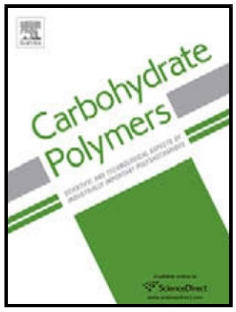

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Author: Henriette Rogstad Nordli Gary Chinga-Carrasco
Anne Mari Rokstad Brita Pukstad

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Producing Ultrapure Wood Cellulose Nanofibrils and Evaluating the Cytotoxicity Using Human Skin Cells

Author list:

Henriette Rogstad Nordli[a], Gary Chinga-Carrasco[b], Anne Mari Rokstad[c,d], Brita Pukstad[a,e]

[a]Department of Cancer Research and Molecular Medicine, Norwegian University of Science and Technology (NTNU), Trondheim, Norway

[b]PFI, Høgskoleringen 6b, NO-7491 Trondheim, Norway (gary.chinga.carrasco@pfi.no)

[c]Centre of Molecular Inflammation Research, and Department of Cancer Research and Molecular Medicine, Norwegian University of Science and Technology (NTNU), Trondheim, Norway (anne.m.rokstad@ntnu.no)

[d]Liaison Committee between the Central Norway Regional Health Authority (RHA) and the Norwegian University of Science and Technology (NTNU), Norway

[e]Department of Dermatology, St. Olavs Hospital HF, Trondheim University Hospital, Trondheim, Norway (brita.pukstad@ntnu.no)

Corresponding author:

Henriette Rogstad Nordli

E-mail: henriette.r.nordli@ntnu.no

Phone work: +4772825285

Mobile: +4791834341

Address: Faculty of Medicine

Department of Cancer Research and Molecular Medicine

Postboks 8905

N-7491 Trondheim

NORWAY

Highlights

- Ultrapure wood cellulose nanofibrils have been produced with a final endotoxin level of 45 endotoxin units/g cellulose.
- Cellulose nanofibril dispersion (50 μg/ml) did not affect the cytotoxicity or metabolic activity of Normal Human Dermal Fibroblasts and Human Epidermal Keratinocytes
- Aerogels made of cellulose nanofibrils induced a reduction of metabolic activity by the fibroblasts and keratinocytes, but no significant cell death
- Cytokine profiling measuring 27 cytokines revealed that the keratinocytes and fibroblasts did not induce cytokines upon direct exposure to the CNF materials.
- Due to the nano dimension of the CNFs, the aerogels had a high moisture-holding capacity (~7500%)

Abstract

Wood cellulose nanofibrils 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 (CNF), 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.

Keywords

Ultrapure cellulose nanofibrils, endotoxins, keratinocytes, fibroblasts, cytotoxicity

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 Jr, 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, Decher, Norgren, Lindström, Ankerfors & Axnäs, 2008). CNF produced with chemical pre-treatments are nanoobjects 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, ultrapure 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 (USP, 2011b), or 5 EU/kg of body weight for parenteral drugs (USP, 2011a). Endotoxins can be destroyed using high temperatures (>250 ºC for more than 30 min), or alkalis or acids of at least 0,1 M strength (Gorbet & Sefton, 2005; Magalhães, Lopes, Mazzola, Rangel-Yagui, Penna & Pessoa Jr, 2007), with NaOH commonly used for purification of bacterial cellulose (Bodin, Bharadwaj, Wu, Gatenholm, Atala & Zhang, 2010; Cherian et al., 2013; Chiaoprakobkij, Sanchavanakit, Subbalekha, Pavasant & Phisalaphong, 2011; Helenius, Bäckdahl, Bodin, Nannmark, Gatenholm & Risberg, 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; 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, Nishiyama, Putaux, Vignon & Isogai, 2006; Wågberg, Decher, Norgren, Lindström, Ankerfors & Axnäs, 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, Khan, Chinga-Carrasco, Wright, Hill & Thomas, 2016; Rees et al., 2014; Syverud, Kirsebom, Hajizadeh & Chinga-Carrasco, 2011; Tehrani, Nordli, Pukstad, Gethin & Chinga-Carrasco). 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 & Methods

2.1 Cellulose nanofibrils (CNF) production

The raw material was a 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, Nishiyama, Putaux, Vignon & Isogai, 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 hour 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 x 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 second) 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 1x1 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 of tweezers and weighed. Water holding capacity was estimated based on Eq. 1.

$$\text{Water holding capacity} = \frac{w_l - w_d}{w_d} \times 100 \qquad (1)$$

where w_l is the weight of the hydrogel immersed in the liquid (water or PBS) and w_d is the weight of the aerogel in its dry form.

2.3 Ultrapure material

2.3.1 Endotoxin testing

The QCL-1000 120 Endpoint Chromogenic LAL Assay (Lonza) was modified to reduce reagent volumes. All volumes were halved and the assay was performed in Costar 96-well half-area plates. The protocol was followed according to the kit. Values are given as endotoxin units/g (EU/g) of cellulose based on a standard curve.

To assess LPS-concentration in the fiber-samples, the filtrate after autoclaving the fibers (2.5 %, 20 min) in sterile water was tested. For CNF dispersions LPS-levels was quantified directly in dispersion after diluting the dispersion in sterile water giving a final concentration of 0.5 %. Aerogels of freeze dried CNF were tested by having punch biopsies (8 mm)

incubated in 500 µl PBS for 72 hours. Samples were removed and the LPS-level in the PBS was tested.

2.4 Cytotoxicity testing

2.4.1 Preparation of samples and controls

CNF dispersions were freeze dried (-20 ºC) in petri dishes yielding a porous material with grammage of 20 g/m^2. Punch biopsies (3 or 8 mm) were used to punch out small circular samples. AquaCel® with and without silver were used as positive and negative controls, respectively.

2.4.2 Cell cultures

Human Epidermal Keratinocytes, adult (Life Technologies) were cultured in EpiLife medium with Human Keratinocyte Growth Supplement (HKGS) and gentamicin/amphotericin (Life Technologies). Normal Human Dermal Fibroblasts (Clonetics), were cultured in Dulbecco modified Eagle's medium (DMEM, Lonza) with 10 % FCS, L-glutamine, 1 µg/ml insulin, penicillin, streptomycin and 1 ng/ml basic fibroblast growth factor (bFGF) (Sigma-Aldrich). Cells were seeded in 75 cm^2 culture flasks with 10 ml medium and trypsinised after reaching 70-90 % cell confluence, for use in experiments or for further culturing. During the cytotoxicity assay cells were grown in low condition medium for keratinocytes, implying the lack of HKGS. For the fibroblasts the amount of FCS was reduced to 1 %. The low condition medium was used to avoid interference of serum with the LDH-assay.

2.4.3 Cytotoxicity assay

The experiments were performed in the line of the ISO 10993-5 standards. Briefly, cells were seeded in 96 well plates in a concentration of 5x10^3 cells/well and further incubated three-four

days until the cells were 80-90 % confluent. Thereafter the medium was replaced by addition of 200 µl/well fresh low condition medium. CNF aerogels and wound dressing samples (3 mm diameter) were washed 3 times in PBS and further soaked in 50 µl PBS. The samples together with presoak solution were added into the wells (six replicates), including the control, giving a final volume of 250 µl/well. The dispersion of CNF (20 mg/ml) was diluted four times in PBS (5 mg/ml), and further in medium and added to the wells at a concentration of 50 µg/ml, 200 µl/well. This was compared to a cell control without CNF dispersion (0 µg/ml). As a control, Triton X-100 was diluted in medium and added to the wells giving a final concentration of 1 %. After two, six and 24 hours incubation the CNF and wound dressing samples were removed and the supernatant transferred to a new well plate.

Lactate dehydrogenase (LDH)

100 µl supernatant was transferred to a well plate. 100 µl of LDH reaction mixture (Cytotoxicity Detection Kit (LDH), Roche) was added to each well. The plate was incubated for 30 min at room temperature before 50 µl of 1 M HCl was added to each well. The absorbance was measured at 490 nm.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT)

The metabolic activity assay MTT was used for viability/proliferation measurements during the 2-24 h incubation. New medium (100 µl/well) and subsequently MTT was added to the cells to a final concentration of 10 %. After incubation for two-three hours, the medium was removed and the formazan crystals were solubilized by addition of isopropanol with HCl (50 µl/well). The absorbance was measured at 570 nm.

2.4.4 Bio-Plex immunoassay

Supernatants were harvested from cell incubated for 24 h in low condition medium using Bio-Plex Human Cytokine 27-Plex Panel (Bio-Rad, Oslo, Norway) for quantification of TNFα, IFN-γ, IL-1β, IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-7, CXCL8 (IL-8), IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, MCP-1, IP-10, RANTES, MIP-1α, MIP-1β, G-CSF, GM-CSF, Eotaxin, bFGF, VEGF and PDGF-BB according to the manufacturer's protocol. Cytokines selected were based on the following criteria; significant difference response between the material and the cell control, independent of a significant difference measured between the medium and cell control. Any of the other detected cytokines that did not fulfill the set criteria's are presented in the supplement (Figure S2).

2.5 Statistical Analysis

Results were analyzed using GraphPad Prism Software, version 5. Each sample in the cytotoxic test was compared with the cell control at the corresponding time-point (n=3, 6 replicates, supplementary Figure S1). A two-way ANOVA followed by Bonferroni post test was chosen to take into account the three time points in addition to the various samples. Each cytokine measured in the different samples were compared with the control (n=3, 2 replicates) and a one-way ANOVA followed by Dunnet's multiple comparison test was chosen for this. Values at $p<0.05$ or less were considered statistically significant.

3. Results

3.1 Ultrapure cellulose nanofibrils (CNF)

The production of the ultrapure CNF material (<100 EU/g) is shown in Table 1. The sodium hydroxide treatment and washing with MQ-water yielded a large decrease in LPS-level from 8240 EU/g to 237 EU/g. The subsequent TEMPO mediated oxidation procedure reduced the

LPS-concentration to 45 EU/g. The final LPS concentration of the freeze-dried material was 2.1 EU/g.

Table 1: Decrease in LPS-level during purification.

Sample	Treatment	LPS conc. (EU/g cellulose)
Fiber	Non	8240
Fiber	3 x 0.1 M NaOH	237
Ultrapure CNF	TEMPO mediated oxidation	45
CNF aerogel	Freeze-dried	2.1

3.2 Structure characterization

According to Chinga-Carrasco et al. (2014) the micro-roughness of air-dried films is an important characteristic as this indicates the degree of fibrillation of the CNF. Considering the micro-roughness of the films (LP roughness=2.3 μm ±0.17 μm) a fraction of approximately 25% residual fibers was thus estimated (Chinga-Carrasco et al., 2014). Additionally, the CNF films were translucent with a light transmittance of 89 %, confirming a highly nanofibrillated material. A major fraction of CNFs with widths of 3.7 nm (±1.3 nm) was quantified by S(T)EM (Figure 1, Table 2).

Table 2: Morphological analysis of CNF films and individualized nanofibrils.

Parameter	Method	Quantity
LP Roughness of films	Laser profilometry	2.3 μm (±0.17 μm)
Light transmittance (600 nm) of films	UV-vis spectrophotometry	89 %
CNF widths	S(T)EM	3.7 nm (±1.3 nm)

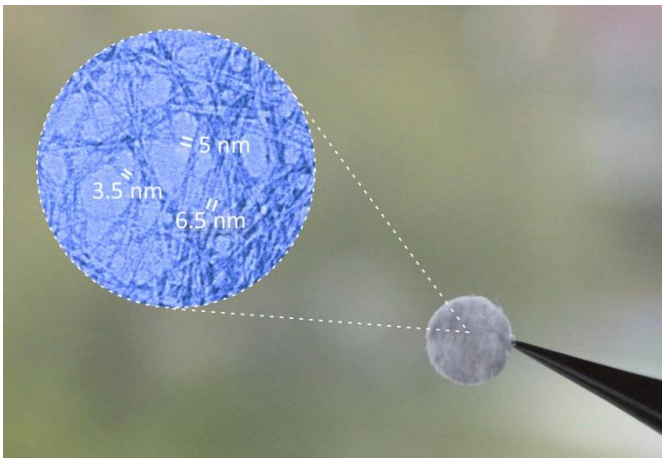

Figure 1: Left: STEM image of the morphology of the CNF. Right: CNF freeze dried aerogel, 8 mm punch biopsy sample.

The water holding capacity of CNF aerogels was compared with AquaCel® (Figure 2). The CNF aerogels were able to hold water or PBS more than 7500 % of the dry weight. AquaCel® samples were able to hold 2500 % PBS of its dry weight. In water, AquaCel® kept on swelling up to over 4000 % of its dry weight, before it disintegrated.

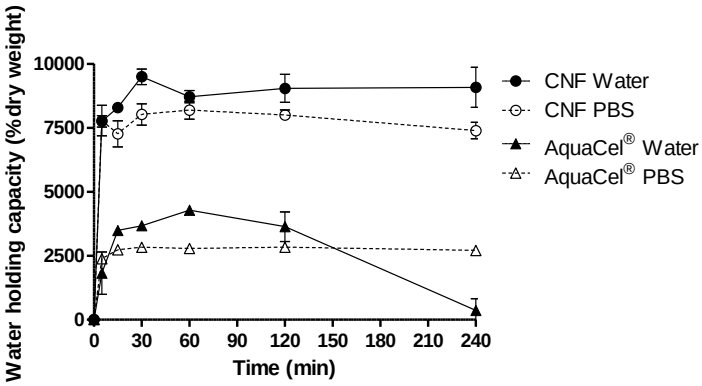

Figure 2: Water holding capacity of CNF aerogel and AquaCel® in water or PBS. The means are given with the corresponding standard errors of the means (SEM).

3.3 Human skin cell assessment

3.3.1 Cytotoxicity testing

The cytotoxic effects of CNF aerogels and dispersion were tested towards Normal Human Dermal Fibroblasts (fibroblasts) and Human Epidermal Keratinocytes (keratinocytes) (Figures 3 and 4), using AquaCel® with and without silver for comparison. It was detected an increase in the metabolic activity between two and 24 h after addition of the CNF aerogel and dispersion by the fibroblasts (Figure 3), and between 6 and 24 h by the keratinocytes (Figure 4). Compared to the control, a slight, though significant ($p<0.001$) decrease in metabolic activity (MTT assay) was found in the fibroblasts in the presence of CNF aerogels and AquaCel® after 6 and 24 h incubation (Figure 3A). However, no increase in LDH-activity, was detected after 24 h incubation (Figure 3B), and thus no indication of cell-death was found. AquaCel Ag® reduced the metabolic activity substantially (Figure 3A), but the LDH-activity was still low (Figure 3B). For the CNF dispersion the metabolic activity was comparable and non-significant different from the control, and no increase in the LDH activity was detected (Figure 3).

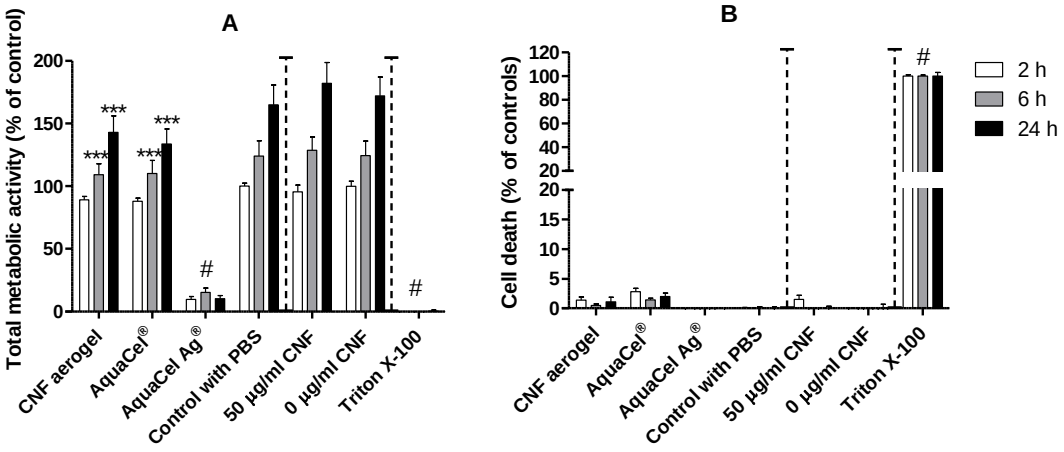

Figure 3: Cytotoxicity of CNF samples and wound dressings towards Normal Human Dermal Fibroblasts. A) MTT assay. Values are given as percentages of the corresponding control at the 2 h time point (n=3, 6 replicates). Significance levels are based on percentages of control in each time point (Supplement Figure S1). B) LDH assay. Values are given as percentages of control (0 %) and triton X-100 (100 %) within each time point (n=3, 6 replicates). The means and standard errors of the means (SEM) are shown. *** p<0.001, # for all three time points p<0.001.

For the keratinocytes, a significant decrease in the metabolic activity was found in the presence of CNF aerogels and AquaCel® (Figure 4A) after 2 hours, as compared to the control. This was also confirmed by the slight increase in LDH-activity (Figure 4B). AquaCel Ag® showed a substantially reduction in metabolic activity, without a correspondingly increase in the LDH activity (Figure 4B). For the CNF dispersion, the metabolic activity was comparable to the control, and no toxic effect was detected for the keratinocytes in the presence of CNF dispersion (Figure 4).

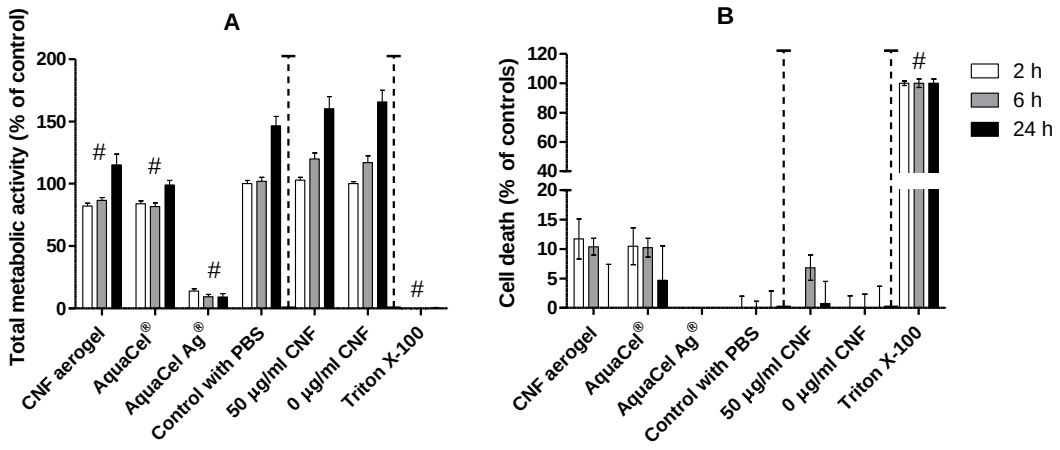

Figure 4: Cytotoxicity of CNF samples and wound dressings towards Human Epidermal Keratinocytes. A) MTT assays. Values are given as percentages of the corresponding control at the 2 h time point (n=3, 6 replicates). Significance levels are based on percentages of control in each time point (Supplement Figure S1). B) LDH assay. Values are given as percentages of control (0 %) and triton X-100 (100 %) within each time point (n=3, 6 replicates). The means and standard errors of the means (SEM) are shown. # for all three time points p<0.001.

Images of fibroblast and keratinocyte cultures after 24 h incubation are shown in Figure 5. In the presence of CNF aerogel and dispersion and AquaCel® a normal morphology was observed, whereas a changed morphology with few fibroblasts and few and rounded keratinocytes was found in the presence of AquaCel Ag®.

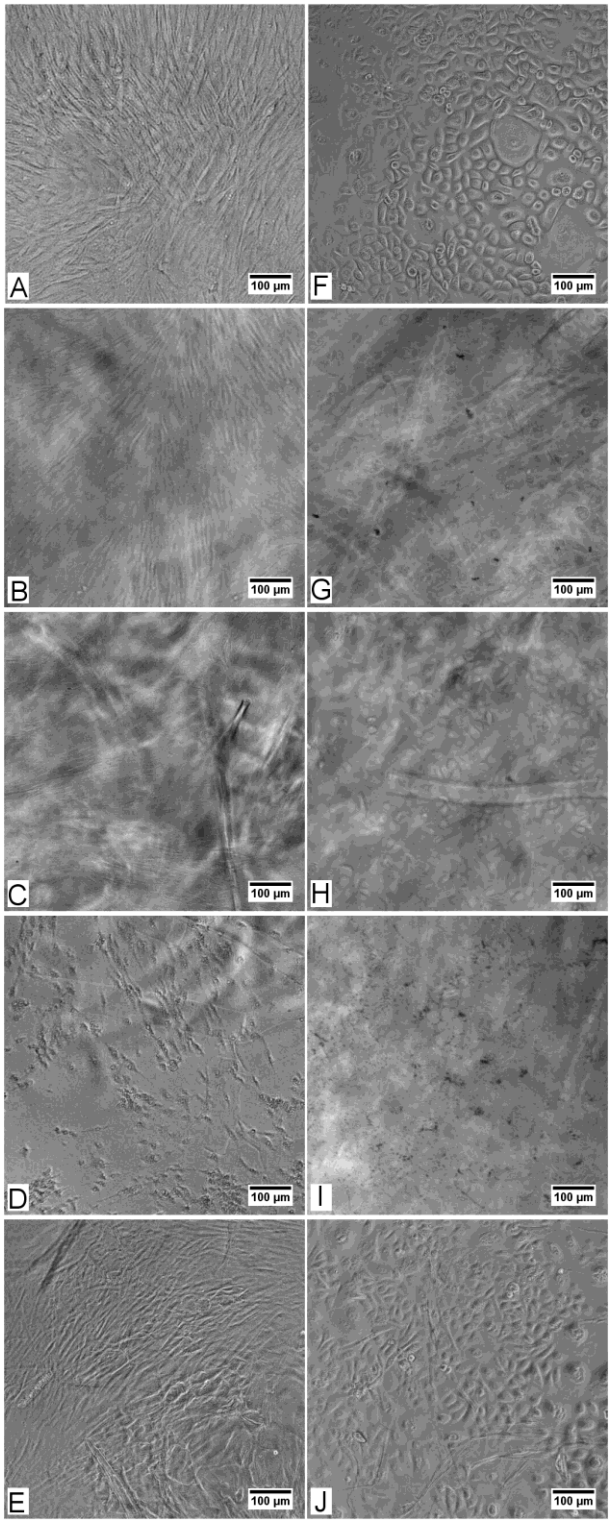

Figure 5: Skin cells after 24 h incubation covered with CNF aerogel and AquaCel®. A-E: Fibroblasts. F-J: Keratinocytes. A and F: Control. B and G: CNF aerogel. C and H: AquaCel®. D and I: AquaCel Ag®. E and J 50 µg/ml CNF. Scale bar, 100 µm.

3.3.2 Cytokine response

Significant difference between the biomaterial and the cell control were only found for some cytokines (Figure 6). For CNF aerogels, a significant lower concentration of IL-10, IL-12(p70), IP-10 and VEGF was found for the keratinocyte. Correspondingly, lower values were detected for the CNF dispersion, with in addition a significant reduction of MIP-1β. For the fibroblasts, no differences were found after addition of CNF aerogels, while the dispersed CNF gave a significant reduction of IL-8. For AquaCel®, the keratinocytes responded with a significantly elevated G-CSF and PDGF-BB, and a significantly lowered VEGF response. Further on, the fibroblast responded with significantly elevated IL-8 and bFGF. The CNF materials did not in any cases give cytokine secretion above the secreted levels from the control cells. For a total overview of all cytokines, and responses to CNF, se Figure S2 (Supplementary).

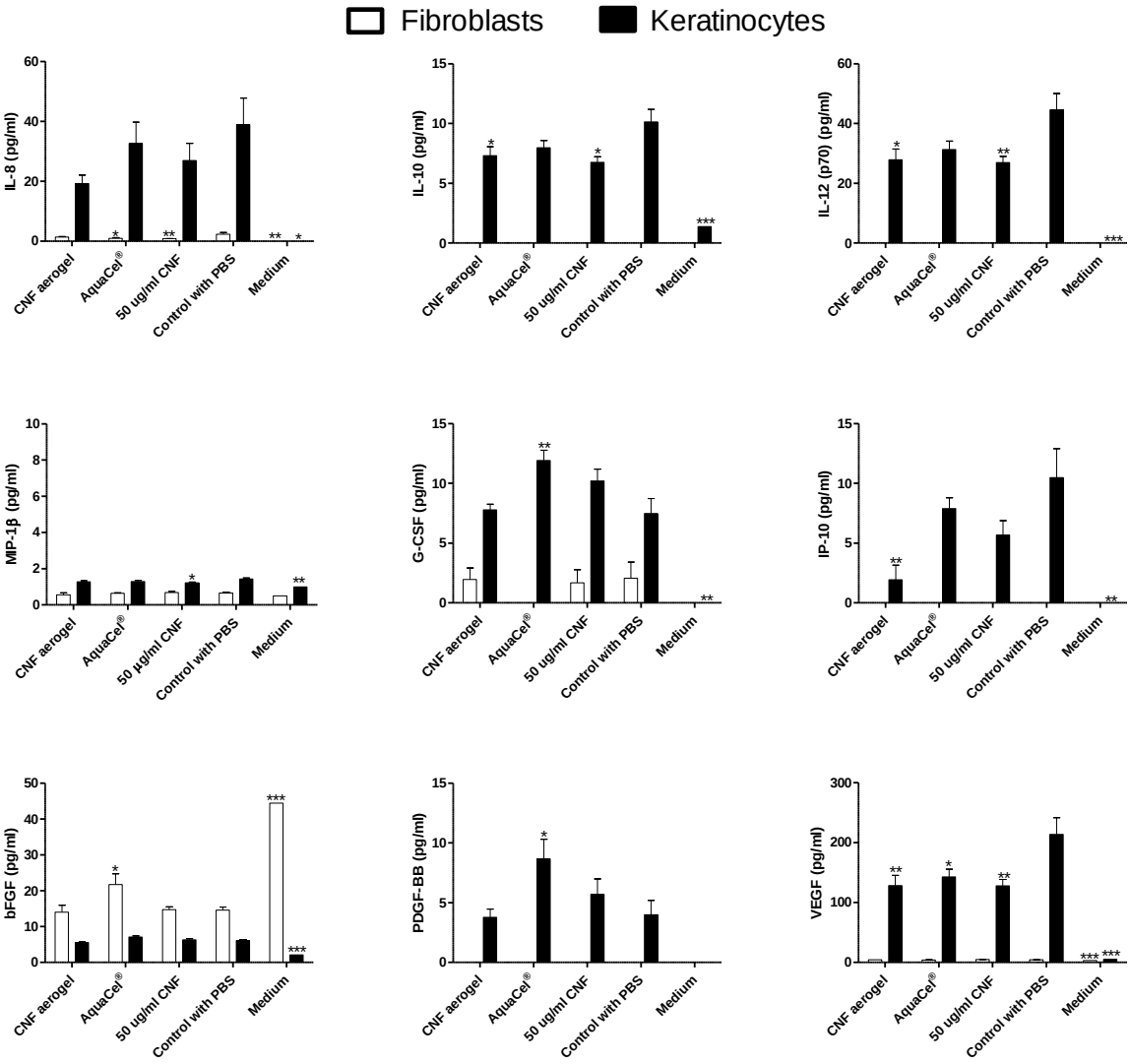

Figure 6: Secreted cytokines by fibroblasts or keratinocytes after 24 hours growth in the presence of CNF aerogels, AquaCel®, CNF dispersion or without material (control). Medium represents the background value without cells. The means of three independent experiments with standard errors of the means (SEM) are shown. Significant different (* p<0.05, ** p<0.01, *** p<0.001) as compared to the control.

4. Discussion

When assessing biomaterial biocompatibility, ideally the material should be free from endotoxins (Gorbet & Sefton, 2005). The term "endotoxin" is mostly used synonymously with LPS, which is a potent inducer of inflammation. In combination with the biomaterial, it tends to escalate a fibrotic response or a foreign body reaction, thus leading to bio-

incompatibility (Franz, Rammelt, Scharnweber & Simon, 2011). FDA has strict regulations on contamination levels allowed in medical application (USP, 2011a, b). In this study, a method based on TEMPO mediated oxidation was modified for producing ultrapure cellulose nanofibrils (CNF) with endotoxin levels <50 EU/g. The achieved LPS levels are thus comparable to ultrapure alginates (<100 EU/g), which are currently commercially available for biomedical applications (NovaMatrix, 2015). Hence, the CNF assessed in this study is considered to be ultrapure. To the best of our knowledge this is the first time that ultrapure nanocellulose from wood is reported, since previous reports have described endotoxin levels above the limit of 100 EU/g (Čolić, Mihajlović, Mathew, Naseri & Kokol, 2015), or materials that are able to trigger TNF mRNA-expression which could indicate the presence of LPS (Vartiainen et al., 2011). This is a major achievement, establishing a concrete procedure which secures the production of CNF from wood with acceptable low LPS levels. Considering the potential application of CNF from wood in eg scaffolds and wound dressings (Chinga-Carrasco & Syverud, 2014; Rees et al., 2014), the production of an ultrapure CNF quality is a significant contribution in this respect.

NaOH treatment of fibers reduced the LPS-concentration (Table 1). However, production of an ultrapure material based only on a NaOH treatment implies that the number of treatments has to be doubled (data not shown here). In addition to facilitating a homogeneous fibrillation of cellulose fibers into CNF (Chinga-Carrasco, Yu & Diserud, 2011; Saito, Nishiyama, Putaux, Vignon & Isogai, 2006), the TEMPO mediated oxidation procedure reduced the LPS-concentration to an acceptable level, which is most valuable. It is important to note that the TEMPO mediated oxidation alone, without the previous NaOH treatment, did not yield an ultrapure CNF. Hence, the treatment introduced in this study implies a more effective process for producing an ultrapure CNF as the time consumption is reduced significantly. One

possible explanation for the positive effect of the TEMPO mediated oxidation procedure on the LPS reduction is that LPS is negatively charged (Gorbet & Sefton, 2005) and may be easier to wash out from the fibers after the oxidation process. In addition, the oxidation is performed under alkali conditions (pH 10.5), which may also be one factor contributing to the LPS-reduction (Gorbet & Sefton, 2005; Magalhães, Lopes, Mazzola, Rangel-Yagui, Penna & Pessoa Jr, 2007). After freeze-drying the LPS-amount released from the sample was further reduced (Table 1). This is probably due to LPS being trapped within the cellulose nano-structures (Figure 1).

To determine the cytotoxic effects of CNF aerogels the commercially available wound dressing AquaCel® was chosen as a control. CNF aerogels was compatible with fibroblasts since it was found an increase in metabolic activity by the cultivation time. The slight decrease in metabolic activity as compared to the controls was not associated with cell death. The decrease in metabolic activity might be related to a reduced cell proliferation. One possible explanation causing a reduced proliferation could be an increased mechanical stress provided by the aerogel itself, as has been demonstrated to affect the proliferative capacity for other cell-types in 3D matrixes (Rokstad et al., 2006; Rokstad et al., 2012). The larger reduction in metabolic activity by the keratinocytes might indicate that this cell type is more sensitive than the fibroblast, which is in consistence with Burd et al. (Burd et al., 2007). The CNF material was however not cytotoxic to the keratinocytes, and the cells showed the same morphology as the control cells, indicating its suitability as a wound dressing material. Our data are in consistence with the work done by Čolić et al (Čolić, Mihajlović, Mathew, Naseri & Kokol, 2015) showing reduced metabolic activity and cell proliferation, but low cytotoxicity for CNF. Also AquaCel® has previously been shown to give a reduction in cell proliferation, but not affecting survival in keratinocytes (Paddle-Ledinek, Nasa & Cleland,

2006). CNF dispersion (50 µg/ml) did not affect the fibroblasts or the keratinocytes during the 24 h incubation, showing that a potential leakage of CNFs in a wound could be acceptable.

The AquaCel Ag® gave large reduction in metabolic activity. However, no cell death could be detected with the LDH-toxicity assay. It has been shown that silver ions bind thiol groups and will inhibit lactate dehydrogenase by giving a conformational change to the enzyme (Rogers, 1972), thus this effect most likely leads to a false lack of LDH-activity in the AquaCel Ag® sample. Based on the MTT data one could expect approximately 100% cell death, which was supported by light microscope pictures. This is also supported by previous work showing that silver ions are toxic to skin cells *in vitro* (Barnea, Weiss & Gur, 2010; Burd et al., 2007; Paddle-Ledinek, Nasa & Cleland, 2006; Poon & Burd, 2004).

The CNF or AquaCel® materials had a low stimulatory ability on the fibroblast and keratinocyte ability to secrete cytokines under the current culturing conditions. In the measured panel of 27 cytokines, the CNF material did not induce any elevated cytokine responses. The findings might also be in consistence with the production of an ultrapure material, although we cannot exclude the possibility of sub-optimal culturing conditions utilizing fetal calf serum instead of human serum. Interestingly, the only detected differences between the materials and the control cells were a lowered response, suggesting a potential adsorption to the cellulose biomaterial. Several of the cytokines are positively charged at neutral pH (Helmy, Carpenter, Skepper, Kirkpatrick, Pickard & Hutchinson, 2009; Nagy, Dvorak & Dvorak, 2007; Yang et al., 2003), whereas the oxidized cellulose material contains carboxyl-groups, and hence is negatively charged (Saito, Nishiyama, Putaux, Vignon & Isogai, 2006). Another explanation could be the reduced metabolic activity as seen in both the keratinocytes and the fibroblasts after 24 h. Previous work has shown that fibroblasts tend to

secrete a range of cytokines upon LPS stimulation, whereas keratinocytes responds to polyI:C (Grimstad et al., 2011), in both cases illustrating their potential to secrete cytokines. The cytokine profile by the keratinocytes in the present study was comparable to the findings by Grimstad et al. (2011). A low inflammatory potential of CNF material has previously also been measured in human peripheral blood mononuclear cells with only IL-6 to be significantly elevated (Čolić, Mihajlović, Mathew, Naseri & Kokol, 2015). AquaCel® was the only material giving significant elevated cytokine responses of G-CSF and PDGF-BB, molecules involved in neutrophil stimulation and wound repair, respectively (Ansel et al., 1993; Sugiyama, Ishii, Ochiai & Esumi, 2008). Thus, it might be beneficial with a material that triggers these growth factors.

The most potent in vitro model for assessing an inflammatory potential of a biomaterial is the whole blood model (Rokstad et al., 2013; Rokstad, Strand, Espevik & Mollnes, 2013), and would be used in follow-up studies. As a third step of evaluation, tissue explant wound models (Kratz, 1998), and animal models would be valuable to assess the wound healing potential.

The CNF has a great capacity to absorb water, being the water holding capacity 3 times as much as the water holding capacity of the AquaCel® dressing used in this study. Note that contrary to AquaCel®, which disintegrated when immersed in water, the CNF aerogels were able to keep their shape and withstand the mechanical stress during the assessment period in the present study (Figure 2).

The water absorption capability of neat CNF aerogels is an interesting and promising characteristic for wound dressings. Highly absorptive wound dressings based on alginates

have a water holding capacity of 10-20 times their own dry weight (Wiegand, Tittelbach, Elsner & Hipler, 2015). Such wound dressings are recommended for management of wounds having moderate to high wound exudates. The CNF-based aerogel developed in this work had a water holding capacity of more than 70 times its own dry weight. CNF-based wound dressings may thus be a good alternative for management of chronic wounds having high amounts of exudates (Wiegand, Tittelbach, Elsner & Hipler, 2015).

5. Conclusion

A method to produce ultrapure wood CNF has been developed, using sodium hydroxide followed by TEMPO mediated oxidation, giving a biomaterial with < 50 endotoxin units/g. Cytotoxic assessment of the material towards Human Epidermal Keratinocytes and Normal Human Dermal Fibroblasts showed that the biomaterial was as safe as a commercially available wound dressing, AquaCel®. The cytotoxic testing, combined with the great capacity of the CNF aerogels to absorb water, confirms that the ultrapure CNF is a good candidate as wound dressing-material for the management of exudating chronic wounds.

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Disclosures

The authors declare that there is no conflict of interest regarding the publication of this paper.

Appendix A. Supplementary data

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