of soft tissues such as articular cartilage (Gold et al., 2003), and is therefore proposed here as a promising imaging technique for ear cartilage.

This study aims to assess whether BNC is a mechanically appropriate implant material for ear cartilage replacement. The objectives are firstly to measure the mechanical properties of native ear cartilage in order to define a preliminary benchmark for BNC implants, and secondly to assess whether BNC properties can be tuned to match these requirements. Finally, a biofabrication process to produce patient-specific BNC auricular implants is described.

2. Materials and methods

2.1. Ear cartilage harvesting

Human ear cartilage samples were obtained according to the ethics regulations of the University Hospital Zurich, UZH, (Zurich, Switzerland), the Ulm University Medical Center, UUMC, (Ulm, Germany) and the Erasmus Medical Center, EMC, (Rotterdam, The Netherlands). Samples were either harvested from patients undergoing ear reconstructive surgery (UZH, UUMC, and EMC) or from complete auricles obtained post mortem from human donors (EMC). The samples were shipped at 4°C in a phosphate buffer saline solution (PBS) supplemented with an antibiotic/antimycotic solution (Antibiotic/Antimicotic, Gibco®, Invitrogen Corporation, Carlsbad, California, USA) to ETH Zurich (Zurich, Switzerland). Upon arrival (~24 hours post-surgery), perichondrium was removed so as to expose the cartilage surface. Cylindrical plugs (Ø5 mm, ~1-2 mm thick) were then cut perpendicular to the cartilage surface. Stress relaxation

\(^4\)magnetic resonance imaging
indentation was performed on each plug. A total of 17 ear cartilage plugs harvested from 4 males (49 -93 years old) were used for mechanical testing.

2.2. BNC synthesis

250 mL conical flasks (wide neck) were inoculated with Gluconacetobacter xylinus ATCC® 700178 (LGC Standards AB, Borås, Sweden). A 3 mL bacteria suspension and 300 mL of sterile culture media (described by Matsuoka et al. (Matsuoka et al., 1996)) was added to each flask. The conical flasks were placed in an incubator at 30°C. The bacterial culture continued until the cellulose pellicle had reached a height of 5 cm, which took approximately 30 days. The protocol used to remove the bacteria and its residues from the cellulose matrix was adapted from previous studies (Helenius et al., 2006; Martinez et al., 2012). BNC pellicles were immersed in 0.1 M NaOH for 21 days, changing the solution every 24 hours, they were then rinsed with large amounts of 60°C deionized water to remove bacterial residues and neutralize the pH.

2.3. Production of high cellulose content BNC pellicles

A sample was cut from each BNC pellicle and its initial cellulose content (CC) was determined using a HB43 halogen moisture analyzer (Mettler-Toledo Inc., Columbus, OH, USA). The initial cellulose content refers to the weight percent of cellulose produced by the bacteria, which is approximately 1 wt%. This was computed using the equation:

\[ \text{CCI} = \left( \frac{\text{Dry weight}}{\text{Wet weight}} \right) \times 100\% \]

and is calculated directly after bacterial synthesis and cleaning. The BNC pellicles were then cut to different initial heights \( H_i \) (equation 1). The cut pellicles were placed inside a stainless steel
tube (Ø = 50 mm) and a stainless steel rod was placed on top, and each pellicle was compressed to a final height $H_f$ of 1.00 ± 0.05 mm at a rate of 0.5 mm/min using an Instron 5565A (Instron, Norwood, MA, USA). $H_i$ is computed from equation 1, where $CC_n$ is the desired nominal cellulose content and $H_f$ is the height after compression (nominally 1 mm):

$$H_i = H_f \cdot \left( \frac{CC_n}{CC_i} \right)$$

(1)

The pellicles were then stored at 4°C in vacuum-sealed bags.

2.4. SEM characterization of BNC

The nanocellulose fiber structure was characterized by scanning electron microscopy (SEM).

Cellulose samples of 1%, 5% and 10% nominal cellulose content ($n = 2$ for each cellulose content) were quenched in liquid nitrogen, lyophilized for 72 hours (Heto PowerDry PL3000, Thermo Fisher Scientific, Waltham, MA, USA), then mounted on SEM studs, sputtered with a gold film and analyzed using a Leo Ultra 55 field emission gun (FEG) SEM (Carl Zeiss AG, Oberkochen, Germany). Images were taken at a magnification of 25000x.

2.5. BNC sample preparation for mechanical testing

For mechanical testing, cylindrical plugs (Ø5 mm, ~1 mm thick) were cut with a biopsy punch from BNC pellicles of 2.5%, 5%, 7.5%, 10%, 12.5% and 15% nominal cellulose content ($n = 13$ for each cellulose content, $n_{total} = 78$). In order to determine the effective cellulose content, the plugs were weighed before mechanical testing. After mechanical testing, the samples were freeze-dried and the dry weight was measured. The effective cellulose content ($CC_e$) refers to
the true cellulose content of the plugs during testing and was computed according to equation 2.

\[ CC_e = \left( \frac{\text{Dry weight after testing}}{\text{Wet weight before testing}} \right) \times 100\% \]  

(2)

2.6. Mechanical testing

Auricular cartilage (n = 17) and BNC samples (n_{total} = 78) with varying cellulose content were placed in close-fitting stainless steel cylindrical wells. Mechanical testing was performed with a materials testing machine (Zwick Z005, Ulm, Germany) equipped with a 10 N load cell, a built-in displacement control, and a cylindrical, plane ended, stainless steel indenter (Ø0.35 mm). During mechanical testing the cartilage samples were immersed in PBS supplemented with antibiotic/antimycotic solution, BNC samples were immersed in Millipore™ water. Stress relaxation testing was performed as described previously (Stok et al., 2010). In short, a preload of 3 mN was first applied on the sample to locate the sample surface and measure sample thickness, and held for 5 minutes. Five successive strain steps were then applied in 5% increments of the original sample thickness, and specimens were left to relax for 1 hour (BNC) and 20 minutes (cartilage) at each step. The hold time was defined as the time necessary to reach equilibrium. An in-house Matlab® script was used to convert force and displacement data to stress and strain. Measurements of maximum stress \( \sigma_{max} \), equilibrium modulus \( E_{eq} \), and instantaneous modulus \( E_{in} \) were determined for every sample, as described previously (Stok et al., 2010). Additionally, a relaxation half-life time \( t_{1/2} \) and a characteristic relaxation time \( \tau \) were computed to estimate the viscoelastic relaxation after the first strain application. \( t_{1/2} \) was defined as the time needed for the stress to decrease to half of its maximum value, and \( \tau \) was
obtained by fitting an exponential decay function to the stress-time data (see Figure 1). In equation 3, \(A\) and \(\tau\) are the two fitted parameters, \(\sigma_i\) is the stress immediately following the first strain application, \(\sigma_{eq}\) is the equilibrium stress at the end of the first strain step and \(T\) is the hold time. Note that by definition \(f(0) = \sigma_i\) and \(f(T) = \sigma_{eq}\).

\[
f(t) = (\sigma_i - A) + \frac{t}{T} \cdot (A + \sigma_{eq} - \sigma_i) + A \cdot \exp(-t/\tau)
\]

(3)

2.7. MRI of human auricles for the biofabrication of patient-specific BNC implants

An MRI scan of the external ear was performed on a male 28 year-old volunteer with a Magnetom Skyra 3T MRI (Siemens AG, Erlangen, Germany). The volunteer gave informed consent for the study. A spoiled gradient-echo sequence (T1 VIBE) with fat saturation was used to scan a 5.76 x 5.76 x 5.64 cm\(^3\) VOI\(^5\) centered on the auricle. The XY plane was chosen parallel to the sagittal plane. An in-plane (XY) resolution of 0.45 mm x 0.45 mm and an out-of-plane (Z) resolution of 0.40 mm were used. Manual segmentation of the external ear shape was performed, and a 3D surface mesh was created and saved in STereoLithography (STL) file format (suitable for rapid prototyping). A nylon ear replica was produced by Materialise (Leuven, Belgium) from the 3D surface mesh using selective laser sintering. A negative ear mold was produced by casting silicone around the ear model. The negative silicone mold consisted of three pieces for easy retrieval of the ear model and, subsequently, the 3D ear-shaped BNC implant prototype.

2.8. 3D biofabrication system

\(^5\)volume of interest
A bioprinter, developed in-house and consisting of a high precision micro-dispensing system - used to dispense controlled volumes of bacterial culture media, and a precision motion system - used for controlling the dispensing location, was used for the biofabrication of a patient-specific ear-shaped BNC implant prototype (see figure 2). The motion system consists of a two-axis linear stepper motor gantry stage (H2W Technologies Inc, Valencia, CA, USA) connected to a NI MID-7604 stepper power drive (National Instruments, Austin, TX, USA), see figure 2a,c, which provides stepper motor control from a NI PCI-7344 controller (National Instruments, Austin, TX, USA). NI Motion Assistant 2.7 (National Instruments, Austin, TX, USA) was used to program the controller for motion control (figure 2c). The micro-dispensing system (figure 2b) consists of an M/2 Very High Speed micro-dispensing valve (The LEE company, Westbrook, CT, USA) and a Spike and Hold Driver (The LEE company, Westbrook, CT, USA) connected to the controller and programmed in NI Motion Assistant. The micro-dispensing valve coupled to an atomizing nozzle (The LEE company), was connected to a pressurized tank (Model 66839, Central Pneumatic) containing sterile culture media (figure 2a). The pressure inside the tank was monitored using a Motorola MPX4250AP absolute pressure sensor (Freescale Semiconductor Inc, Austin, TX, USA) connected to a NI USB-6009 data acquisition device (National Instruments, Austin, TX, USA). The pressure was monitored and recorded using NI LabVIEW Signal Express 2009 (National Instruments, Austin, TX, USA). A silicone rubber heater connected to an EHG® SL10 process controller (Watlow Electric Manufacturing Company, Columbia, MO, USA) was used to maintain culture media temperature at 30°C. The micro-dispensing valve and gantry stage were placed inside a laminar airflow bench during bacterial culture. The pressure tank, silicone tubing,
fittings and atomizing nozzle were steam sterilized (1 bar, 121°C) for 20 min. The VHS micro-dispensing valve was sterilized by flushing it repeatedly with 70% ethanol.

2.9. Synthesis of patient-specific ear-shaped BNC implant prototype

The negative silicone mold was inoculated with 1 ml of bacteria suspension and positioned in the bioprinter. After 24 hours, a thin film of BNC had grown. Thereafter, the micro-dispensing valve was programmed to spray 500 μL of sterile culture media into the mold every 6 hours and the motion system was programmed to move along the helix of the mold during dispensing. The bacterial culture continued for 18 days until the cellulose layer filled the silicone mold. The ear-shaped BNC implant prototype was removed from the mold and cleaned following the same protocol described for the BNC pellicles.

2.10. Statistical analysis

All statistical analyses were performed with R (version 2.13.0, R Foundation for Statistical Computing, Vienna, Austria). A univariate analysis of variance was also used to test significant differences (p < 0.05) in the measured mechanical parameters between BNC and ear cartilage. The relationships between effective cellulose content and the measured mechanical parameters were evaluated using Pearson’s linear regression coefficient of correlation, R. All results are displayed as mean ± standard deviation (SD).

3. Results

3.1. Mechanical testing of ear cartilage
The results obtained from the stress relaxation testing show an expected viscoelastic behavior of human ear cartilage (see figure 1b). After processing the data from the stress-strain and stress-time curves, the following measurements were determined: 6.4 ± 3.2 MPa for $E_{in}$, 3.3 ± 1.3 MPa for $E_{eq}$, 1.6 ± 0.5 MPa for $\sigma_{max}$, 2.1 ± 1.3 s for $t_{1/2}$ and 6.0 ± 2.6 s for $\tau$ (Figure 4).

3.2. Production and mechanical testing of high cellulose content BNC

BNC pellicles with effective cellulose content ranging from 4.1% up to 13.7% were produced. The network structure of native (1% cellulose content) and compressed (5% and 10%) BNC was characterized by SEM. As seen in figure 3, the spacing between the nanocellulose fibers decreases with increasing cellulose concentration. Compressive properties, $E_{in}$, $E_{eq}$, and $\sigma_{max}$, were correlated to effective cellulose content with $R$ values of 0.66, 0.79, and 0.78, respectively ($p < 0.05$ in all cases), see figure 4. The $E_{eq}$ of BNC could be tuned to values around 3.2 MPa at the highest effective cellulose content (13.7%). Despite observing a typical stress relaxation behavior in all BNC samples, the relaxation half-life, $t_{1/2}$, of BNC (0.4 ± 0.1 s) was significantly less than ear cartilage (2.1 ± 1.3 s; $p < 0.05$). The same observation was made for $\tau$ (3.7 ± 1.0 s and 6.0 ± 2.6 s, respectively; $p < 0.05$).

3.3. MRI of human auricles for the biofabrication of patient-specific BNC implants

The ear cartilage and external ear volume of a volunteer was successfully segmented and reconstructed from the MR image data (Figure 5a-b). A negative silicone mold was used to guide the bacteria during bacterial culture to reproduce the large-scale features of the outer ear; a process taking about 18 days (Figure 5c). Subsequently, a 3D patient-specific BNC implant
prototype (1% effective cellulose content), mimicking the external ear shape, was successfully synthesized using this mold in a novel biofabrication process (figure 5d).

4. Discussion

In this study, values for $E_{\text{in}}$, $E_{\text{eq}}$, $\sigma_{\text{max}}$, $t_{1/2}$ and $\tau$ of ear cartilage were measured using stress-relaxation indentation. These values represent a preliminary benchmark for BNC implants aimed at ear cartilage TE. Moderate standard deviations are observed. These variations are expected to result from factors such as donor age, gender and harvesting location within the auricle. A detailed investigation of these factors is out of the scope of the present study and will be performed in future work, in order to provide a standard for ear cartilage replacement strategies. The observed variability of cartilage mechanical properties emphasizes the need of an ear cartilage replacement material with spatially tunable mechanical properties.

The compressive mechanical properties of BNC were demonstrated to be tunable and correlated to effective cellulose content. The computed correlation coefficients ($R = 0.66, 0.79, 0.78$ for $E_{\text{in}}$, $E_{\text{eq}}$, $\sigma_{\text{max}}$, respectively) are moderate. This is likely due to an imperfect reproducibility of the cellulose content measurement protocol. Nonetheless, by varying effective cellulose content in BNC, an increase in $E_{\text{eq}}$ from 0.06 to 3.2 MPa was observed; partly covering the range of values observed for ear cartilage ($3.3 \pm 1.3$ MPa, see figure 4). The same observation holds for $E_{\text{in}}$ and $\sigma_{\text{max}}$. These parameters of tunability make BNC a promising candidate as a non-biodegradable implant material for ear cartilage. Firstly, a further increase in $E_{\text{in}}$, $E_{\text{eq}}$, and $\sigma_{\text{max}}$ of BNC could be achieved through escalation of the cellulose content, so as to cover the whole range of equilibrium modulus observed in ear cartilage. In future, implants with
varying local mechanical properties could be produced to more closely match the local mechanical properties of the native tissue, as explained above.

Although similar equilibrium moduli were measured for BNC and ear cartilage (see figure 4), the relaxation measures - namely $t_{1/2}$ (0.4 ± 0.1 s and 2.1 ± 1.3 s, respectively) and $\tau$ (3.7 ± 1.0 s and 6.0 ± 2.6 s, respectively), were significantly shorter for BNC. As opposed to BNC which is electrically neutral, cartilage contains charged groups by way of the GAG$^6$ content trapped in its collagen matrix (Mow and Guo, 2002). These charged groups play a role in the relaxation behavior of cartilage, via charge-dependent osmotic swelling pressures (i.e. Donnan osmotic pressure) enhanced during compression (Mow and Guo, 2002). Therefore, the introduction of electrical charge into the BNC network could provide mechanical properties that better mimic those of native cartilage, in particular improved relaxation kinetics (higher relaxation half time and characteristic relaxation time). In chondrocyte-seeded BNC constructs this introduction of electrical charges could be achieved by the GAG produced by the seeded cells; which would require adequate seeding and distribution throughout the scaffold. Alternatively this could be obtained by chemical modification of BNC. Numerous chemical modifications of BNC have been reported in literature, aimed at promoting cell adhesion on BNC (Bodin et al., 2007a; Kalaskar et al., 2008), functionalizing it with drugs (Barud et al., 2008; Klechkovskaya et al., 2008; Nguyen et al., 2008) or tuning its biodegradability (Kusuhara et al., 2009) and producing nanocomposites (Grande et al., 2009; Wan et al., 2007). Interestingly Svensson et al. (Svensson et al., 2005) demonstrated that chemical modifications such as sulfonation or phosphorylation could also increase BNC compressive modulus (i.e. $E_{in}$), thus artificially mimicking the presence of charged GAG in the BNC matrix.

$^6$glycosaminoglycan
In addition to appropriate mechanical behavior, penetration and proliferation of seeded chondrocytes throughout the material is a key aspect for the use of BNC as a scaffold material. Native BNC is known to be impenetrable to cells (Backdahl et al., 2006; Klemm et al., 2005). To circumvent this issue, Bäckdahl et al. (Backdahl et al., 2006) synthesized porous BNC scaffolds by introducing porogens as space holders in the culture medium, and subsequently removing them; leaving empty pores in the BNC network. They reported tunable pore size and pore interconnectivity by controlling the porogen size and density, as well as better ingrowth of cells in comparison to similar studies on non-porous BNC pellicles. However, the tensile mechanical properties of these porous BNC scaffolds were drastically reduced (~5 MPa in native BNC compared to ~2 MPa in porous BNC) (Bodin et al., 2010). In future work, the approach developed by Bäckdahl et al. (Bäckdahl et al., 2008) will be combined with the one presented in this study, in order to create bilayer BNC scaffolds. These scaffolds will contain a high cellulose content BNC layer, tuned to the specific application, and a porous layer to support the cells and enhance ECM formation. As chondrocytes have been reported to proliferate in native BNC (Andersson et al., 2010; Svensson et al., 2005), these scaffolds will provide an adequate environment for chondrocyte proliferation in the porous region, as well as mechanical stability throughout the high cellulose content layer.

Besides the control of the implant porosity, it is key in the field of ear cartilage replacement, to control the external shape of the implant to obtain satisfying aesthetic results. Several investigators reported the production of BNC scaffolds with customized shapes (Bäckdahl et al., 2008; Bodin et al., 2007b; Czaja et al., 2004). For example, a tube-like cultivation bioreactor with oxygen-permeable walls has been developed to create hollow BNC tubes for use in blood vessel...
replacement (Bäckdahl et al., 2008; Klemm et al., 2001). The 3D BNC implant prototype (1% cellulose content) in the shape of a human auricle, presented in this study, demonstrates the ability of BNC to be produced in more complex 3D shapes. This was made possible through the use of a bottom-up biofabrication approach, which requires a mold to guide the bacteria to reproduce the large-scale features of the tissue of interest (figure 5). Most efforts in ear cartilage TE have used standardized auricle shapes (Lee et al., 2011; Ruszymah et al., 2011; Shieh et al., 2004; Zhou et al., 2011). The BNC implant prototype presented in this study was obtained from patient-specific MR image data which produces a result compatible with patient expectations. In order to match the mechanical properties of ear cartilage as measured in this study, one can scale-up the dimensions of the mold in the z-direction. This would allow the bacteria to synthesize enlarged ear-shaped BNC implants, which can then be compressed to the original ear size, and desired cellulose content. An alternate method for the creation of patient-specific ear scaffolds was presented by Liu et al. (Liu et al., 2010) who described the 3D printing of an ear scaffold using computed-tomography data. The advantages of using MRI are, firstly the absence of potential radiation damage to the patient, and secondly a better soft tissue contrast and hence the direct visualization of cartilage (figure 5). This makes it possible to produce BNC implants which mimic the shape of ear cartilage, rather than the whole ear.

An important caveat however, is the time required to produce patient-specific ear-shaped BNC implants, which is significantly long. One alternative would be to produce the ear-shaped BNC implants by compressing BNC pellicles in an ear mold to shape the pellicle into an ear. Once the pellicle is molded to the final shape, it can then be freeze-dried to physically crosslink the BNC network. This method of producing ear-shaped BNC implants will significantly shorten the
production time, as the BNC pellicles can be pre-produced and kept as an off the shelf product, which can then be compressed to the patient’s ear shape. This way the BNC production will not cause a delay in the production process of the implant. Consequently, future work will aim at producing 3D ear scaffolds mimicking the shape of ear cartilage only. These scaffolds will consist of a high cellulose content core surrounded by a porous layer, so as to combine mechanical stability and porosity to allow chondrocytes to proliferate throughout the porous part and synthesize ear cartilage matrix.

5. Conclusion

This study aimed to assess whether BNC is a mechanically appropriate implant material for ear cartilage replacement. Firstly, mechanical properties of native ear cartilage were measured in order to define a preliminary benchmark required for BNC ear cartilage replacement implants. Secondly, the compressive mechanical properties of BNC materials could be tuned to match the mechanical requirements of native human ear cartilage by increasing the effective cellulose content. The difference in relaxation kinetics is likely due to the lack of charge-dependent osmotic swelling pressure in the BNC construct, as opposed to the native ECM. This important property of fluid flow resistance in the BNC network may be improved by chemical modification or even after implantation; where a native ECM would be produced by the cells in the porous layer of the BNC scaffold. Additionally the ability of the material to be produced in patient-specific and complex 3D shapes, such as a human auricle, was demonstrated. Ongoing work focuses on further enhancing BNC mechanics, and on developing BNC scaffolds surrounded by a porous layer to provide adequate environment for chondrocyte proliferation. In conclusion, BNC
shows promise for engineering non-biodegradable ear implants with suitable mechanical properties and patient-specific shapes.

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9. Conflict of interest

All authors have no conflict of interest.

10. References


**Figure Captions**

Figure 1: (a) Schematic representation of a stress-time curve measured on a viscoelastic sample during stress-relaxation indentation. Two stain steps are represented. This curve displays the typical behavior of cartilage during stress-relaxation testing: at each strain step, a sudden stress increase is observed during compression, followed by relaxation to an equilibrium value. Relaxation half-life time $t_{1/2}$ is defined as the time needed for the stress to decrease from its maximum value at the first strain step ($\sigma_i$) to $(\sigma_i + \sigma_{eq})/2$, where $\sigma_{eq}$ is the equilibrium stress at the end of the first strain step. Characteristic relaxation time $\tau$ is obtained by fitting an exponential decay function $\sigma_{fitted}$ to the stress-time curve. $t_{1/2}$ and $\tau$ provide measures of fluid flow through the matrix network. (b) Example of a stress-time curve measured on one human auricular cartilage sample (male, 85 year old) during stress-relaxation indentation with 5 successive strain steps (5%, 10%, 15%, 20%, 25%) and a Ø0.35 mm indenter.

Figure 2: (a) The bioprinter was placed inside a laminar air flow hood during the biofabrication of a patient-specific ear-shaped BNC implant. The bioprinter consists of (a) a high precision motion system and (b) a micro-dispensing system. (c) The software NI Motion Assistant 2.7 was used to program the controllers of the motion and micro-dispensing systems.

Figure 3: SEM images of BNC with (a) 1% effective cellulose content, (b) 5% and (c) 10%. Cellulose fiber (approximately 30 nm wide) network is visible and fiber spacing is observed to decrease with increasing cellulose concentration.
Figure 4: (a) Instantaneous modulus, $E_{in}$, of BNC ($n_{total} = 78$) correlates with effective cellulose content ($R = 0.66$), $6.4 \pm 3.2$ MPa is measured for $E_{in}$ of ear cartilage ($n = 17$). (b) Equilibrium modulus, $E_{eq}$, of BNC correlates with effective cellulose content ($R = 0.79$), $3.3 \pm 1.3$ MPa is measured for $E_{eq}$ of ear cartilage. (c) Maximum stress, $\sigma_{max}$, of BNC correlates with effective cellulose content ($R = 0.78$), $1.6 \pm 0.5$ MPa is measured for $\sigma_{max}$ of ear cartilage. (d) Relaxation half-life time, $t_{1/2}$, of BNC is not influenced by the effective cellulose content, significant differences ($p < 0.05$) are observed between BNC and ear cartilage ($0.4 \pm 0.1$ s and $2.1 \pm 1.3$ s, respectively). (e) Characteristic relaxation time, $\tau$, of BNC is not influenced by the effective cellulose content, significant differences ($p < 0.05$) are observed between BNC and ear cartilage ($3.7 \pm 1.0$ s and $6.0 \pm 2.6$ s, respectively).

Figure 5: (a) Transverse slice isolated from a spoiled gradient-echo MRI scan of volunteer’s left ear. Auricular cartilage is visible (dashed contour) surrounded by adipose tissue and skin. Plain contour indicates the external outline of the auricle. (b) 3D rendering of the external ear shape (white color) and of the auricular cartilage shape (red color) obtained by manual segmentation of the previous MRI scan. (c) Negative silicone mold used to guide the bacteria during the bacterial culture to reproduce the large-scale features of the outer ear. (d) 3D BNC implant prototype (1% effective cellulose content) produced in the shape of the whole outer ear using the dataset presented in (b).
Figure 1

(a) \[ \sigma(t) \]

1\textsuperscript{st} strain step

2\textsuperscript{nd} strain step

\[
\sigma_fitted(t) = \frac{\sigma_i + \sigma_{eq}}{2}
\]

(b) Stress (MPa)

Stress vs. time (min)

5\% strain

10\% strain

15\% strain

20\% strain

25\% strain
Figure 4

(a) Instantaneous modulus (MPa) vs. Effective cellulose content $CC_e$

(b) Equilibrium modulus (MPa) vs. Effective cellulose content $CC_e$

(c) Maximum stress (MPa) vs. Effective cellulose content $CC_e$

(d) Relaxation half-life time (s) vs. Effective cellulose content $CC_e$

(e) Characteristic relaxation time (s) vs. Effective cellulose content $CC_e$

- BNC
- Auricular cartilage
Figure 5