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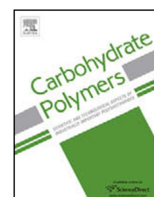

# Producing ultrapure wood cellulose nanofibrils and evaluating the cytotoxicity using human skin cells

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ABSTRACT

Wood cellulose nanofibrils (CNF) have been suggested as a potential wound healing material, but its utilization is limited by FDA requirements regarding endotoxin levels. In this study a method using sodium hydroxide followed by TEMPO mediated oxidation was developed to produce ultrapure cellulose nanofibrils, with an endotoxin level of 45 endotoxin units/g (EU/g) cellulose. Scanning transmission electron microscopy (S(T)EM) revealed a highly nanofibrillated structure (lateral width of 3.7 ± 1.3 nm).

Assessment of cytotoxicity and metabolic activity on Normal Human Dermal Fibroblasts and Human Epidermal Keratinocytes was done. CNF-dispersion of 50 μg/ml did not affect the cells. CNF-aerogels induced a reduction of metabolic activity by the fibroblasts and keratinocytes, but no significant cell death. Cytokine profiling revealed no induction of the 27 cytokines tested upon exposure to CNF. The moisture-holding capacity of aerogels was relatively high (~7500%), compared to a commercially available wound dressing (~2500%), indicating that the CNF material is promising as dressing material for management of wounds with a moderate to high amount of exudate.

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## 1. Introduction

Wound healing is a complex process involving several phases and a range of cells and cytokines (Reinke & Sorg, 2011). In order to facilitate the wound healing process, a range of natural biomaterials have been developed, based on cellulose, alginate, collagen and chitin, among others (Nakagawa et al., 2003). Bacterial cellulose is produced by some types of bacteria, such as *Gluconacetobacter* (Inder & Brown, 2012), and has been suggested for various medical applications, such as wound dressings (Czaja, Krystynowicz, Bielecki, & Brown, 2006; Jorfi & Foster, 2015; Petersen & Gatenholm, 2011; Portal, Clark, & Levinson, 2009). The production is currently not economically competitive compared with other more established polymers in most applications (Dana & Nadine, 2012). Hence, nanocellulose from wood could be a good alternative to bacterial cellulose, but needs better characterization regarding cytotoxicity and inflammatory potential since it in addition to cellulose also contains lignin and hemicellulose. Contamination of bacterial products (endotoxins) is known to trigger inflammation, and need evaluation.

Nanocellulose from wood is a novel material, including cellulose nanocrystals (CNC) and cellulose nanofibrils (CNF). It can be efficiently produced in large quantities, following a series of well-established procedures (Klemm et al., 2011; Saito, Nishiyama, Putaux, Vignon, & Isogai, 2006; Wågberg et al., 2008). CNF produced with chemical pre-treatments are nano-objects with widths less than 20 nm and lengths in the micrometer scale (Chinga-Carrasco, 2011), with advantages such as maintaining a moist environment, being strong and forming translucent structures. CNF has thus attracted much attention as a biomaterial for biomedical applications (Klemm et al., 2011; Kollar et al., 2011; Lin & Dufresne, 2014). However, in order to use CNF in contact with the human body, ultra-

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pure CNF qualities are required, with low levels of endotoxins such as bacterially derived lipopolysaccharides (LPS).

LPS are common contaminators of naturally derived materials and potent activators of inflammatory cytokines in various human cells (Gorbet & Sefton, 2005; Grimstad et al., 2011). Unpurified or inadequately purified materials containing LPS might lead to unwanted inflammatory responses, and further compromise the biocompatibility (Gorbet & Sefton, 2005). The current FDA limits for endotoxin contamination is <20,0 EU/medical device (United States Pharmacopeia and National Formulary, 2006b), or 5 EU/kg of body weight for parenteral drugs (United States Pharmacopeia and National Formulary, 2006a). Endotoxins can be destroyed using high temperatures (>250 °C for more than 30 min), or alkalis or acids of at least 0,1 M strength (Gorbet & Sefton, 2005; Magalhães et al., 2007), with NaOH commonly used for purification of bacterial cellulose (Bodin et al., 2010; Cherian et al., 2013; Chiaoprakobkij, Sanchavanakit, Subbalekha, Pavasant, & Phisalaphong, 2011; Helenius et al., 2006; Maneerung, Tokura, & Rujiravanit, 2008; Saska et al., 2012).

Another important aspect when producing materials for medical application is the potential cytotoxicity, which might compromise a healing process. While CNF materials have been compatible with different cell lines (no cytotoxicity) (Alexandrescu, Syverud, Gatti & Chinga-Carrasco, 2013; Bhattacharya et al., 2012; Hua et al., 2014; Lou et al., 2014; Malinen et al., 2014; Tehrani, Nordli, Pukstad, Gethin & Chinga-Carrasco, 2016; Vartiainen et al., 2011), a concentration dependent reduction in metabolic activity and/or cell proliferation has also been seen (Čolić, Mihajlović, Mathew, Naseri, & Kokol, 2015).

TEMPO-mediated oxidation and carboxymethylation are relatively common pre-treatments applied to facilitate the deconstruction of the fibre cell wall and thus the production of CNF (Saito et al., 2006; Wågberg et al., 2008). TEMPO and carboxymethylated CNF from *Pinus radiata* pulp fibres have been applied in a series of studies focusing on biomedical applications (Chinga-Carrasco & Syverud, 2014; Powell et al., 2016; Rees et al., 2014; Syverud, Kirsebom, Hajizadeh, & Chinga-Carrasco, 2011; Tehrani, Nordli, Pukstad, Gethin & Chinga-Carrasco, 2016). However, to the best of our knowledge, the production of ultrapure CNF and its effect on cytokine stimulatory responses from human skin cells have not yet been reported, aspects that are highly relevant for wound dressing materials.

In the present study we describe an updated method, based on a TEMPO mediated oxidation pretreatment, for the production of ultrapure CNF. Further, cytotoxicity and metabolic activity of primary Human Epidermal Keratinocytes, and Normal Human Dermal Fibroblasts exposed to the purified material was evaluated, both cell types of relevance for wound healing. Water holding capacity, an important property of wound dressings, was also assessed.

## 2. Materials and methods

### 2.1. Cellulose nanofibrils (CNF) production

The raw material was never dried, fully bleached, 100% *Pinus radiata* pulp fibers.

Carbohydrate composition of the pulp fibres have been reported previously by Chinga-Carrasco et al. (2012), being composed by 87% cellulose, 12.2% hemicellulose and 0.8% lignin. The fibers were washed with MQ water, filtrated and deionized water with resistance of 18.2 MΩ/cm, (25 l) on a Büchner funnel with filter cloth. Fibers (2.5%) were autoclaved in 0.1 M NaOH for two hours and then washed with MQ water (25 l). This was done a total of three times.

2,2,6,6-Tetramethylpiperidinyl-1-oxyl (TEMPO) mediated oxidation, using 3.8 mmol hypochlorite (NaClO) per gram cellulose was performed, which has been suggested as an appropriate amount of NaClO for an effective oxidation and fibrillation (Saito et al., 2006). The reaction time was approximately 30 min and was performed at room temperature. Following the same TEMPO mediated oxidation procedure, the degree of polymerization (DP), and the carboxyl and aldehyde content have been reported to be 709, 855 μmol/g cellulose and 71 μmol/g cellulose, respectively (Rees et al., 2014). Oxidized fibers were washed with MQ-water (30 l) on a Büchner funnel with filter cloth, before homogenization using an ultra-turrax, with 24 000 rpm for 6 min. The process yielded a translucent and viscous gel, which is a clear indication of CNF production. The concentration of the dispersion was 2%. Although the ultra-turrax is not as effective as e.g. high-pressure homogenizers for producing CNF, it is important to emphasize that the ultra-turrax equipment was used instead of a laboratory homogenizer in order to secure sterile conditions and avoid contamination of the produced ultrapure CNF quality. The ultra-turrax equipment was washed and left in 70% ethanol overnight, and further left in 0.1 M HCl for 1 h before rinsing with sterile water. For clarity purposes we will refer to the cellulose nanofibril material produced in this study as CNF, which is based on a TEMPO mediated oxidation.

AquaCel® (Convatec inc), a commercially available wound dressing consisting of sodium carboxymethylcellulose (hydrofibres), and AquaCel Ag® (AquaCel® with silver) was used as controls.

### 2.2. Structure characterization

From the suspension of CNF, films with a grammage of 20 g/m$^2$ were made in plastic petri dishes, and allowed to dry in room temperature. The manufactured films were considered an appropriate substrate for characterization, yielding a comprehensive assessment of the CNF components (Chinga-Carrasco et al., 2014; Chinga-Carrasco, Yu, & Diserud, 2011). CNF aerogels (20 g/m$^2$) were made by freeze-drying 0.2% suspension in petri dishes.

#### 2.2.1. Structural and optical quantification

Two samples (1 cm × 1 cm) were cut from the CNF film and mounted on the surface of glass slide, using a double-sided tape. The sample was sputtered (120 s) with a thin layer of gold (Agar Auto Sputter Coater). Ten Laser profilometry (LP) topography images were acquired from the top side of the film sample using a LP (Lehmann, Lehman Mess-Systeme AG Baden-Dättwil, Germany). The lateral and z-resolution of the LP system was 1 μm and 10 nm, respectively. The size of the local areas was 1 mm x 1 mm. The LP micro-roughness was described by the root-mean square (*Sq*). This method has been proven to be adequate to assess the micro-roughness of CNF films as described by (Chinga-Carrasco et al., 2014).

Scanning (transmission) electron microscopy (S(T)EM) was used to assess the nanofibril morphology. Cobber grids were immersed in a 0.01% suspension of the CNF sample and stained with uranyl acetate. The S(T)EM was a Hitachi S-5500 electron microscope. The acceleration voltage was 30 kV. The images were acquired in bright field mode. Totally, 104 randomized measurements of single CNFs were undertaken from 13 S(T)EM images.

Ultraviolet–visible (UV–vis) transmittance of light through the CNF film was quantified with a UV–vis spectrophotometer (Cary 300 Conc., Varian). In the analysis the wavelengths between 200 and 800 nm were included with two replicates.

#### 2.2.2. Water holding capacity

CNF aerogels and AquaCel® were cut into squares of approximately 1 × 1 cm. Samples were soaked in either phosphate-buffered saline (PBS) or MQ-water. At given time-points (5, 15, 30, 60, 120 and 240 min) the samples were lifted up with a pair

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