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Evaluating the Genotoxicity of Cellulose Nanofibrils in a Co-Culture of Human Lung Epithelial Cells and Monocyte-derived Macrophages

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Highlights

- Cellulose nanofibrils (CNF) produced from *Eucalyptus globulus* were characterized.
- A co-culture of lung alveolar cells and macrophages was used to analyse CNF toxicity.
- No secretion of proinflammatory cytokine IL-1β was detected in exposed co-cultures.
- Low CNF concentrations induced lung alveolar cells overgrowth.
- CNF was genotoxic at low, but not at high concentrations (micronucleus assay).

Abstract

Cellulose nanofibrils (CNF) are manufactured nanofibres that hold impressive expectations in forest, food, pharmaceutical, and biomedical industries. CNF production and applications are leading to an increased human exposure and thereby it is of utmost importance to assess its safety to health. In this study, we screened the cytotoxic, immunotoxic and genotoxic effects of a CNF produced by TEMPO-mediated oxidation of an industrial bleached *Eucalyptus globulus* kraft pulp on a co-culture of lung epithelial alveolar (A549) cells and monocyte-derived macrophages (THP-1 cells). The results indicated that low CNF concentrations can stimulate A549 cells proliferation, whereas higher concentrations are moderately toxic. Moreover, no proinflammatory cytokine IL-1β was detected in the co-culture medium suggesting no immunotoxicity. Although CNF treatment did not induce sizable levels of DNA damage in A549 cells, it leaded to micronuclei formation at 1.5 and 3 $\mu g/cm^2$. These findings suggest that this type of CNF is genotoxic through aneugenic or clastogenic mechanisms. Noteworthy, cell overgrowth and genotoxicity, which are events relevant for cell malignant transformation, were observed at low CNF concentration levels, which are more realistic and relevant for human exposure, e.g., in occupational settings.

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Keywords: Cellulose nanofibrils, safety assessment, immunotoxicity, comet assay, micronucleus assay

1. Introduction

Nanocellulose is an advanced material that exhibits unique characteristics, depending on the source and production method. These include high specific surface area, high aspect ratio (length to width ratio) and high tensile strength and stiffness, besides being renewable and biodegradable in nature (Eichhorn et al. 2010, Khalil et al. 2014, Nechyporchuk et al. 2016). Cellulose nanofibrils (CNF), also referred to as cellulose nanofibres or nanofibrillated cellulose, are usually obtained from wood, cotton, hemp, flax, sugar beet or potato tuber. Depending on the source and on the production method, the size of the fibrils can vary significantly, but usually nanofibrils are defined as materials with diameters inferior to 100 nm and lengths in the micrometer scale (TAPPI standard proposal WI3021, Chinga-Carrasco et al. 2011, Kangas et al. 2014). CNF are produced by intensive mechanical treatment, such as in a high-pressure homogenizer (Li et al. 2012, Osong et al. 2016; Siró et al. 2010), usually combined with a chemical or enzymatic pre-treatment to reduce energy consumption. One of the most effective pre-treatments is an oxidation mediated by 2,2,6,6-tetramethylpiperidine-1-oxyl radical (TEMPO) that introduces carboxylate and aldehyde functional groups in the cellulose fibres, making them highly negative and more suitable for their deconstruction process (Isogai et al. 2011; Lourenço et al. 2017; Saito and Isogai 2007). CNF exhibits exceptional high mechanical resistance and low density, being a prime candidate for strength-enhancement of the mechanical properties of other composite materials, such as paper, carton and packaging materials. They also have a wide array of applications in the form of gels or emulsions, e.g., as a rheology modifier. Due to its likely biocompatibility, CNF have been investigated in regenerative

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medicine as scaffolds for tissue-engineered meniscus, blood vessels, ligaments or tendons (Jia et al. 2013; Lin and Dufresne 2014; Mathew et al. 2012, 2013). Other biomedical applications of CNF are on wound healing (Basu et al. 2017; Hakkarainen et al. 2016; Jack et al. 2017; Sun et al. 2017; Syverud et al. 2011), stem cell decorated threads for surgical suturing (Mertaniemi et al. 2016), haemodialysis membranes (Ferraz et al. 2013), long-lasting sustained drug delivery systems (Kolakovic et al. 2012) or 3D cell culture scaffolds (Bhattacharya et al. 2012; Lou et al. 2014; Malinen et al. 2014).

The production of CNF at an industrial scale and its application in a multiplicity of products and biomedical devices can represent a potential hazard to workers along the lifecycle and to consumers, as well. Vartiainen et al. (2011) concluded that workers' exposure to particles in the air during grinding and spray drying of birch cellulose was low or non-existent with the implementation of appropriate protection equipment and proper handling. However, the high aspect ratio of CNF and its biodurability in the human lungs (Stefaniak et al. 2014) resembles the fibre paradigm that has been associated to the adverse effects of other fibrous nanomaterials (e.g., carbon nanotubes, CNT). Therefore, to ensure the safety of CNF to humans prior to their large-scale commercialization, it is of utmost importance to investigate their potential toxicological properties, particularly their genotoxicity that is closely associated to carcinogenicity.

Most toxicological studies have focused on nanocellulose types with morphological and surface chemical characteristics different from the above-mentioned CNF. These include bacterial nanocellulose (Jeong et al. 2010; Lin et al. 2014; Moreira et al. 2009; Pertile et al. 2012; Saska et al. 2012; Scarel-Caminaga et al. 2014) and nanocrystalline cellulose (Catalán et al. 2015; Clift et al. 2011; Dong et al. 2012; Kovacs et al. 2010; Shvedova et al. 2016; Yanamala et al. 2014). These nanocellulose types are generally considered as nontoxic, although nanocrystalline cellulose could induce low cytotoxicity and immunotoxicity *in vitro* and *in vivo*

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(Clift et al. 2011; Yanamala et al. 2014). Regarding CNF, the few published studies mainly indicate no relevant cytotoxic, genotoxic or immunotoxic effects (Alexandrescu et al. 2013; Colić et al. 2015; Nordli et al. 2016; Pitkanen et al. 2014; Vartiainen et al. 2011). Nevertheless, a recent study by Catalán et al. (2017) showed that C57Bl/6 mice exposure by pharyngeal aspiration to CNF produced through TEMPO oxidation led to an acute lung inflammatory response and induced DNA damage in lung cells. However, it cannot be completely excluded that the effects observed were related to the presence of LPS, given that no information about this issue is provided. Moreover, Lopes et al. (2017) reported that an unmodified CNF induced a pro-inflammatory effect in THP-1 macrophages that could be moderated by the introduction of surface modifications (Lopes et al. 2017). Thus, more investigation is clearly required to create a knowledge basis to assess the human health risk from exposure to CNF.

The present study aimed at investigating the immunotoxic and genotoxic effects of a CNF produced from industrial bleached *Eucalyptus globulus* kraft pulp fibrillated by a combination of high pressure homogenization with a preliminar oxidation mediated by TEMPO on a co-culture of A549 human lung epithelial alveolar cells and THP-1 monocyte-derived macrophages. Nowadays, it is recognized that co-culture systems best mimic the *in vivo* toxicological potential of nanomaterials and are more realistic models as compared to monocultures (Snyder-Talkington et al. 2012, 2015). Macrophages are well-recognized primary immune cells in the forefront of the defence system through the engulfment of foreign material from tissues, and alveolar macrophages can play a key role in the biological response to inhaled nanofibres. Histological analysis of mouse lung tissue has demonstrated CNF accumulation in the cytoplasm of lung macrophages (Catalán et al. 2017), and it has been suggested that the acidic pH of the macrophage phagolysosome is insufficient to degrade nanocellulose (Stefaniak et al. 2014). Thus, nanocelluloses are likely to be cleared by mechanical movement of macrophages out of the alveoli and eventually to the mucociliary escalator. This knowledge

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reinforces the relevance of incorporating THP-1 monocyte-derived macrophages in the A549 cell culture to reflect more realistically, in an *in vitro* system, the *in vivo* biological response to CNF exposure.

2. Materials and Methods

2.1 Nanocellulose production and characterization

Nanocelluloses were produced from industrial bleached *Eucalyptus globulus* kraft pulp. In order to fibrillate the pulp, an oxidation mediated by TEMPO was applied, according to a procedure described elsewhere (Lourenço et al. 2017; Saito and Isogai 2007), followed by mechanical treatment in a high-pressure homogenizer. For that, the pulp, previously refined at 4000 rev. PFI, was mixed with TEMPO (0.016 g/g of fibres) and NaBr (0.1 g/g of fibres) in demineralized water and, after proper mixing, a NaClO solution (9.7 % active chlorine) was slowly added (5 mM/g of fibre). The reaction was carried out for 2 hours with pH constant at 10 by adding NaOH 0.1 M. The resultant fibres were thoroughly washed with demineralized water until the suspension final conductivity was low (20 µS/cm). Finally, the pre-treated fibres were passed 2 times in the homogenizer (GEA Niro Soavi Model Panther NS3006L), the first one at 500 bar and the second one at 1000 bar, to reduce the size of the fibrillated fibres to the nanoscale. The final consistency of the nanocellulose aqueous suspension was 0.83 wt % exhibiting a gel-like behaviour.

The nanocellulose was characterized for its fibrillation yield, amount of carboxylic groups, degree of substitution, degree of polymerization and size. The yield was determined in duplicate by submitting a 0.2 wt % nanocellulose suspension to centrifugation at 9000 r/min for 30 min (8965 g) in a Hettich Universal 32. The yield was calculated as the percentage of supernatant material (w/w), corresponding to the nanofibrillated fraction of the sample (Gamelas et al. 2015). The concentration of carboxyl groups (C_{COOH}) was determined by a conductometric

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titration according to a methodology reported elsewhere (Lourenço et al. 2017): briefly, an aqueous suspension of nanocellulose (0.1 g dry weight) was well stirred and its pH was set to 3.0 with HCl. Then, a 0.01 M NaOH solution was added until pH 11. The carboxylate content was determined in triplicate from the conductivity curve and, from this value, it was possible to estimate the degree of substitution (DS), taking into account the molar masses of the anhydroglucose units and of units substituted at the C6 position by COO^-Na^+ groups, as explained elsewhere (Lourenço et al. 2017). The degree of polymerization (DP) was calculated using the Mark-Houwink equation with the parameters reported by Henriksson et al (2008). The intrinsic viscosity necessary for the calculations was determined by the cupri-ethylenediamine methodology (ISO standard 5351). The structure of the fibrils was assessed by Field emission-SEM (FE-SEM) on 20 g/m^2 films prepared by air-drying of a 0.2 % (w/v) nanocellulose suspension. The images were acquired at 500x magnification in a Carl Zeiss Merlin microscope, in secondary electron mode, using 1 kV voltage. Gold sputtering (3s) was previously performed. The fibrils diameter was assessed by atomic force microscopy (AFM) in a Bruker Innova microscope using the aforementioned films. The peak force-tapping mode was used with a tip radius of 8 nm. Several 2 x 2 µm scans were acquired and a mean diameter was computed using the Gwyddion software. As a complement to this measurement, a non-operator dependent technique, providing results more representative of the whole sample, since thousands of fibrils are analysed, was used - the Dynamic Light Scattering (DLS). For that, the supernatant of the aforementioned centrifugation was analysed in a Zetasizer Nano ZS equipment (Malvern Instruments) at a scattering angle of 173° and using the CONTIN algorithm to obtain the size distribution. The value reported corresponds to the smaller peak of the distribution. However, some caution needs to be taken considering that this technique is not suitable for particles with such a high aspect ratio as that of the nanocellulose. Nevertheless, it provides information that may be used for comparison purposes, namely with samples under

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similar conditions. Although the CNF was not tested for LPS contamination, previous studies by Nordli et al. (2016) showed that the TEMPO-mediated oxidation performed in alkaline conditions strongly reduces the LPS content in the sample, becoming easier to wash out from the fibres after the oxidation process. Because washing was performed exhaustively in this CNF production it is unlikely that LPS still persists in the sample.

2.2 Cell culture

The human alveolar epithelial cell line A549 (ATCC, Manassas, VA, USA, CCL-185) and the human monocytic leukaemia cell line THP-1 (ATCC, TIB-202) were both grown in RPMI 1640 medium (Gibco, Waltham, MA, USA) supplemented with 10 % heat-inactivated foetal bovine serum (FBS*i*) (Gibco), 1 % penicillin/streptomycin (1.000 U/mL penicillin and 10 mg/ml streptomycin), Gibco) and 1 % fungizone (0.25 mg/mL, Gibco,) at 37 ºC in an atmosphere of 5 % CO_2. The THP-1 monocytes were grown on transwell inserts with a nominal pore size of 0.4 µm (Greiner Bio-One GmbH, Kremsmünster, Austria) at a density of $0.2x10^5$ cells/mL and differentiated into macrophages by 48 hours incubation with 100 ng/mL of 12-O-tetradecanoylphorbol-13-acetate (TPA, Sigma-Aldrich). The medium was then removed and substituted by serum-free RPMI 1640 medium for further 48 hours, to allow cells to recover from the TPA effect. The A549 cells were cultured on 12–well plates at a density of 0.5×10^5 cells/mL and the inserts with differentiated THP-1 cells were placed directly on the top of the A549 cells. The resulting co-culture was incubated for further 24 hours in RPMI 1640 medium. To ensure that THP-1 and A549 cells were exposed to the same CNF concentrations (1.5, 3, 6, 12.5, and 25 µg/cm^2) the dispersions were added to the apical and basolateral sides of the insert whenever co-cultures were used.

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2.3 MTT assay

The MTT assay was performed according to Mossmann (1983) using three independent experiments. A549 cells were plated in 96-well plates and allowed to attach for 24 hours at 37 ºC and 5 % CO_2. The cells were then exposed for 24 hours or 48 hours to 1.5, 3, 6, 12.5, and 25 µg/cm^2 of CNF in culture medium. These concentrations were prepared from a stock solution at 1.5 mg/mL of a 0.872% CNF gel diluted in phosphate buffered saline (PBS) and correspond to the dry weight of the CNF. SDS (1 µg/mL, Sigma), 1 hour exposure, was used as a positive control. After washing twice with PBS, the cells were incubated for 2 hours with fresh growth medium containing 10 % of the MTT solution (5 mg/mL, Calbiochem, Darmstadt, Germany). The MTT-containing medium was discharged and DMSO (Sigma) was added for 20 min under shaking. The absorbance was recorded at 570 nm against a reference filter set at 690 nm using a Multiscan Ascent spectrophotometer (Labsystems, Helsinki, Finland). The relative cell survival of exposed cultures was expressed as the ratio between the absorbance of the exposed and unexposed cultures, assuming that the absorbance of the latter represents 100% cell survival.

2.4 Lactate dehydrogenase (LDH) assay

LDH determination was conducted in the supernatant removed from CNF-exposed cultures for 48 hours, used for the MTT assay. After centrifugation of the supernatant at 4000 g for 10 minutes, LDH concentration was measured using the CytoTox-ONE homogeneous membrane integrity assay (Promega, Madison, USA). A maximum LDH release control was performed by the addition of lysis solution to the untreated control cells before adding Cyto-tox ONE. The percentage of cytotoxicity was calculated as the ratio between the concentration of LDH in each

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supernatant (subtracting the culture medium background) and the maximum LDH release (subtracting the culture medium background) multiplied by 100.

2.5 Clonogenic assay

The clonogenic assay was performed as described by Herzog et al. (2007). Briefly, a very low density of A549 cells (100 cells) was plated in each well of a 6-well plate and allowed to attach for approximately 16 hours, at 37 ºC and 5 % CO_2. The cells were then exposed to 1.5, 3, 6, 12.5, 25, 50, and 100 µg/cm^2 of CNF. For each experiment, negative (non-treated cells) and positive (0.004 µg/mL mitomycin C, Sigma) controls were included. Cells were incubated for 8 days, at 37 ºC and 5 % CO_2 to allow colonies formation. The wells were then washed twice with PBS, fixed in absolute methanol (Sigma) and stained with 10 % Giemsa (Merck, Darmstadt, Germany) in phosphate buffer, pH 6.8. The number of colonies formed was counted and the plating efficiency (CE) was determined using the following equation (Herzog et al. 2007):

CE = 100 x (no. colonies in negative control / no. of plated cells)

The surviving fraction (SF) for each CNF concentration was calculated as follows:

SF = no. colonies formed after exposure / (no. of plated cells x CE/ 100)

The cytotoxicity was determined as the decrease in the SF in relation to the negative control, based on the results from three independent experiments.

2.6 Determination of IL-1β secretion

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Cell culture supernatants were collected after the 24 hour treatment for the comet assay and stored at −80 °C until analysis. The IL-1β concentration in the supernatants of the 1.5, 6 and 25 µg/cm^2 treatment with CNF was determined using a colorimetric sandwich ELISA method (IL-1β-EASIA Kit, Source, Louvain-la-Neuve, Belgium), according to the manufacturer's protocol. A positive control was prepared adding 100 ng/µL of lipopolysaccharide (LPS; Sigma) and 5 mM adenosine 5'-triphosphate disodium salt (ATP; Sigma) to the supernatant of the cell culture in the inserts (Park et al. 2007).

2.7 Comet assay

The A549 and THP-1 cells in co-culture were equally exposed for 24 hours to 1.5, 3, 6, 12.5, and 25 µg/cm^2 of CNF by adding the corresponding volume of the dispersion medium to both sides of the transwell inserts placed on 12-well plates. Ethyl methanesuphonate (EMS, 5mM, Sigma-Aldrich) with an exposure time of 1 hour was used as a positive control. The plates were washed with PBS and harvested after tripsinization. The comet assay was performed as described in Louro et al. (2016). Briefly, the cell suspensions were centrifuged (1200 r/min, 10 min, 4 ºC) and the pellets resuspended and embedded in 0.8 % low melting point agarose, then spread onto 1 % agarose-precoated microscope slides (2 gels per slide). Slides were immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10 % DMSO and 1 % Triton X-100, pH 10) for a minimum of 1 hour and washed twice with enzyme buffer (40 mM HEPES, 100 mM KCl, 0.5 mM EDTA, 0.2 mg/mL BSA, pH 8). The resultant agarose-embedded nucleoids were then treated either with enzyme buffer or with 50 µL of formamidopyrimidine DNA glycosylase (FpG, kindly provided by Dr. A. R. Collins, University of Oslo, Norway), for 30 min, at 37ºC. The slides were immersed into cold electrophoresis buffer (0.3 M NaOH, 1 mM hydrated Na_2EDTA; pH 13) for 30 min to allow DNA unwinding under alkaline conditions followed by a 25 min electrophoresis at 0.8 V/cm. Finally, after 10 min

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neutralization with PBS, slides were rinsed another 10 min with destilled water, dried overnight and stained with ethidium bromide (0.125 µg/µL). Three independent experiments were carried out, each with two replicates per treatment condition. In each experiment, a total of 100 randomly selected nucleoids (i.e., 50 nucleoids per gel) were analysed in FpG-treated and untreated gels for each culture, using an Axioplan2 Imaging epifluorescence microscope equipped with a high resolution camera (Carl Zeiss Microscopy, Gottingen, Germany). Scoring was done with the Comet Imager 2.2 software (MetaSystems, Althlussheim, Germany), choosing the percentage of DNA in the tail as a measure of DNA damage. The results represent the Mean ± Standard Deviation (M ± SD) of three independent experiments.

2.8 Micronucleus assay

The cytokinesis-blocked micronucleus assay was carried out as described by Louro et al. (2014). Following the A549/THP-1 cells co-culture exposure to 1.5, 3, 6, 12.5, and 25 µg/cm^2 of CNF for 6 hours, cytochalasin B (Sigma) was added to each well at a final concentration of 6 µg/mL. For each experiment, negative (non-treated cells) and positive (50 µg/mL mitomycin C, Sigma) controls were included. Briefly, at the end of the 48 hour treatment, cells were washed twice with PBS and, following detachment with trypsin-EDTA, cells were submitted to a hypotonic shock with a RPMI 1640:dH2O:FBS (37.5:12.5:1) solution, centrifuged and the pellet spread onto microscope slides. The slides were dried, fixed in absolute methanol (Sigma), stained with 4% Giemsa (Merck, Darmstadt, Germany) and air-dried at room temperature. Slides were scored under a bright field microscope for the presence of micronuclei (MN), using the criteria described by Fenech et al. (2007). At least 2000 binucleated cells from two independent cultures were scored per treatment condition and the results of the frequency of micronucleated binucleated cells (MNBC) are presented as the M ± SD. In addition, nuclear

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buds and nucleoplasmic bridges were also scored in those binucleated cells and their mean frequency determined. The proportion of mono- (MC), bi- (BC) or multinucleated-cells (MTC) was calculated by scoring 1000 cells per treatment and the cytokinesis-blocked proliferation index (CBPI) was calculated as follows (OECD, 2010): CBPI = (MC + 2BC + 3MTC)/Total cells. The Replication Index (RI) was calculated using the following equation:

RI = [(BC + 2MTC)/Total cells, in treated cells]/[(BC + 2MTC)/Total cells, in untreated cells]

2.9 Statistical analysis

Statistical comparisons of the clonogenic, MTT and comet assays data between treated and control cells were performed through a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test, after testing for the data normality . Then, in the Comet assay, the two-tailed Student's *t*-test was used to compare the differences between the results obtained with and without FpG treatment. The same test was also used to compare the CBPI results between the treated and control cells. The 2-tailed Fisher's exact test was applied to analyse the results of the frequency of micronucleated cells. All analyses were performed with the SPSS statistical package (version 22, SPSS Inc. Chicago, IL).

3. Results

3.1 Nanocellulose characterization

The nanocellulose sample was fully characterized in order to assess the properties that could be more significant for the cytotoxicity and genotoxicity tests. For that, their chemical and physical characteristics (Table 1) were evaluated by measuring the amount of carboxylic groups attached to the cellulose chain after the TEMPO-mediated oxidation, as well as the obtained degree of substitution and degree of polymerization.

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Table 1

A FE-SEM image of low magnification is presented to show the structure organization of the cellulose nanofibrils (Figure 1), in which agglomeration of the fibrils is observable. The size of the fibrils was assessed by AFM (Figure 2). As visible in the Figure 2B, the sample presents a wide distribution of diameters, with the mode in the 20-25 nm range. A value for the length of the nanofibrils could not be assessed by AFM since they are several micrometers long. In fact, for nano-objects with such a high aspect ratio, this is a common limitation. In order to analyse a larger number of nanofibrils, a dynamic analyser based on light scattering was also used. Although not adequate for non-spherical particles, it provides a comparison between different samples. The result obtained in this study is of the < 50 nm magnitude.

Figure 1

Figure 2

3.2 Cytotoxic effects

Figure 3 presents the results of the MTT assay and shows that none of the CNF concentrations tested during a 24-hour exposure period induced a significant cytotoxic effect in A549 cells, as compared to controls ($p > 0.05$, one-way ANOVA). Following 48 hours of exposure to the same CNF dose-range, an increase in cells viability was observed for the two lowest concentrations

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(1.5 and 3 µg/cm^2) and a significant decrease in cell viability was observed for the two highest concentration tested (p = 0.003 and p = 0.0004, respectively). Nevertheless, according to the ISO 10995-5, a clear cytotoxic effect was observed for the 25 µg/cm^2 concentration only (51 ± 1.42 %), whereas the 12.5 µg/cm^2 concentration decreased cells viability to 72 ± 2.13 %, i.e., slightly above the 70 % imposed by international standards. A dose-response effect was obtained following a potential function (r^2 = 0.987). The positive control showed a relative cell viability of 2.1% and 2.6% at 24 hours and 48 hours, respectively.

Regarding the results of the LDH assay following a 48-hour incubation time, a statistically significant membrane integrity loss was observed only for the highest CNF concentration tested (p = 0.03). Nevertheless, it should be highlighted that this difference corresponds merely to a 4% increase in cytotoxicity relatively to control cells (Figure 4).

The clonogenic assay was additionally used to assess the CNF ability to impair cell proliferation following a longer exposure period (8 days). A statistically significant increase in the number of A549 colonies formed was observed for the 1.5 µg/cm^2 of CNF, as compared to controls (p < 0.05, Tukey HDS), followed by a slight non-significant decrease in cell proliferation ability for higher CNF concentrations. The dose-response relationship was fitted to a sigmoidal curve (r^2=0.987, Figure 5). The positive control produced a decrease of cells surviving fraction to 50% relatively to control.

Figure 3

Figure 4

Figure 5

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3.3 Immunotoxic effects

Cells exposure to three CNF concentrations (1.5, 6 and 25 µg/cm^2) during 24h did not induce the release of detectable levels of IL-1β to the culture medium (detection limit of 0.35 pg/mL). The positive control had a determination of 57 pg/mL of IL-1β in the cell culture supernatant.

3.4 Genotoxic effects

The genotoxic effects of the CNF under study were assessed by the comet and the micronucleus assays performed in A549 cells cultured in the above described co-culture system. The selection of the concentration-range was based on the results of the cytotoxicity tests with A549 cells monocultures, assuming that the presence of THP-1 cells would not negatively affect A549 cells viability. The results of the comet assay (without FpG treatment) revealed a significant genotoxic effect of CNF in the co-culture of A549 cells and THP-1 differentiated macrophages, following exposure to the highest concentration tested (25 µg/cm^2), as compared to controls (p = 0.019 one-way ANOVA, Tukey post-hoc test) (Figure 6). However, even for this concentration, the level of DNA damage measured is quite low (6.68%) and may not have biological relevance. The DNA damage detected by the comet assay with FpG apart from DNA single- and double-strand breaks includes also oxidative lesions that are converted in DNA breaks. For CNF-treated cells the overall level of DNA breaks was not statistically different from that of control cells (p > 0.05, ANOVA). The comparison between the mean percentage of DNA in tail obtained with and without FpG treatment was only statistically significant for cells exposed to 12.5 µg/cm^2 of CNF (p < 0.05) but still within very low levels. The mean percentage of DNA in tail obtained for EMS, the positive control, was 30.0 % and 38.2 %, without and with FpG treatment, respectively.

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Figure 6

Figure 7

The frequencies of MNBCs and the CBPI estimated following co-cultures exposure to CNF are presented in Figure 8 (and Table 1 supplementary material). Nanocellulose induced a statistically significant increase in the frequency of MNBCs at the two lowest concentrations (1.5 and 3 µg/cm^2) tested (p = 0.035 and 0.001, respectively); the frequency of nuclear buds was also significantly increased by 3 µg/cm^2 of CNF (p = 0.05). No significant induction of nucleoplasmic bridges was observed in all CNF concentrations tested.

Figure 8

4. Discussion

The substantial number and variety of nanocellulose applications has raised the likelihood of human exposure in environmental and occupational settings, or as consumers and, consequently, has increased the concern about their potential adverse health effects. All high aspect-ratio nanomaterials, as nanofibrillated cellulose is, are recommended to be tested for their toxicity at the first phase of a flow chart developed by Dekkers et al. (2016) that attempts to prioritize the hazard assessment of nanomaterials and develop a nanospecific approach for their risk assessment. For this purpose, several complementary *in vitro assays* covering

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biological effects relevant for the occurrence of long-term effects, particularly cancer, are used. Compared with *in vivo* approaches, *in vitro* assays to characterize nanomaterials toxicity have shown to generate results in a simpler, faster and economic manner. Moreover, they can provide a basis for evaluating potential health risks of exposure and they can give insights into the mechanisms underlying the effects of nanomaterials on cells (Collins et al. 2016). For example, measuring the levels of pro-inflammatory cytokines may give a first indication on the ability of the nanomaterials to cause immunotoxic effects *in vivo* whereas the cytotoxicity is central for a good interpretation of the results of the *in vitro* genotoxicity assays and can provide also mechanistic information about the interactions with the intracellular organelles, e.g., mitochondria or lysosomes. The strategy for in *vitro* genotoxicity testing of nanomaterials needs to include the detection of the most relevant events for the multistep process of malignancy, i.e., DNA damage, clastogenicity and aneugenicity, which are covered by the combination of the comet assay and the *in vitro* micronucleus assay (Louro et al. 2015).

In this study, a preliminary safety assessment of a CNF produced by TEMPO-mediated oxidation of an industrial bleached *Eucalyptus globulus* was conducted before its production is scaled-up. Following CNF production, its physicochemical characterisation showed that a high fibrillation degree (yield) was achieved, with 100 % of the material in the nanoscale. In fact, with the pre-treatment, a high amount of carboxylic groups was introduced in the cellulose molecules, confirmed by the degree of substitution close to 0.2. The high charge caused repulsion between the nanofibrils that compose the fibre wall. The subsequent high-pressure homogenization was therefore able to effectively separate and break the fibre chains, producing a nanocellulose sample with a small degree of polymerization. The results are in accordance with the literature for CNF produced by TEMPO-mediated oxidation applying *ca.* 5 mM NaClO / g of cellulose fibre (Jin et al. 2014; Lourenço et al. 2017; Saito and Isogai 2007). As mentioned before, the diameter of the nanofibres was assessed by AFM and compared to that obtained

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from DLS measurements. The achieved mode of diameter is a common value for this type of material (Gamelas et al. 2015; Hanninen et al. 2015; Lourenço et al. 2017), and the DLS result is of the same magnitude of those obtained for identical nanofibrils (Gamelas et al. 2015; Lourenço et al. 2017; Mandal and Chakrabarty 2011).

The cytotoxicity of the CNF was assessed in A549 cells by three assays spanning different endpoints, from the alteration of cells metabolic activity (MTT assay) or loss of membrane integrity (LDH assay) to the cells proliferative ability in the presence of the nanofibres. All assays revealed the capacity of the highest CNF concentration (25 µg/cm^2) to induce alveolar cells death, following a 48-hour or 8 days exposure (Figures 3 – 5). In contrast, the 48 hours of treatment with the lowest CNF concentration (1.5 µg/cm^2) resulted in a significant increase in cell viability (MTT assay) and the 8 days treatment stimulated cells proliferation and their capacity to form colonies (clonogenic assay). Thus, the results of the MTT and the clonogenic assays are in general agreement in that the effect of CNF on cell viability is concentration-dependent whereas the LDH assay revealed a lower sensitivity to detect CNF influence on cells viability. These findings agree with those reported by Colić et al. (2015), showing a dose-dependent decrease in L929 fibroblastic cell proliferation and metabolic activity after an incubation of 48 hours with a high CNF concentration (250 µg/mL - 1 mg/mL). However, as the cell proliferation inhibition was less than 30% and not associated with cell death or oxidative stress, the CNF was considered as non-cytotoxic (Colić et al. 2015). It should be noted, however, that the concentration-range tested in the referred work was much higher than that herein used and does not encompass the ones that increased cell proliferation. Other studies have addressed the cytotoxic potential of nanocelluloses in several cell lines and the majority showed non-toxic effects following a 24-hour exposure, similarly to the data obtained in this study for the same exposure length. Kollar et al. (2011) observed no significant effect on THP-1 cell growth and viability after treatment with six variously modified types of non-nanosized

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cellulose after 24-hour incubation, except for dialdehyde cellulose that significantly decreased cell viability. Likewise, Lopes et al. (2017) reported the absence of cytotoxic effects in THP-1 differentiated macrophages, HDF and MRC-5 cells exposed to three types of CNF for 24 hours (Lopes et al. 2017). Pitkanen et al. (2014) reported no cytotoxic effect of a finest fraction of CNF in human cervix carcinoma (HeLa229) cells, as assessed by the highest tolerated dose (HTD) test. However, they reported the inhibition of cellular growth and viability decrease at the highest dose using the total protein content (TPC) test (24- and 72-hours exposures). Hua et al. (2015) also reported the absence of toxicity in the indirect cytotoxicity test performed in THP-1 cells exposed for 24 hours to the extract medium of three differently functionalized CNF films. No toxic effects in indirect cytotoxicity assays (crystal violet, MTT and LDH) were found in mouse fibroblasts incubated with the extracts of TEMPO-oxidized CNF and carboxymethylated CNF during 1, 4, and 7 days (Rashad et al. 2017). On the other hand, very high concentrations (2 – 5 mg/mL) of a needle-like cellulose nanowhisker from cotton cellulose were cytotoxic to bovine fibroblasts exposed for 24 hours (Pereira et al. 2013); an upregulation of the expression of stress- and apoptosis-related genes (*HSP70.1*, *PRDX1* and *BAX*) was also identified.

None of those studies has reported a stimulation of cell metabolism upon nanocellulose exposure. A hypothesis for the effect herein observed is that CNF, at low concentrations, may stimulate alveolar cells to proliferate because they are biocompatible, and they mimic endogenous fibrous structures that may facilitate cell adhesion. Indeed, the observation under phase contrast microscopy of cells grown during 48 hours in the presence of CNF confirmed that they were attached to CNF aggregates/agglomerates in the bottom of the culture well. At higher concentrations, however, CNF may slowdown cell proliferation due to increased mechanical stress, mimicking what has been observed in CNF hydrogels for 3D cell cultures (Nordli et al. 2016; Malinen et al. 2014). On the other hand, uncontrolled cell proliferation may

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also be a consequence of the nanofibre interaction with the mitotic spindle apparatus, as it has been described *in vitro* for asbestos fibres in various types of cells (Huang et al. 2011), for 1 to 4 nm width single-walled CNT in BEAS-2B and SAEC cells (Sargent et al. 2009), for 10 to 20 nm thin multi-walled CNT in BEAS-2B cells (Siegrist et al. 2014), and hypothesised for 13 and 14 nm multi-walled CNT in A549 and BEAS-2B cells (Louro et al. 2016). These diameters are close to the ones of the CNF under study and thereby interference with the mitotic spindle can be also a plausible explanation for the observed increase in cell viability at low concentrations. At high concentrations agglomeration/aggregation occurs and the availability of single CNF to be uptaken by cells and interact with the spindle fibres greatly decreases. Interestingly, this effect resembles the induction of fibroblasts proliferation *in vitro* following exposure to thin and curled dispersed single-walled CNT (Vietti et al. 2013; Wang et al. 2010) as this CNF is. The fibroblastic response to CNT is known to play a key role in tissue fibrosis that, in turn, may result in a carcinogenic effect on the long-term.

Overall, the referred studies evidence the gap that exists regarding long-term toxicity studies of CNF. Once we observed a significant cytotoxic effect only with an exposure length of 48 hours or more, there is still a need of focusing the CNF toxicity studies on longer exposure times and encompassing the low-dose range. This is particularly important since low-dose chronic studies are nowadays considered more suitable experimental models for risk assessment than single acute exposure studies in that they better mimic human exposure (Oberdörster 2010).

Immunotoxicity of CNF was assessed by determining the concentration of the proinflammatory cytokine IL-1β in the A549 cell co-culture supernatant. Our finding of unchanged levels of IL-1β in the co-culture of A549 and THP-1 is in agreement with the overall results from more comprehensive studies available in the literature that CNF has no proinflammatory effect (Basu et al. 2017; Colić et al. 2015; Hua et al. 2015; Mertaniemi et al. 2016; Nordli et al. 2016; Vartiainen et al. 2011). In addition, toxicological studies on the pulmonary toxicity of carbon

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nanotubes have suggested that when the nanofibre length exceeds the macrophages length, it triggers frustrated phagocytosis, which in turn stimulates a cytokine proinflammatory response (Brown et al. 2007; Murphy et al. 2012). However, Clift et al. (2011) reported that cellulose nanowhiskers from cotton, a nanocellulose that more resembles the needle-like structure of asbestos, did not cause any form of frustrated phagocytosis in macrophages, being instead internalized within vesicles. No signs of phagocytosis were also found in THP-1 macrophages exposed to three different modified CNF (Lopes et al. 2017). Nevertheless, Catalán et al. (2017) reported that CNF produced using TEMPO oxidation trigged the recruitment of neutrophils, macrophages, lymphocytes and eosinophils to the lungs of C57Bl/6 mice exposed by pharyngeal aspiration, indicating an acute inflammatory response. A significant dose-dependent increase in mRNA of the pro-inflammatory cytokines IL-1β and IL-6, tumour necrosis factor α (TNF-α) and chemokine (C-X-C motif) ligand 5 (CXCL5) was detected in the lung tissue, but without an increase in their protein levels. Lopes et al. (2017) also reported increased levels of Il-1β and TNF-α in THP-1 macrophages treated with 250 and 500 μg/mL, and 500 μg/mL, respectively, of unmodified CNF (Lopes et al. 2017). These CNF concentrations are in the range of those tested in the Cólic et al. (2015) study that, conversely, reported no induction of those pro-inflammatory cytokines in peripheral blood mononuclear cells (PBMCs) cultures stimulated with phytohemagglutinin (31.25 – 1000 μg/mL). Still, the CNF concentrations herein used are below 31.25 μg/mL and, therefore, a negative result was not unexpected. Non-nanometric cellulose fibres also trigger an inflammatory response in Wistar rats by inhalation, but it appears to be transient, declining in a 14-day period (Cullen et al. 2000).

Concerning the genotoxic effects of exposure to nanocellulose, the present results showed that the CNF under study induced a low but significant level of DNA damage in A549 cells in co-culture with THP-1 cells, at the 25 μg/cm^2 concentration. In addition, two CNF concentrations (1.5 and 12.5 μg/cm^2) caused a slight induction of the level of oxidative DNA lesions detected

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as FpG-sensitive sites, e.g, oxidised 8-oxoGua. It is known that oxidative stress can be rapidly repaired by the cell repair systems and thereby the 24-hour timepoint might have been too long to allow the detection of this type of DNA damage in exposed cells. In fact, there are studies e.g., with TiO_2 nanomaterials, that showed an induction of FpG-sensitive sites in A549 cells at 2 or 3 hours, which were not apparent at 24 hours exposure (El Yamani et al. 2017; Ursini et al. 2014) indicating that from this study data the induction of oxidative DNA damage cannot be completely excluded. In the study by Stefaniak et al. (2014) nanocellulose, including CNF, induced significantly more free radicals than that of the essentially inert cellulose microcrystals, which could lead to reactive oxygen species formation and DNA damage. Low, but significant values of DNA damage, detected by the comet assay, were also obtained *in vitro* in human lymphocytes exposed to brown cotton and curauá nanofibres (Lima et al. 2012). Regarding *in vivo* studies, Catalán et al. (2017) reported significant positive comet assay results ($p < 0.001$) in the lung cells of mice exposed to 10 and 40 µg/mouse of CNF by pharyngeal aspiration, but non-significant values of % DNA in tail compared to the zero dose for the highest concentrations tested, 80 and 200 µg/mouse. This observation agrees with the present *in vitro* results in that low CNF concentrations seem to induce more toxicity in lung cells than the higher ones, either *in vitro* or *in vivo*.

Interestingly, the two lowest CNF concentrations tested by the *in vitro* micronucleus assay were also able to increase significantly the frequency of chromosome numerical or structural anomalies in A549 cells, while for the highest does no effects were observed. On the other hand, the results of the CBPI did not show any significant decrease of A549 cells capacity to divide at those higher dose levels of CNF and thus an influence of toxicity on the micronucleus frequency is not likely. This result confirms the incidence of the CNF biological effects on the low-concentration range. A decrease in the genotoxic effect associated with a dosage increase has been reported for other nanomaterials, e.g. carbon nanotubes, and it is thought to be related

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with the aggregation or agglomeration of nanomaterials at the highest dose levels that decreases the bioavailability of nano-objects (Brown et al. 2008; Rittinghausen et al. 2014; Shvedova et al. 2005). A hypothesis associated with the decreased toxicity observed with functionalized carbon nanotubes is that their functionalization, with either carboxyl or amino groups, increase the adsorption of proteins in protein-rich biological media, which promotes their agglomeration (Allegri et al. 2016). Cellulose nanofibres also show a strong tendency to agglomerate, especially after drying and in highly concentrated aqueous solutions due to strong inter- and intra-molecular hydrogen bonding; in nonpolar media they tend to form aggregates (Lima et al. 2012). These authors observed an inverse association between CNF aggregation and toxicity while Pereira et al. (2013) observed that high concentrations of cotton CNF resulted in large CNF aggregates and increased cytotoxicity. In the present study, an aggregation/agglomeration of CNF in the cell culture medium was clearly observed under the optical microscope (Figure 1 supplementary material) 24 hours after cells treatment with the highest concentrations (25 µg/cm^2), supporting the hypothesis that the decreased toxicity is related to a lower bioavailability of CNF in its nanosized form.

Catalán et al. (2017) reported no micronucleus induction in the bone marrow erythrocytes of mice exposed to CNF by pharyngeal aspiration, but the time between the exposure of mice to CNF and the bone marrow sampling might not have been sufficient to allow a systemic genotoxic effect (Catalán et al. 2017). Even though we observed micronuclei induction by the two lowest CNF concentrations tested, whether they were mediated by clastogenic or aneugenic mechanisms, both leading to irreversible chromosome damage linked to early events in carcinogenesis, was not investigated (Bonassi et al. 2011). Clastogenic events can often be associated to the formation of DNA adducts and to oxidative stress that result in DNA breakage that should have been distinguished by the comet assay. On the other hand, loss of chromosomes may be explained by a direct interaction of the CNF with tubulin from the mitotic

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spindle, or with proteins involved in the segregation of the chromosomes in metaphase, events that are not detected in the comet assay. Likewise, a significant disruption of the mitotic spindle by multi-walled CNT has been previously reported (Siegrist et al. 2014). Several studies have stated that the micronucleus assay is more sensitive to detect genotoxic effects of nanomaterials than the comet assay (Louro et al. 2016), but the type and repair capacities of target cells, the stage of cell cycle, and the time elapsed between exposure and analysis are additional factors that may contribute to the different sensitivities of these assays (Valentin-Severin et al. 2003).

5. Conclusions

Overall, the data of the present work suggests that CNF produced with an oxidative pre-treatment mediated by TEMPO is able to produce concentration-dependent effects in the viability and proliferation of human alveolar cells and genotoxic effects in these cells co-cultured with THP-1 macrophages, particularly at a low concentration-range. The results of cytotoxicity assessment also suggest that CNF exposures longer than 24 hours are needed to yield detectable effects. The use of A549 cells co-cultured with THP-1 monocyte-derived macrophages allowed a preliminary assessment of CNF immunotoxicity that confirmed the absence of a proinflammatory effect at a low CNF concentration. Concerning CNF genotoxicity assessed in the same *in vitro* system, although no biologically relevant DNA damage was detected in A549 cells by the comet assay, the formation of micronuclei at the two lowest concentrations tested raised some concern about the safety of this nanofibre. Further studies should be performed to complement these findings, since they suggest that low CNF doses, which are the most realistic exposure doses to humans, may stimulate cell proliferation and induce aneugenic/clastogenic events in alveolar cells, representing a potential risk for human health. Given that this toxicity assessment of a newly produced CNF was conducted in an early

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phase of the nanofibre development, the present findings are expected to stimulate its modification towards a safer material.

Statement of Author Contributions

All authors contributed to the manuscript writing. In addition, Maria João Silva contributed to the study design and supervised the scientific work, Ana Filipa Lourenço and Paulo Ferreira produced and characterized the nanocellulose investigated in this study, and Célia Ventura performed the *in vitro* toxicological experiments, data analysis and discussion.

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Figure captions

Figure 1. Field-Emission SEM of films of cellulose nanofibrils

Figure 2. Cellulose nanofibrils AFM image in phase imaging mode (A) and nanofibrils diameter distribution obtained by AFM (B).

Figure 3. Relative viability of A549 cells after exposure to different concentrations of CNF (24 hours and 48 hours) as assessed by the MTT assay. Results are expressed as M ± SD of 3 independent experiments.

Figure 4. Cytotoxicity results after a 48 hour exposure of A549 cells to a concentration-range of CNF, as assessed by the LDH release assay. Results are expressed as M ± SD. * $p < 0.05$.

Figure 5. Colony forming ability of A549 cells after 8 days exposure to different concentrations of CNF, as assessed by the clonogenic assay. Results are expressed as M ± SD of the cells surviving fraction relative to control.

Figure 6. Comet assay results obtained in the co-culture of A549 epithelial cells and THP-1 differentiated macrophages exposed to CNF, without and with FpG addition. Results are expressed as M ± SD. * $p < 0.05$.

Figure 7. A549 cell nucleoids observed under the fluorescence microscope in the comet assay with FpG addition. (A) Non-exposed cells control (B) A549 cells exposed to 12.5 μg/cm^2 of CNF (C) Positive EMS exposed cells control.

Figure 8. Results of the micronucleus assay after A549 cells exposure to CNF. In columns, frequency of micronucleated binucleated cells (MNBCs) per 1000 binucleate cells (BNC); the dotted line represents the cytokinesis-blocked proliferation index (CBPI). Mitomycin C was

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used as a positive control and induced 54.5 MNBNC/1000BNC (p = 0.000). Results are expressed as M ± SD. * $p < 0.05$.

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Fig 1

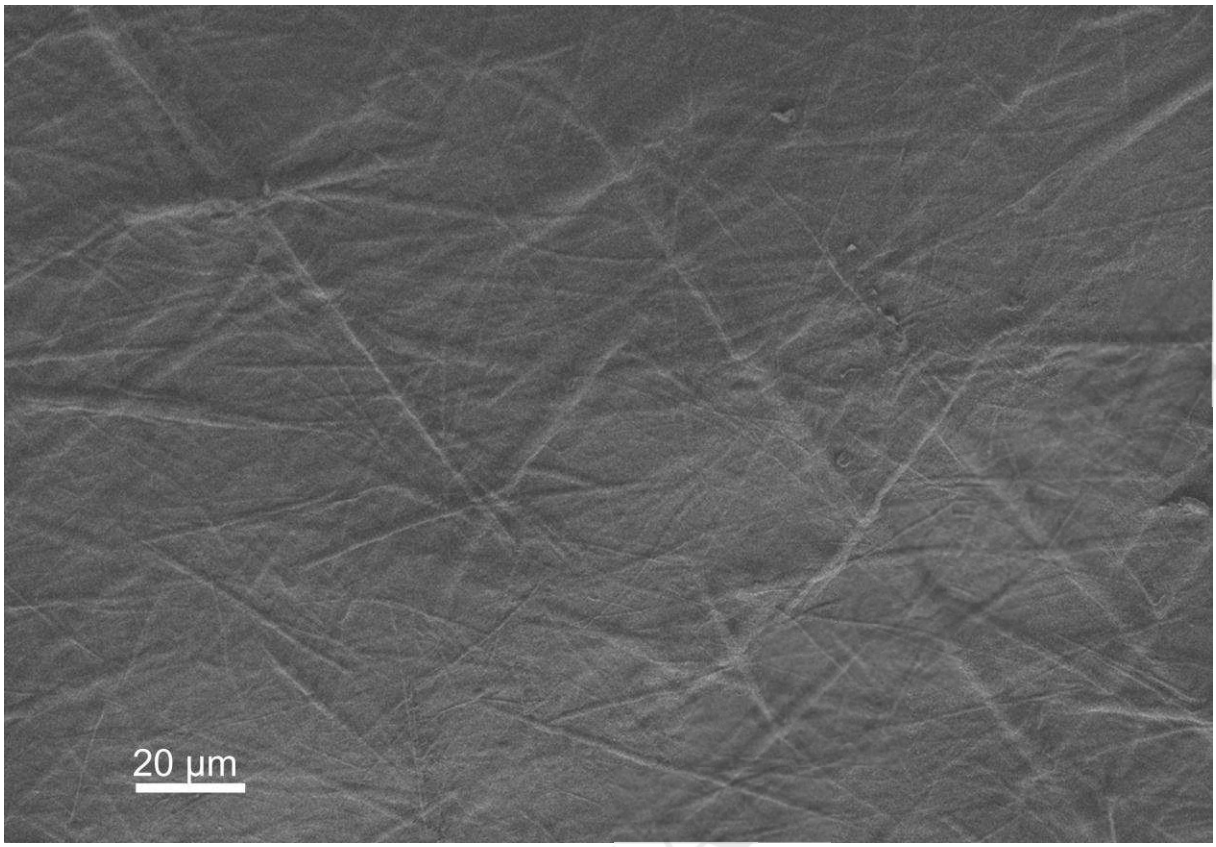

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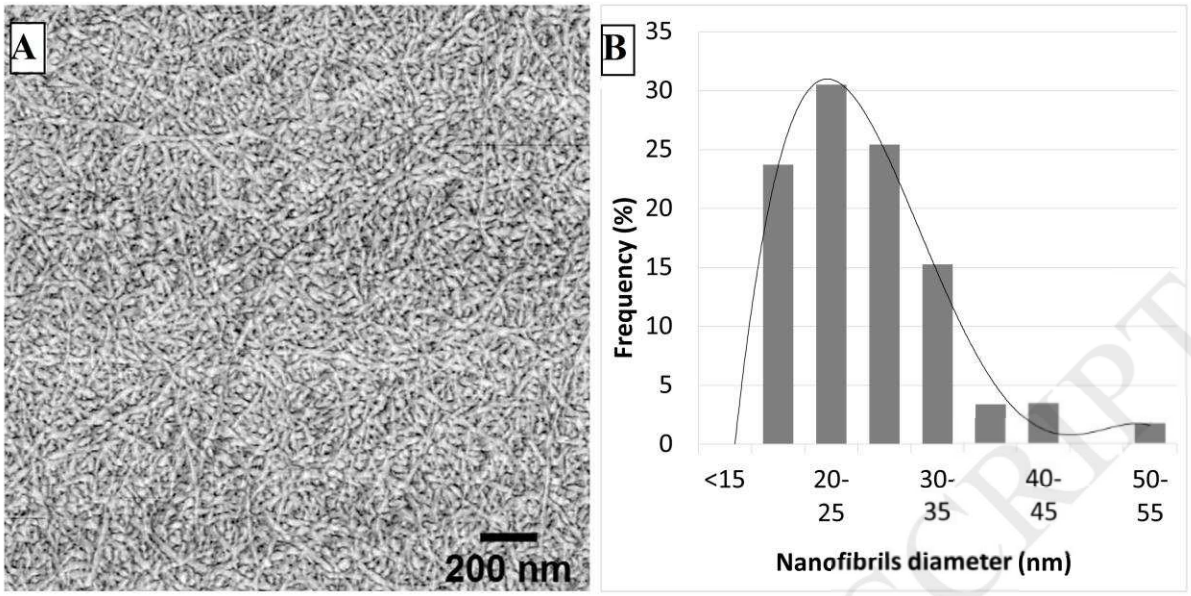
Fig 2



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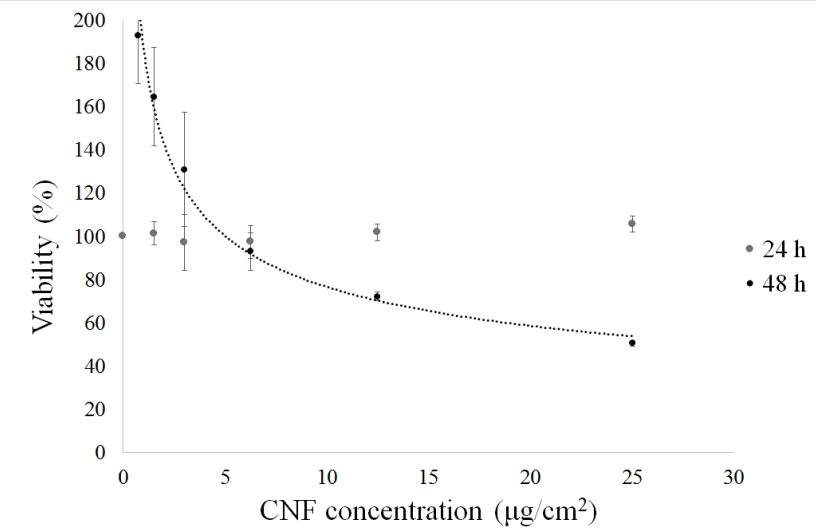

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Fig 4

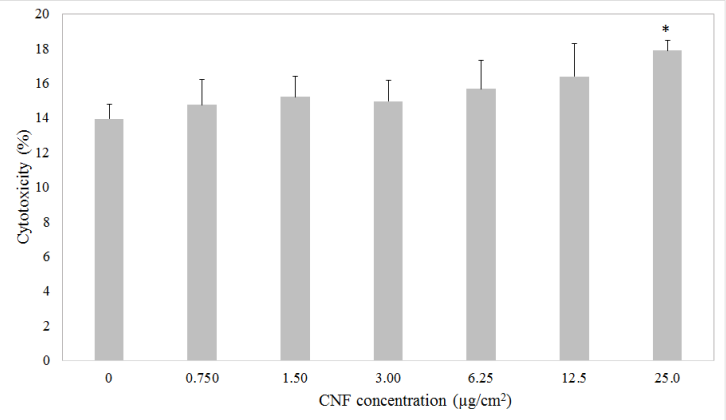

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Fig 5

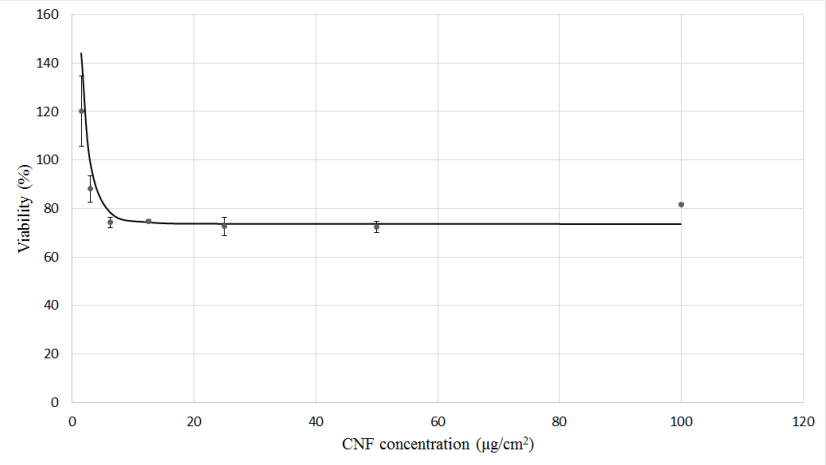

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Fig 6

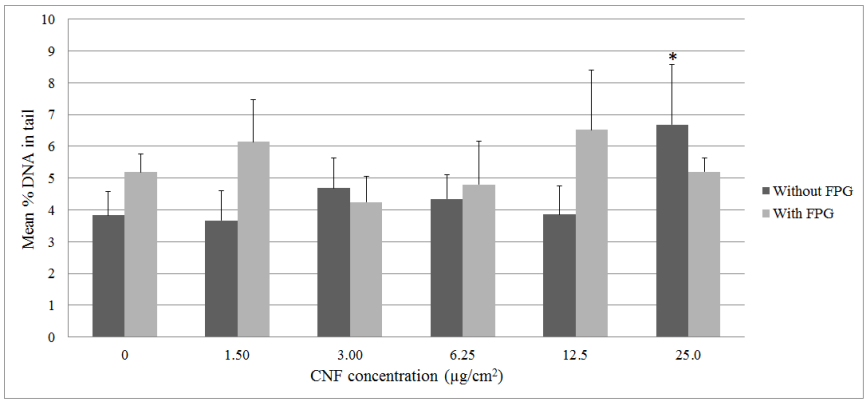

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Fig 7

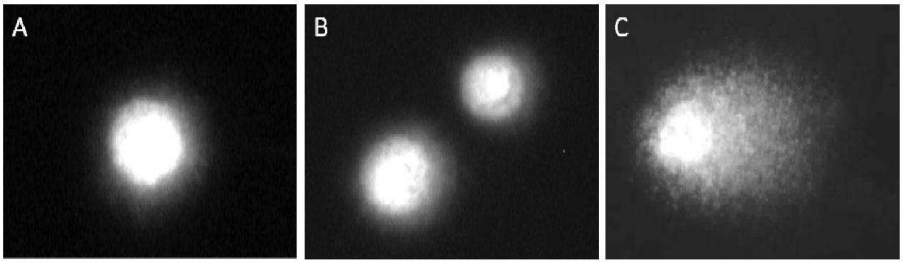

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Fig 8

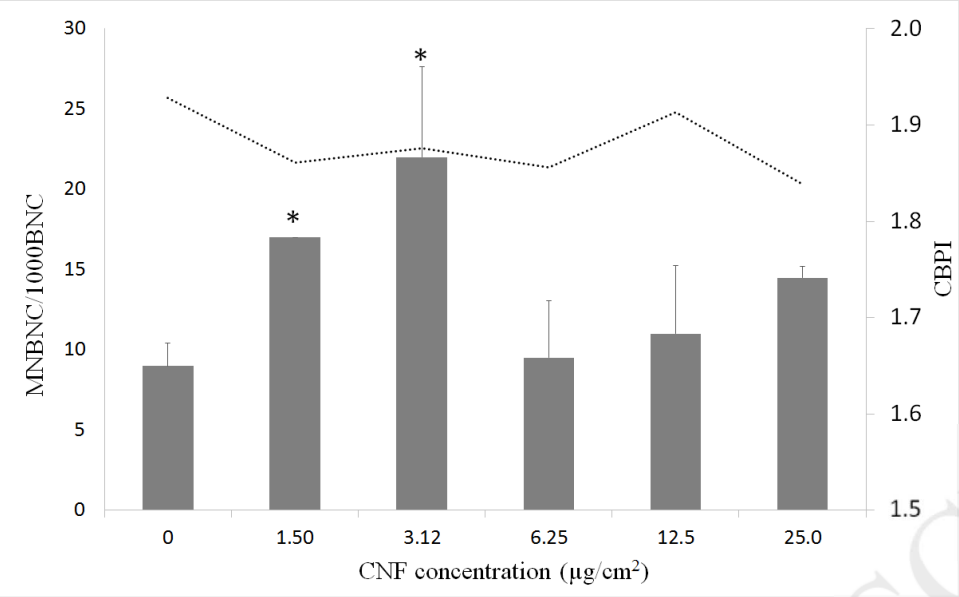

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Table 1. Characterization of the nanocellulose sample.

Yield (%)	C_{COOH} (µeq·g/g)	DS	DP	d_{AFM} (nm)	d_{DLS} (nm)
82.4	1177	0.19	289	25.9	18.5

C_{COOH}: Carboxyl group content; DS: Degree of substitution; DP: Degree of polymerization; $d_{AFM,DLS}$: Diameter (obtained by AFM or DLS)

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