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Hazard assessment of nanocellulose – the role of physico-chemical properties

FINAL REPORT OF PROJECT NUMBER 117146 OF THE FINNISH WORK ENVIRONMENT FUND

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Photos: Azovskaya Valeria (Figure 1)

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TIIVISTELMÄ

Puukuituperäinen nanoselluloosa on uusiutuva materiaali, jolla on runsaasti potentiaalisia teollisia sovelluksia. Nanokokoon saattaa kuitenkin liittyä erityisiä ominaisuuksia ja biologisia vaikutuksia, jotka ovat haitallisia terveydelle. Materiaalien ja prosessien turvallisuuskysymysten huomioon ottaminen onkin tärkeää ennen nanoselluloosan laajamittaista tuotantoa. Nanoselluloosista on vain vähän toksisuustietoja. Aiemmissa koe-eläintutkimuksissa on ilmennyt, että nanofibrillaarisen selluloosan (NFC) jotkut muodot aiheuttavat keuhkoissa akuutin tulehduksen ja perimämyrkyllisiä vaikutuksia, joilla katsotaan olevan merkitystä fibroosin ja syövän synnyssä. Muiden kuitumaisten nanomateriaalien haittavaikutukset riippuvat usein materiaalin fysikaaliskemiallisista ominaisuuksista ja näin voi olla myös NFC:n kohdalla. Tutkimustulokset NFC:n perimämyrkyllisyydestä ovat olleet ristiriitaisia, sillä soluviljelmillä tehdyissä kokeissa perimämyrkyllisyyttä ei ole yleensä havaittu. NFC:n perimyrkyllisyys *in vivo* voi ehkä selittyä sekundaarisella genotoksisella mekanismilla, joka liittyy NFC:n tai siinä olevan endotoksiinin aikaansaamaan tulehdukseen.

Tämän hankkeen tarkoituksena oli selvittää, voivatko fysikaaliskemialliset ominaisuudet, endotoksiinikontaminaatio, oksidatiivinen stressi ja tulehdus selittää NFC:n perimämyrkyllisyyttä. Testasimme 12 kuitukooltaan ja pintaominaisuuksiltaan erilaisen, Aalto-yliopiston tuottaman NFC-materiaalin toksisuutta ihmisen epiteelisolujen (BEAS-2B) viljelmissä. Kolme materiaaleista tutkittiin myös hengitystiealtistuksessa hiirillä. Lisäksi tarkastelimme viiden muun, Uppsalan yliopistosta saadun NFC-näytteen toksikologisia vaikutuksia BEAS-2B-solujen viljelmissä.

Tulostemme mukaan NFC:n pintakemialliset ominaisuudet vaikuttavat merkittävästi materiaalien toksisuuteen. Anioniset ja kationiset toiminnalliset ryhmät liittyvät genotoksisiin vaikutuksiin. EPTMAC- (epoksipropyylitrimetyyliammoniumkloridi) ja karboksimetyloitu NFC näyttävät toimivan primaarisella mekanismilla luultavasti hapen reaktiivisten välituotteiden kautta. TEMPO- (2,2,6,6-tetrametyylipiperidiini-1-oksyyli) oksidoidun NFC:n genotoksisuus saattaa selittyä tulehdukseen liittyvällä sekundaarisella mekanismilla. Nanofibrillien koko vaikuttaa genotoksisuuteen yhdessä pintakemiallisten ominaisuuksien kanssa. Bakteeriperäisellä endotoksiinikontaminaatiolla ei näytä olevan merkitystä genotoksisuuden määräytymisessä. Tuloksia ei voi kuitenkaan yleistää, sillä NFC-synteesi ja toksisuustestauksessa käytetty dispersiomenetelmä vaikuttavat materiaalin fysikaaliskemiallisiin ominaisuuksiin ja sitä kautta toksisuuteen. Vaikka NFC:n toksikologisten toimintatapojen ja materiaaliominaisuuksien merkityksen ymmärtäminen vaatii vielä lisätutkimuksia, nyt saatuja tuloksia voidaan hyödyntää kehitettäessä turvallisten NFC-materiaalien suunnitteluperiaatteita ja prediktiivistä toksikologiaa.

ABSTRACT

Wood-derived nanofibrillated cellulose (NFC) is a renewable material that has been the subject of increased industrial interest. However, some of the nanoscale features of NFC may endow novel properties and biological effects, raising concerns about possible harmful effects on human health. Hence, safety in working environments needs to be addressed. Although the scarce knowledge on the toxicity of NFC precludes a thorough hazard assessment, previous studies have suggested that pulmonary exposure to some NFC types is able to induce genotoxic effects and acute inflammation, which are considered to contribute to the development of malignancy and fibrosis. However, as with other fibrous nanomaterials, the *in vivo* effects of NFC may partly depend on the physico-chemical properties of NFC. Conflicting results have been observed in genotoxicity assays *in vitro* (negative) and *in vivo* (positive), which suggests the involvement of a secondary genotoxic mechanism *in vivo*, triggered by inflammation due to NFC itself or its possible endotoxin contamination.

In the present project, we investigated whether physico-chemical properties, endotoxin contamination, oxidative stress, and inflammation could explain the genotoxicity of NFC. We examined the toxicological potential of 12 NFC samples of varying fiber length and width and different surface chemistry, synthetized by Aalto University. The effects of the NFCs were examined using an *in vitro* cell system (BEAS-2B cells), and three NFCs were also studied in mice *in vivo*. In addition, 5 NFC samples provided by Uppsala University were assessed *in vitro*, to gain insight in the potential effects of the source of the materials and the dispersion method used.

Our findings suggest that surface chemistry is a central property in determining the toxic effects of NFCs. Both cationic and anionic functionalization are associated with genotoxic effects. EPTMAC (epoxypropyl trimethylammonium chloride) and carboxymethylated NFC appear to operate by a primary mechanism of action, probably through the generation of reactive oxygen species. An inflammation-mediated mechanism may be involved in the genotoxicity of TEMPO (2,2,6,6-tetramethyl-piperidin-1-oxyl) oxidized NFC. The size of the nanofibrils modulates the genotoxic effect of NFCs, although the effect varies depending on surface chemistry. On the other hand, contamination with bacterial endotoxin does not seem to play a role in the observed genotoxic effects. However, the results cannot be generalized to all types of NFCs, as the synthesis process and the dispersion method used for testing them may influence their physico-chemical properties and, hence, their toxic effects. Further investigations are needed to better comprehend the toxicological mechanisms of NFC and the possible connections between the properties of the materials and their toxicity. The present findings can aid in developing safer-by-design principles for NFC and our understanding of predictive toxicological outcomes.

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1 BACKGROUND

Cellulose is the most abundant natural polysaccharide polymer in the world (Čolić et al., 2020). It has a semi-crystalline conformation that encompasses a broad range of nano-scale fibrous structures (Ventura et al., 2020). The increasing demand for sustainable and environmentally friendly resources has contributed to considering cellulosic-based materials as desirable renewable resources in the production of biopolymers in nanoscale form (Catalán & Norppa, 2017; Čolić et al., 2020; Lopes et al., 2017). Cellulosic materials have long been used in the paper and packing industrial sector, as well as in healthcare and food products, and cosmetic formulations (Endes et al., 2016; Ong et al., 2017). However, the emergence of nanotechnology has opened new and multiple possibilities not only in the existing industrial sectors but also for completely novel applications, such as biofuels, aerogels for insulation, electronics, nanocomposite formulation and reinforcement, and several biomedical applications, such as drug delivery systems and tissue engineering (Chinga-Carrasco, 2018; Li et al., 2021; Lin & Dufresne, 2014; Thomas et al., 2018; Ventura et al., 2020).

Wood is the most significant renewable natural resource in Finland that is processed at an industrial scale, contributing to a low-carbon bioeconomy. Finland has set up ambitious growth targets for the bioeconomy in its National Bioeconomy Strategy[1] targeting to push the Finnish bioeconomy output up to EUR 100 billion by 2025, and to create 100,000 new jobs. The paper and pulp industries have long been the foundation of Finnish exports and they have accounted for ~2/3 of the value of the Finnish forest industry[2]. Although paper products are declining, there is an increasing demand for other pulp products. New smart paper and packaging products, as well as construction materials are being developed with the help of nano-scale technology. Products gain novel characteristics, allowing new types of applications in, for example, the food and pharmaceutical industries. Finnish nanocellulose production is currently at pre-commercial scale, which creates an important opportunity to address safety concerns prior to commercialization, contributing to safer product design and manufacturing.

1.1 Nanofibrillar cellulose

Cellulose nanomaterials (CNMs) can be derived from a variety of sources including wood, annual plants, agro-industrial side streams, bacteria and marine resources

[1] Updated Finnish Bioeconomy Strategy aims to promote sustainable growth and climate objectives | Biotalous - Bioeconomy

[2] https://www.forestindustries.fi/statistics/forest-industry/

(Chinga-Carrasco et al., 2021; Ventura et al., 2020). Wood pulp fibers are processed with chemical and enzymatic pre-treatments to facilitate the structural deconstruction of the fibers into two main types of CNMs: cellulose nanocrystals (CNC) and nanofibrillated celluloses (NFC) (Chinga-Carrasco et al., 2021; Foster et al., 2018). The former is obtained by strong chemical treatment (e.g. sulfuric acid hydrolysis), leading to the short and highly crystalline cellulose nanocrystals, whereas the extraction of the latter consists of pre-treatment steps followed by mechanical fibrillation (Foster et al., 2018; Klemm et al., 2006; Kondo et al., 2014).

NFCs, which are also called cellulose nanofibrils, microfibrillated cellulose or cellulose microfibrils (Chinga-Carrasco et al., 2021; Foster et al., 2018), have dimensions of roughly >1 µm in length and <100 nm in width (ISO, 2017). They consist of long flexible and entangled nanofibers that, in water-based dispersions, form a highly viscous gel already at very low concentrations (Figure 1).

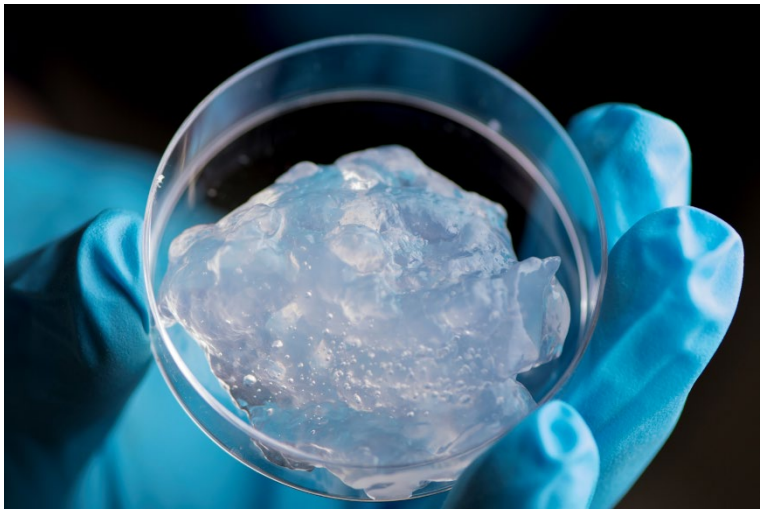

Figure 1. Water-based (1 %) dispersion of nanofibrillar cellulose (NFC) (Photo: courtesy of Azovskaya Valeria).

NFCs exhibit unique characteristics due to their nanoscale size, fibril morphology and large surface area. It allows them to be used in new applications where conventional cellulosic materials are not suitable for (Foster et al., 2018; Ventura et al., 2020). However, the nanoscale features may impart novel chemical and biological properties, affecting their safety use (Catalán & Norppa, 2017; Lopes et al., 2017). According to the fiber paradigm, fibers of high aspect ratio and high lung biopersistence - two features that

characterize NFC (Knudsen et al., 2015; Stefaniak et al., 2014; Vartiainen et al., 2011)- may cause fibrosis and malignancy upon inhalation (Donaldson & Poland, 2009).Inhalation exposure to CNMs in occupational settings has been identified as the most relevant exposure scenario (Shatkin & Kim, 2015). Therefore, the release and inhalation of cellulose nanopowders during processing steps (e.g. drilling, cutting, and sanding of polymer nanocomposites), or airborne droplets during the production and manufacturing of wet slurry, might be of concern (Catalán & Norppa, 2017). Consequently, it is necessary to address the human health and environmental safety aspects of nanocelluloses before scaling up their production (Catalán & Norppa, 2017). Safety characterization has, however, been hindered by scarce knowledge on the toxicity of NFC, which has precluded a thorough hazard assessment of these materials.

1.2 Physico-chemical and biological properties of nanofibrillar cellulose

Knowledge on the potential adverse health effects of NFC is still scarce, despite the increasing number of studies performed during the last few years. As summarized in recent reviews on this topic (Čolić et al., 2020; Endes et al., 2016; Stoudmann et al., 2020; Ventura et al., 2020), toxicological studies show contradictory results. Factors such as cellulose source, fibrillation process or pre-treatments, which can affect the properties of the materials, could partly explain these controversies (Stoudmann et al., 2020). The physico-chemical features of nanomaterials may affect their toxicity (Bitounis et al., 2019; M. V. Park et al., 2018). For instance, the interaction of NFC and dendritic cells depended on the thickness and length of the material (Tomić et al., 2016). In the case of nanocelluloses, surface chemistry is one of the most relevant features (Roman, 2015).

Surface modifications can impart new beneficial properties to nanocelluloses, increasing their applicability in, e.g., healthcare products and food packaging (Lopes et al., 2020). Chemical pre-treatments (e.g. carboxymethylation, phosphorylation, etc.) are usually applied to ease the fibrillation process (Chinga-Carrasco et al., 2021). However, such pretreatments affect not only the surface charge of the nanofibrils, but also properties like fiber dimensions, specific surface area, and degree of branching of the nanofibrils (Lavoine et al., 2012). Surface chemistry was reported to drive *in vitro* inflammatory response to NFC (Lopes et al., 2017), while no differences in cell metabolic activity or cell membrane integrity were observed when diverse *in vitro* cell models where exposed to differently functionalized NFC materials (Ilves et al., 2018; Lopes et al., 2017; Lopes et al., 2020). However, most of the studies that have compared the toxicological effects of NFCs with different surface chemistries have been hindered by the diversity of variables included in the investigations (Hadrup et al., 2019; Ilves et al., 2018; Lindberg et al., 2017; Menas et al., 2017).

Nanomaterials can be contaminated by microbial components, such as bacterial endotoxin (also known as lipopolysaccharide- LPS) that can trigger toxicological responses when cells and animals are exposed to particulate materials (Giannakou et al., 2019; Li & Boraschi, 2016). Therefore, the presence of endotoxins can generate misleading results when evaluating the toxic effects of nanomaterials (Li & Boraschi, 2016; Neun et al., 2020). Due to the higher surface area, nano-sized materials can have higher contamination levels for the same dose than corresponding larger materials. For this reason, the European Chemicals Agency (ECHA) recommends checking for the presence of endotoxins when investigating the toxicity of nanomaterials in general (ECHA, 2017a). Contamination with endotoxins can happen at any step of the manufacturing and handling process, since most nanomaterials are not produced under sterile conditions (Giannakou et al., 2019). In the case of cellulosic materials that are water-based dispersions, endotoxin contamination may become critical, as bacteria easily grow in a cellulose-rich environment (Liu et al., 2018).

Despite intensive research, no directly reproducible relationships between specific physico-chemical properties of nanomaterials and biological effects have been agreed to be predictive. This type of comparative studies is even more scarce with nanocellulose. The identification of such a relationship could help in developing safer-by-design principles and our understanding of predictive toxicological outcomes (Chinga-Carrasco et al., 2021; Lopes et al., 2017; Shatkin & Kim, 2015). Furthermore, this knowledge can support strategies in grouping nanoforms to fulfill the regulatory requirements when registering nanomaterials (ECHA, 2017c). Currently, nanocelluloses are exempted from being registered under the Registration, Evaluation, Authorization and Restriction of Chemical (REACH) regulation (EC No. 1907/2006[3]). Cellulose is a natural polymer, and polymers as such, including all their forms (also nanoforms) are exempted from the REACH registration[4]. However, due to concerns about environmental contamination with micro- and nano-plastics (Prata et al., 2020), the European Commission is planning to bring at least some polymers under REACH by 2022.

Although polymers are exempted from the REACH registration, they should comply with food related regulations. Both cellulose (millimetric scale) and micro-cellulose (micrometric scale) have been evaluated by the European Food and Safety Agency, and they were considered as safe (EFSA, 2018). In the case of nanocelluloses, a specific assessment is required during their safety evaluation, as described by the EFSA Guidance on Nanomaterials (EFSA Scientific Committee, 2018), which is currently under revision.

[3] https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32006R1907&from=EN
[4] https://echa.europa.eu/support/qas-support/qas/-/q-and-a/d2363260-9bb2-1c9b-f2da-a416fbc457d9

To date, no specific regulatory framework exists for nanomaterial-based medical products and devices. Instead, nanotechnology-enabled health products follow current regulatory frameworks for medicinal products or medical devices. However, they may require additional quality and safety assessments triggered by the unique characteristics of the nanomaterial (Halamoda Kenzaoui et al., 2019).

1.3 Mechanisms of genotoxicity

A main safety concern related to nanomaterials is their possible genotoxicity (Catalán, Stockmann-Juvala, et al., 2017; Kohl et al., 2020). Genotoxicity describes the capacity of a chemical or physical agent to produce genetic damage, altering the genetic information. Genotoxic events can still be repaired or can lead to permanent changes (mutations) in the amount or structure of the genetic material of cells or organisms (ECHA, 2017b). If mutations occur in critical genes, they may lead to cancer (Hartwig et al., 2020). Therefore, every mutagen is considered to be potentially carcinogenic (Kohl et al., 2020).

Chemical substances (including nanomaterials) can be genotoxic through a primary mechanism, executed by the substance itself, or a secondary mechanism involving an inflammatory response (Figure 2). The primary mechanism can involve a direct interaction with the DNA, or an indirect effect mediated by other molecules (induction of reactive oxygen species (ROS), inhibition of DNA repair mechanisms, etc.) (Alenius et al., 2014; Evans et al., 2017; Gonzalez & Kirsch-Volders, 2016).

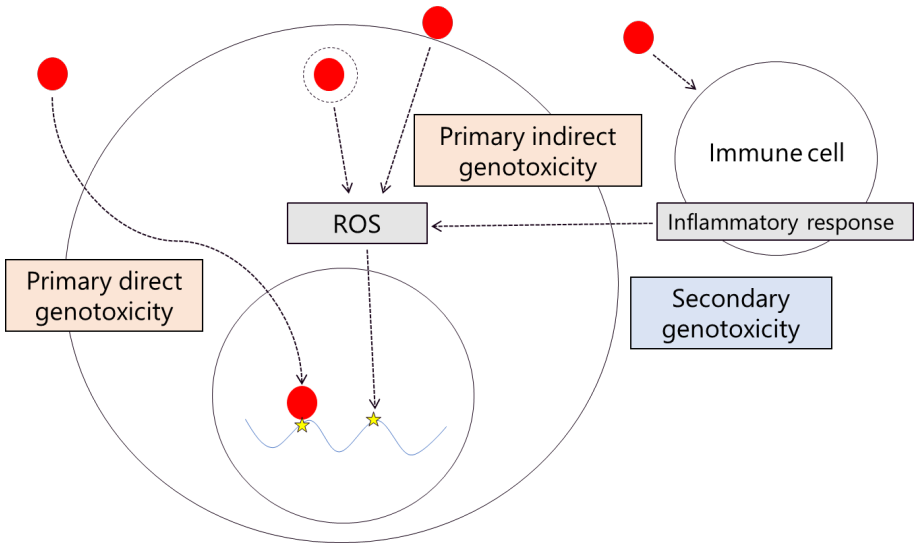

Figure 2. Mechanisms of particle genotoxicity (modified from Alenius et al. 2014). ROS, reactive oxygen species.

Current genotoxicity assessment is based on identifying a substance as genotoxic or non-genotoxic, assuming that the genotoxicity response does not have a threshold value (ECHA, 2017b). However, recent findings in genetic toxicology are moving this paradigm forward to a more semi-quantitative approach, where a threshold mechanism of action (MoA) is assumed when genotoxicity is mediated by secondary mechanisms. Furthermore, primary indirect effects are likely to have a thresholded MoA. However, the difficulties for defining such thresholds and for distinguishing direct and indirect effects preclude such differentiation (Nohmi, 2018; SCOEL, 2017).

Currently, no specific occupational exposure limit (OEL) values exist for nanocelluloses. The Permissible Exposure Limit allowable by the US Occupational Safety and Health Administration (OSHA) for cellulose dust is 5 mg/m^3 for the respirable fraction, expressed as 8-h time-weighted average, TWA (Shatkin & Oberdörster, 2016). No OEL values for cellulose dust are available at the EU. Instead, several countries (e.g. Finland) use the OEL value of unspecific organic dust as inhalable fraction (5 mg/m^3, TWA). On the other hand, an OEL value of 0.01 fibers/cm^3 has been recommended for nanocelluloses (Stockmann-Juvala et al., 2014) which is the same value as suggested for other biopersistent fibrous nanomaterials, e.g., carbon nanofibers.

OEL values are set to any chemical agent relevant for the work environment, except for carcinogens (which are regulated by the carcinogens and mutagens directive, 2004/37/EC). As explained above, OELs cannot be applied to genotoxic carcinogens, as single genotoxic events can induce a carcinogenic response. Hence, no dose can be considered as 'safe'. However, if the carcinogenic substance operates through secondary mechanisms, it is likely to have a MoA threshold, which may allow the derivation of OEL values (SCOEL, 2017). Furthermore, if threshold values are assumed to be lower for the nanosized than larger forms of a substance, different exposure limits could be established for the different forms of the same substance.

2 AIMS OF THE STUDY

The main aim of this project was to establish which of the physico-chemical properties shown to be relevant for the hazard potential of other fibrous materials could modulate toxicological response to NFC, and how the toxic effects could arise. This aim was achieved through the following objectives:

1. Evaluation of the effect of **length**, **width** and **surface chemistry** on the toxic potential of NFC.
2. Evaluation of the possible role of **endotoxin contamination** on the toxic responses induced by NFC.
3. Elucidation of the **primary and secondary mechanisms involved in the genotoxic potential** of NFC.

Identification of the critical physico-chemical properties that could modulate the toxicity of NFC is a key issue in the hazard and risk assessment of nanocellulose. This information can be used to develop safer materials by implementing safe-by-design strategies. It is also important to know whether endotoxin contamination may act as a confounding factor inducing toxic effects, as nano-sized cellulose may accumulate higher levels of endotoxin than conventional cellulose because of its higher specific surface area. Finally, it is of paramount importance to elucidate whether the genotoxicity, and thereby carcinogenicity, of nanocellulose arises by primary or secondary mechanisms of action. The former mechanism is executed by the material itself, whereas the latter mechanism involves an inflammatory response. As a threshold mode of action is assumed when genotoxicity is mediated by secondary mechanisms, occupational exposure limit (OEL) values could be derived for materials acting through inflammation.

3 MATERIALS AND METHODS

3.1 Experimental design

To achieve the objectives of our project, the following experimental design (Figure 3) was established.

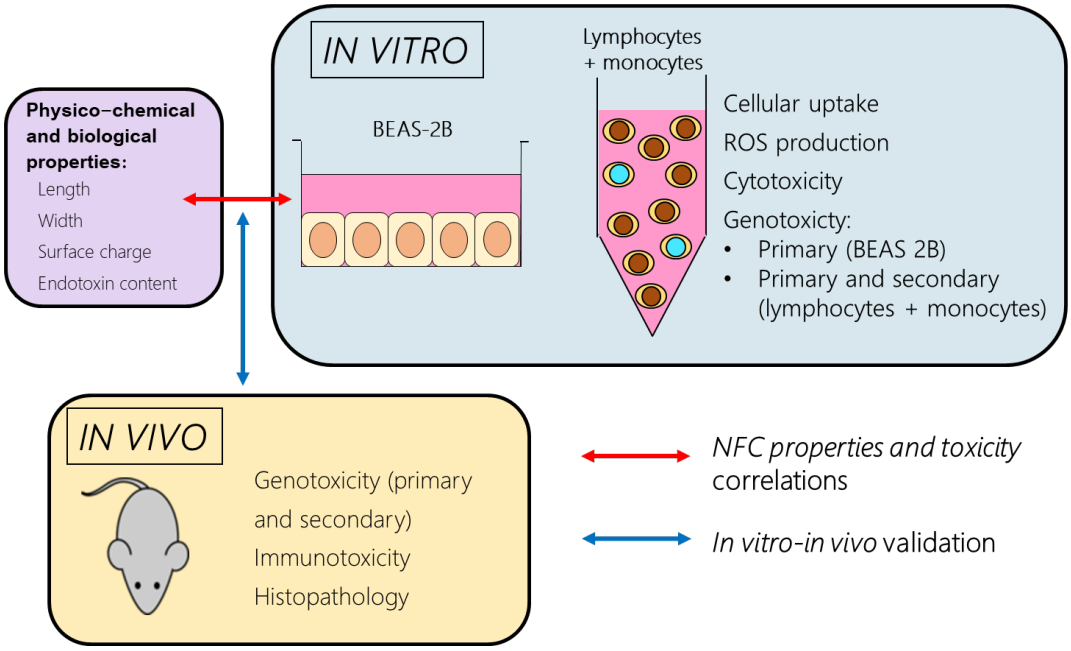

Figure 3. Experimental design of the study.

NFCs with different physico-chemical properties, and different endotoxin levels, were planned to be toxicologically tested using both *in vitro* and *in vivo* models. The *in vitro* models would include monolayer cultures of bronchial epithelial BEAS-2B cells and cultures of peripheral blood cells. The former can only detect primary genotoxic effects, as no inflammatory cells are present in the cultures. Secondary genotoxicity can be demonstrated in animal experiments, but its detection *in vitro* requires co-cultures of target cells and inflammatory cells. As lymphocytes and monocytes (which are precursors of macrophages) are present in whole blood cultures, this system was chosen for detecting *in vitro* secondary genotoxicity. Findings from the *in vitro* models were

expected to identify correlations between material properties and toxicological responses, which could be validated by the *in vivo* experiments.

Unfortunately, whole blood cultures were not suitable for testing nanocelluloses, because the branched fibers trapped the cells in suspension, hindering proper cell division. Hence, we had to abandon this approach. Instead, the complexity of the *in vivo* experiments was increased to fulfill the objectives.

A more detailed description of the materials studied, and the methods used for assessing the different toxicological endpoints is provided in the following sections.

3.2 Nanofibrillated celluloses

3.2.1 Synthesis and surface modification

Different NFCs were specifically synthetized for this study by Aalto University, according to the scheme shown in Figure 4.

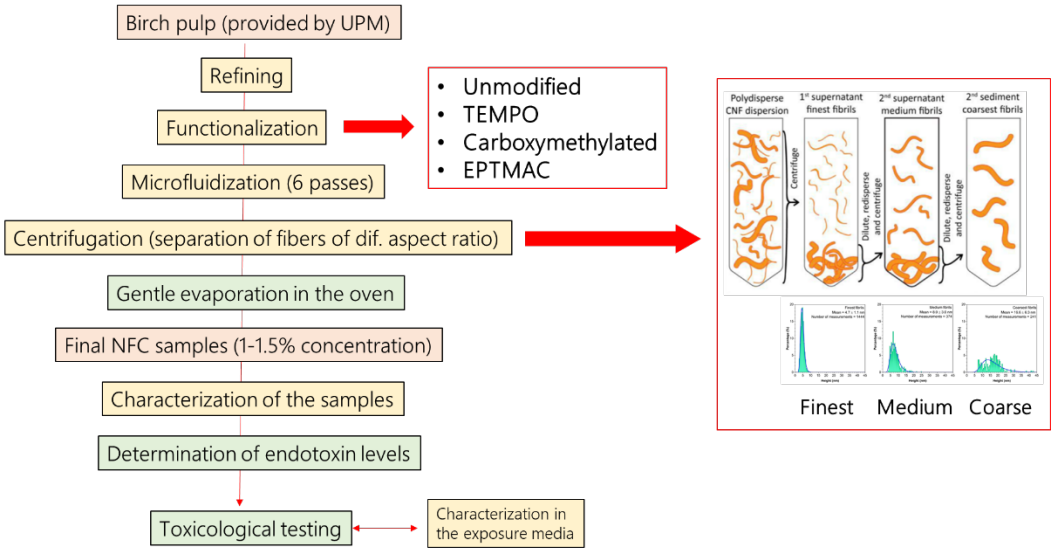

Figure 4. Synthesis of nanofibrillated celluloses (NFC) (partly adapted from Toivonen et al. 2018).

The NFCs were produced from a commercial bleached sulfite birch dissolving pulp provided by UPM Kymmene Oyj (Finland). The pulp fibers were refined and, then, either left unmodified, or pretreated by TEMPO-(2,2,6,6-tetramethyl-piperidin-1-oxyl)

oxidation, carboxymethylation or epoxypropyl trimethylammonium chloride (EPTMAC) quaternization, as explained below. Next, fibers were diluted to 1.5 % consistency, and nanofibrils were obtained by disintegrating the pulp fibers six times through a microfluidizer, as previously reported (Imani et al., 2020; Imani et al., 2019). In this way, four different surface functionalized NFCs - unmodified (U-NFC) and pretreated- TEMPO oxidized (T-NFC), carboxymethylated (C-NFC) and EPTMAC quaternized (E-NFC)- were obtained. Finally, three different size fractions (fine, medium and coarse) of each NFC type were obtained by subsequent centrifugations according to the procedure described by Toivonen and collaborators (Toivonen et al., 2018) and Imani and collaborators (Imani et al., 2020; Imani et al., 2019). The resulting dispersions were gently evaporated in an oven until reaching a 1.0-1.5 % concentration, which was considered adequate for toxicity testing.

The preparations of TEMPO oxidized, carboxymethylated and EPTMAC quaternized fibers have previously been described by Imani and collaborators (Imani et al., 2020; Imani et al., 2019), Im and collaborators (Im et al., 2018), and Kono and Kusumoto (Kono & Kusumoto, 2014), respectively.

As a result, twelve different NFC samples, with four different surface modifications (U-NFC, T-NFC, C-NFC and E-NFC), and three size fractions of each (coarse, medium and fine), were synthetized.

The 12 NFC samples were tested in the *in vitro* model. The original pulp was also included in these experiments as a non-nano-sized reference material. As the amounts of the fractionated samples that could be obtained were too little for performing *in vivo* studies, only unfractionated samples with different surface chemistry were testing in the *in vivo* model.

Water-based 1.0-1.5 % NFC suspensions were diluted to stock dispersions either in the cell culture medium (2 mg/ml) used in the *in vitro* experiments, or in water (2 mg/ml) for use in the *in vivo* studies. The stock dispersions were then mixed vigorously by high speed vortexing for 20 seconds. Then, serial dilutions were prepared in culture medium or water, and mixed with vortex for 20 seconds, before being added to the cells or administrated to the animals. In the case of the T-NFC fractions, due to the presence of bacterial contamination, the stock solution was sterilized by autoclaving before further dilutions in culture medium.

To gain further insight into the role that surface chemistry may play in the toxicological profile of NFCs, especially in the induction of genotoxic effects, five additional NFC materials were also assessed in our *in vitro* system. The materials were provided by Dr Natalia Ferraz, from Uppsala University, who had previously tested their toxicity in different cell lines (Lopes et al., 2017; Lopes et al., 2020).

The NFC materials had been synthetized by RISE Bioeconomy (Stockholm, Sweden) from commercial never-dried bleached sulfite softwood dissolving pulp. Non-functionalized NFC herein referred as enzymatically modified NFC (EZ-NFC) was produced by enzymatic pretreatment of the wood pulp following the protocol previously described (Pääkkö et al., 2007). Carboxymethylated (CM-NFC), hydroxypropyltrimethylammonium (H-NFC), phosphorylated (P-NFC) and sulfoethylated NFCs (S-NFC) were prepared as described by Lopes and collaborators (Lopes et al., 2020). It is worth mentioning that H-NFC and E-NFC (EPTMAC quaternized NFC) correspond to the same surface modification, as EPTMAC is the reagent used for the preparation of H-NFC.

As we aimed to compare our results with the previous findings of Uppsala University, the solutions of the NFCs were prepared and dispersed as they have previously described (Lopes et al., 2017). Hence, the stock solutions were dispersed by sonication and sterilized by autoclaving (except for H-NFC which was subjected to ultraviolet radiation). Then, the stock solutions were diluted in cell culture medium and sonicated again before being added to the cells.

3.2.2 Characteristics of the nanofibrillated celluloses

3.2.2.1 Physico-chemical characterization

The morphology of the different NFC samples synthetized by Aalto University was investigated by atomic force microscopy (AFM) and scanning electron microscopy (SEM) analyses. The images obtained from the AFM and SEM analyses were then analyzed using ImageJ, to extract the size distribution profile of 100 nanofibrils from each size fraction. An example of the SEM images is provided in Figure 5.

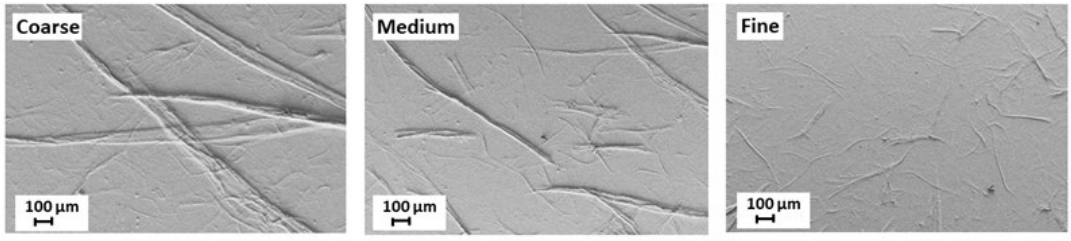

Figure 5. Scanning electron microscopy images of different size fractions of TEMPO oxidized nanofibrillated cellulose (T-NFC).

The NFC samples in the culture medium were characterized by assessing z-potential values in a ZetaSizer Nano instrument. The NFC samples provided by Uppsala University were characterized as described in Lopes et al. (2020).

The characteristics of all the NFC samples tested in this project is summarized in Table 1.

Table 1. Characteristics of the nanofibrillated cellulose (NFC) samples

NFC sample	Surface modification	Size fraction	Fiber diameter (nm)[a]	z-potential (mV)[b]
Materials synthetized by Aalto University for the in vitro studies				
U-NFC	None	Coarse	18.84 ± 1.41	−9.8 ± 0.8
		Medium	8.60 ± 0.13	−11.3 ± 1.1
		Fine	5.73 ± 0.09	−7.2 ± 0.6
T-NFC	TEMPO oxidation	Coarse	15.39 ± 0.21	−2.5 ± 0.3
		Medium	7.84 ± 0.28	−3.9 ± 0.8
		Fine	4.68 ± 0.90	−1.8 ± 0.1
C-NFC	Carboxymethylation	Coarse	17.48 ± 0.10	−11.4 ± 1.0
		Medium	8.95 ± 0.21	−16.1 ± 1.5
		Fine	4.94 ± 0.07	−14.2 ± 1.3
E-NFC	Hydroxypropyl-trimethylammonium substitution	Coarse	16.30 ± 1.04	12.8 ± 1.4
		Medium	7.56 ± 0.29	11.5 ± 0.9
		Fine	4.79 ± 0.14	3.3 ± 0.5
Materials synthetized by Aalto University for the in vivo studies				
U-NFC	None		6-29	-20.1±1.1
T-NFC	TEMPO oxidation		5-21	-21±0.31
C-NFC	Carboxymethylation		5-27	-27.2±0.7
Materials provided by Uppsala University				
EZ-NFC	None	10–30 nm aggregates		−14.1 ± 5.2
CM-NFC	Carboxymethylation	Some individual fibrils, fiber aggregates (10–15 nm)		−20.8 ± 0.6
H-NFC	Hydroxypropyl-trimethylammonium substitution	4–5 nm individual fibrils		18.7 ± 1.0

NFC sample	Surface modification	Fiber diameter (nm)[a]	z-potential (mV)[b]
P-NFC	Phosphorylation	4–5 nm individual fibrils	−29.6 ± 1.1
S-NFC	Sulfoethylation	Some individual fibrils, fiber aggregates (10–12 nm)	−17.8 ± 0.7

[a]Mean ± SD (Aalto's materials for *in vitro* studies), range (Aalto's materials for *in vivo* studies), description (Uppsala's materials)
[b]Mean ± SD

3.2.2.2 Evaluation of the levels of endotoxin contamination

The functionalized NFCs synthetized by Aalto University were analyzed using the Pierce™ Chromogenic Endotoxin Quant Kit, which takes into consideration possible interference with β-glucans. Leachable β-glucans are another important group of contaminants in cellulosic materials that can also have an immunogenic effect (Liu et al., 2018). The pulp, the three size fractions of U-NFC and the NFCs provided by Uppsala University were analyzed by the Pierce™ LAL Chromogenic Endotoxin Quantitation Kit, which does not take into consideration the role of β-glucans. However, separate analyses of β-glucans performed with the Uppsala's materials showed levels of β-glucans below the accepted limit (data not shown).

Table 2 summarizes the results of the endotoxin evaluation of all the cellulosic materials considered in this project.

Table 2. Levels of endotoxin contamination in the different cellulosic materials.

Cellulosic sample	Size	Endotoxin level (EU/mL)
Materials synthetized by Aalto University for the in vitro studies		
Pulp[a]		0.17
U-NFC[a]	Coarse	0.35
	Medium	0.48
	Fine	0.83[d]
T-NFC[b]	Coarse	> 1.2[c,d]
	Medium	> 1.2[c,d]
	Fine	> 1.2[c,d]

Cellulosic sample	Size	Endotoxin level (EU/mL)
C-NFC[b]	Coarse	0.15
	Medium	0.15
	Fine	0.14
E-NFC[b]	Coarse	0.07
	Medium	0.07
	Fine	0.08
Materials synthetized by Aalto University for the in vivo studies[b]		
U-NFC		> 1.2[c,d]
T-NFC		0.22
C-NFC		0.15
Materials provided by Uppsala University[a]		
EZ-NFC		> 1.2[c,d]
CM-NFC		> 1.2[c,d]
H-NFC		0.12
P-NFC		> 1.2[c,d]
S-NFC		> 1.2[c,d]

[a]Measured by the Pierce™ LAL Chromogenic Endotoxin Quantitation Kit
[b]Measured by the Pierce™ Chromogenic Endotoxin Quant Kit
[c]1.2 EU/mL was the detection limit allowed by the kit
[d]Levels above the accepted contamination limit (0.5 EU/mL)

All the functionalized NFC samples synthetized by Aalto University, except the three size fractions of T-NFC, showed endotoxin levels that were below the 0.5 EU/mL limit value established by the US Food Drug Agency for inhalation studies (FDA, 2014). Conversely, the fine fraction of U-NFC and the unfractionated U-NFC sample from Aalto University exceeded this limit. On the other hand, H-NFC was the only sample that showed an acceptable level among the NFCs provided by Uppsala University.

3.3 *In vitro* studies

Transformed human bronchial epithelial BEAS-2B cells constituted the *in vitro* model used to investigate cellular response to the cellulosic materials in the present study. This cell line has been reported to be a good model of the human lung tissue (Garcia-Canton et al., 2013), and it has extensively been used in asbestos and nanotoxicological research (Haniu et al., 2011; Nymark et al., 2015).

The BEAS-2B cells were obtained from the American Type Culture Collection through LGC Promochem AB (Borås, Sweden). The cells were grown in serum-free LHC-9 medium at 37 °C in a humidified atmosphere of 5 % CO_2. Log-phase BEAS-2B cells were plated on 48-well plates (comet assay), 96-well plates (cytotoxicity and radical oxygen species) and 2-well chamber slides (micronucleus assay) from one to three days prior to exposure to the NFC samples.

3.3.1 Cellular uptake

The potential internalization of NFC by BEAS-2B cells was assessed using the methods described below. The calcofluor white staining was applied to all NFCs studied in this project. A more thorough analyses by the hyperspectral imaging system and transmission electron microscopy (TEM) was only done for the NFCs synthetized by Aalto University.

3.3.1.1 Calcofluor white staining

NFCs were stained with the Calcofluor White Stain (Merck KGaA, Darmstadt, Germany). Calcofluor white is a fluorescence dye that stains cellulose and chitin fibers, and it has been reported to be an excellent tool for the microscopic examination of NFCs (Čolić et al., 2015).

The staining was performed on the same slides that were used for the scoring of the micronucleus (MN) frequency (for slide preparation, see section 3.3.3), which had been treated with cellulase (8.7 µl/ml; 15 min at room temperature) to remove excess NFC outside the cells. The staining was performed according to the protocol previously described (Tomić et al., 2016).

3.3.1.2 Hyperspectral image analyses

Fibers taken up by the cells were identified by hyperspectral imaging. The hyperspectral imaging system (CytoViva, Inc, Auburn, AL, USA) consisted of a dark-field microscope connected to an equipment that enabled a label-free spectral identification of nanomaterials within a sample, using a spectral library created from the material. This

technology has previously been utilized, e.g., to recognize various types of nanoparticles in tissues (Cabellos et al., 2020) and in cell cultures (Siivola et al., 2020; Vales et al., 2020).

BEAS-2B cells were exposed to NFC dispersions (12 and 111 µg/ml) for 6 and 24 h. The processing of samples, fiber recognition, and image analyses were performed as previously described (Siivola et al., 2020).

3.3.1.3 Transmission electron microscopy analyses

As none of the previous techniques allowed us to clearly distinguish whether nanofibrils are located on the cell membrane or within the cell, transmission electron microscopy (TEM) was utilised to assess cellular internalization.

Parallel cultures of BEAS-2B cells, treated as described for the hyperspectral image analyses, were processed for TEM analyses as previously described (Lindberg et al., 2013).

3.3.2 Cytotoxicity and production of reactive oxygen species

The purpose of the cytotoxicity assays was to choose the range of NFC and pulp doses to be tested in the genotoxicity assays. According to OECD guidelines on the *in vitro* MN assay (OECD, 2016b), the highest test substance concentration to be included in the assay (in the absence of MN induction at earlier doses) should produce 55 ± 5 % cytotoxicity. Higher levels may induce chromosome damage as a secondary effect of cytotoxicity (Galloway, 2000) and should, therefore, be avoided.

BEAS-2B cells were seeded in 96-well plates and grown to semiconfluency, after which they were exposed to 4-1000 µg/ml of the cellulosic material dispersions for 24 and 48 h. A positive control treatment (0,1 % Triton X-100) was included in the test, while untreated cells served as a negative control at each time point. All treatments were performed in quadruplicates, and the experiments were repeated twice.

Cytotoxicity was measured using the CellTiter-GloVR luminescent cell viability assay as previously described (Catalán et al., 2015). This assay reflects all treatment-related effects (necrosis, cell cycle delay and apoptosis) that reduce the number of viable or living cells. Cytotoxicity was expressed as the relative luminescence in the treated cultures in comparison with the control cultures. In addition, as requested by the OECD TG 487 (OECD, 2016b), cytokinesis was always measured when performing the MN assay. In this way, the adequacy of the chosen dose range based on the luminometric assay could be confirmed.

The levels of intracellular ROS were measured using the chloromethyl derivative of 2',7'-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) (Invitrogen, Eugene, OR, USA) according to the manufacturer's guidelines.

BEAS-2B cells were plated on 96-well plates and grown to semiconfluency for two days. After being washed with phosphate buffered saline (PBS), the cells were loaded with 2.5 µM CM-DCFDA in PBS for 30 min at 37 °C. Thereafter, the loading buffer was removed, and the cells were washed with PBS. Then the cells were treated with the cellulosic material dispersions at 4-1000 µg/ml. Hydrogen peroxide (2 mM) was used as a positive control, while untreated cells served as a negative control at each time point. Fluorescence was recorded at 3, 6 and 24 h with a plate reader. The average fluorescent intensity was calculated by subtracting background values. All treatments were performed in quadruplicates, and the experiments were repeated twice.

3.3.3 Genotoxicity

The comet (single cell gel electrophoresis) assay was used to study DNA strand breaks and alkaline labile sites in BEAS-2B cells after the cellulose exposures. BEAS-2B cells in log phase were plated in 48-well plates two days prior to exposure. Exposure time was 24 h, and the cells were exposed to 4- 1000 µg/ml of each cellulose material. Untreated controls and positive controls (20 mM H_2O_2) were included in all series. All treatments were performed in duplicates, and the experiments were repeated twice.

The comet assay was performed in alkaline conditions (pH > 13) as described previously (Vales et al., 2020). The slides were coded, and one scorer performed the comet analysis using a fluorescence microscope and an interactive automated analysis software. The percentage of DNA in the comet tail from 200 cells per dose and experiment (two replicates per dose, two slides per replicate, 50 cells/slide) was used as a measure of the amount of DNA damage.

The cytokinesis-block micronucleus (CBMN) assay was applied to study chromosomal damage in BEAS-2B cells after exposure to the cellulosic samples. The cells were plated on 2-well chamber slides and incubated for 48 h, to reach semi-confluence, prior to the treatment.

Based on the cytotoxicity assay, the cells were exposed for 48 h to 4-1000 µg/ml. Cytochalasin B (9 µg/ml) was added to the cell cultures 6 h after starting the treatment, to induce binucleation of dividing cells. Untreated cultures and cultures treated with the positive control (150 ng/ml Mitomycin C) were included in all experiments. All cultures were prepared in duplicate.

After the exposure, cells were treated for 15 min at room temperature with cellulase enzyme blend (8.7 µl/ml) to get rid of the non-internalized nanofibrils that could interfere with MN scoring. Then, the slides were fixed and stained with acridine orange and 4,6-diamidino-2- phenylindole (DAPI) and kept at 4 °C protected from light until being analyzed.

All analyses were performed by one scorer. Cytostasis was measured as previously done (Siivola et al., 2020) in the same cultures that were used for the CBMN assay, as a means to ensure that the treatments were conducted at appropriate levels of cytotoxicity.

To evaluate MN frequencies, MN were scored in 4000 binucleate cells per treatment (2000 binucleate cells per replicate; two replicates per treatment) using a fluorescence microscope. Binucleate cells and micronuclei (MNi) in them were identified using a 40× objective lens and a FITC/TRITC double filter for acridine orange, and the MNi were verified with DAPI.

3.4 *In vivo* studies

Occupational exposure to nanomaterials mainly occurs by inhalation. Simplified *in vitro* models are not able to fully predict the effects and fate of nanomaterials in the pulmonary system, and systemic effects of the materials can only be studied in an animal model. *In vivo* studies also allow testing of the health effects on a longer time span than cell culture -based assays. In the current project, *in vivo* studies were utilized to examine both the acute and sub-chronic effects of NFCs, and to unravel the MoA involved in the induction of such effects.

The *in vivo* experiments were carefully planned to fulfill the 3R requirements. The study was approved by the Animal Experiment Board and the State Provincial Office of Southern Finland. The *in vivo* experiments were performed in C57BL/6 female mice (age 7-8 weeks, weight ~20 g) at the Laboratory Animal Center of Helsinki University. The animals were purchased from Scanbur AB and they were housed in individually ventilated plastic cages bedded with aspen chip and were provided with standard mouse chow diet and tap water ad libitum. The environment of the animal room was carefully controlled, with a 12-h dark/light cycle, temperature of 20-21 °C, and relative humidity of 40-45 %. The mice were weighed at the beginning and in the end of the experiment and their health was carefully monitored throughout the experiment.

Three NFCs synthetized by Aalto University - U-NFC, T-NFC and C-NFC - were chosen to be tested *in vivo* for pulmonary and systemic toxic effects. Three different doses (14, 28 and 56 µg/mouse/administration) of each NFC were administrated to mice by repeated (3x) pharyngeal aspiration, and the effects were assessed 1, 28 and 90-day post-exposure (Figure 6).

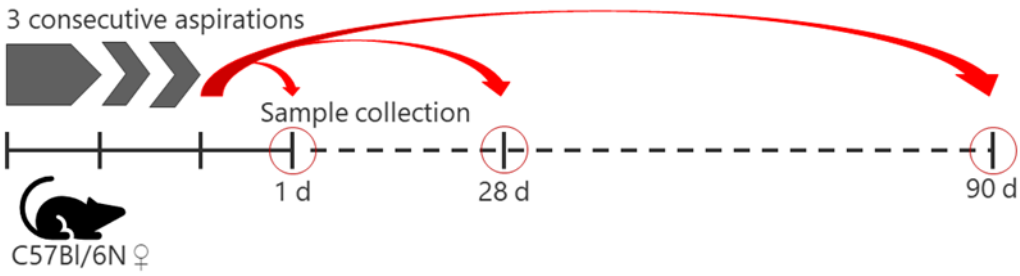

Figure 6. Experimental design of the *in vivo* studies.

To assess the potential toxicological effects of endotoxin contamination, additional groups of mice were treated with T-NFC samples contaminated with increasing amounts of LPS (0.02-50 ng/mouse/administration). Besides, another group of mice were treated with multiwall carbon nanotubes (MWCNTs, 28 µg/mouse/administration) and with Mitomycin C (40 µg/mouse, intraperitoneally injected). These animals served as positive controls for the local pulmonary effects and the micronucleus assay. A group of mice treated with water was included at each post-administration time as negative controls.

Material dispersions were administered by pharyngeal aspiration as previously described (Ilves et al., 2018). This is a safe and reliable method for exposing rodents, and the dose delivered into the lungs can be ascertained. The mice were anesthetized with vaporized isoflurane (4.5 %, 10 min), and 50 µl of the NFC dispersion was delivered onto the vocal folds by pipetting under visual control. Immediately after the delivery, the mouse nostrils were covered, enforcing the mouse to inspire the instilled dispersion. Recovery of the animals was monitored for 30 min after each aspiration.

The mice were sacrificed using an overdose of isoflurane. Blood from the vena cava was collected with 5 % EDTA and stored on ice until being processed in the laboratory. The lungs were lavaged several times with PBS and NaCl via the tracheal tube, and the bronchoalveolar lavage (BAL) samples were collected. Lungs and liver were collected for further preparations.

3.4.1 Inflammatory reaction

Inflammatory reaction was assessed in lung tissue and BAL fluid at all three timepoints. The first BAL sample collected (800 µl) was cytocentrifuged on a slide, and the cells were stained with May Grünwald-Giemsa (MGG). The numbers of inflammatory cells (macrophages, neutrophils, eosinophils and lymphocytes) were counted under a light microscope at 40x magnification. The rest of the sample was fixed in 2.5 % glutaraldehyde and stored refrigerated in PBS for TEM analysis.

Tissue sections of the left lung lobe and liver were collected in 10 % formalin and allowed to fixate for 24 h at room temperature. The samples were thereafter rehydrated and embedded in paraffin. For histological examination, the tissue sections were stained with hematoxylin and eosin (H&E).

3.4.2 Genotoxicity

Biomarkers of genotoxic effects were assessed in mouse samples collected at 28-d and 90-day post-administration. One day post-administration samples were not analyzed for genotoxic effects, as secondary effects, mediated by an inflammatory reaction, may not still be detectable.

Blood samples, collected with 5 % EDTA,were diluted 1:5 in fetal bovine serum on a microscopy slide, to form a smear, dried overnight and fixated in methanol for MN analysis. The slides were stained with MGG, and MN analysis was performed in accordance with TG 474 (OECD, 2016a). The frequencies of micronucleated polychromatic erythrocytes (MNPCEs) and micronucleated normochromatic erythrocytes (MNNCEs), in 2000 polychromatic (PCEs) and normochromatic erythrocytes (NCEs) per mouse, respectively, were analyzed using a light microscope.

Pieces of the right lung lobes and liver were minced in chilled mincing solution (Hank's balanced salt solution with 20 mM EDTA) and mechanically dispersed into a single cell suspension by using a cell strainer (40 µm Ø). The cell suspensions and the rest of the BAL samples collected were centrifuged at 400 ×g for 5 min. The comet assay was performed in alkaline conditions (pH > 13) as described above (section 3.3.3). The percentage of DNA in the comet tail from 150 cells per animal (two replicates, 75 cells each) was used as a measure of the amount of DNA damage.

A small proportion of the cell suspensions were exposed to hydrogen peroxide (100 µM) *ex vivo* and used as an internal positive control to verify the performance of the comet assay.

3.4.3 Biopersistence

The H&E stained slides prepared for histopathological examination were also used to assess the presence and biopersistence of the cellulosic materials in the lungs. In addition, part of the left lung lobe was fixed in glutaraldehyde and processed for TEM analysis. Tissue samples were collected on uncoated copper grids and post-fixed in osmium tetroxide. The samples were stained with uranyl acetate and lead citrate and the presence or absence of cellulose materials was qualitatively determined using TEM (Jeol JEM 1220).

4 RESULTS

4.1 Role of size and surface chemistry on the *in vitro* toxicological effects induced by NFCs

A summary of the toxicological outcomes for the cellulosic materials analyzed in this project is provided in Table 3.

Table 3. Summary of the *in vitro* toxicological responses displayed by the cellulosic materials analyzed in BEAS-2B cells.

		Cytotoxicity		**ROS**			**Comet**	**Micronucleus**
Material	**Size**	**24 h**	**48 h**	**3 h**	**6 h**	**24 h**	**24 h**	**48 h**
Materials synthetized by Aalto University								
Pulp		-	-	+	+	+	-	-
U-NFC	Coarse	-	-	+	+	+	-	-
	Medium	-	-	-	-	-	-	-
	Fine	-	-	+	+	+	-	-
T-NFC	Coarse	-	-		+	+	-	-
	Medium	-	-	+	+	+	-	-
	Fine	-	-	+	+	+	-	-
C-NFC	Coarse	-	±	+	+	+	-	+
	Medium	-	±	+	+	+	-	-
	Fine	-	±	+	+	+	-	-
E-NFC	Coarse	-	±	+	+	+	-	-
	Medium	-	±	+	+	+	-	-
	Fine	-	±	+	+	+	+	+
Materials provided by Uppsala University								
EZ-NFC		-	-	-	-	+	-	-

Material Size	Cytotoxicity 24 h	Cytotoxicity 48 h	ROS 3 h	ROS 6 h	ROS 24 h	Comet 24 h	Micronucleus 48 h
CM-NFC	-	-	+	+	+	-	-
H-NFC	-	-	-	-	-	-	-
P-NFC	-	-	-	-	-	-	-
S-NFC	-	-	-	-	-	-	-

4.1.1 Cytotoxicity and induction of intracellular reactive oxygen species

The purpose of the cytotoxicity assay was to choose the range of doses of each NFC to be tested in the genotoxicity assays. The highest tested doses were 500 µg/ml for the NFCs provided by Uppsala University, and 1 mg/ml for the NFCs synthetized by Aalto University. None of the NFCs provided by Uppsala University reached the cytotoxicity limit at any of the treatment times (24 and 48 h). On the other hand, neither the original pulp nor the NFCs synthetized by Aalto University were cytotoxic in BEAS-2B cells up to 1 mg/ml at the 24-h treatment. After the 48-h treatment, the highest tested dose (1 mg/ml) decreased the number of living cells for all NFCs, but not for the pulp. The effect was especially clear for all size fractions of C-NFC and E-NFC, which showed values below the 55 ± 5 % cytotoxicity limit. However, when cytostasis was assessed in the cultures used for scoring MN induction, the 55 ± 5 % upper limit set by the guidelines was exceeded by none of the cellulosic materials, at none of the tested doses.

The results of the induction of intracellular ROS were quite different depending on the source of the NFCs. EZ-NFC and CM-NFC were the only materials provided by Uppsala University that produced a significant linear dose-dependent increase of ROS. EZ-NFC showed a significant linear dose-response at 24 h ($p < 0.01$), whereas CM-NFC induced a significant linear dose-response at 3 h ($p < 0.01$), 6 h ($p < 0.001$) and 24 h ($p < 0.0001$) of exposure. On the other hand, the medium size fraction of U-NFC was the only material in the Aalto study that did not induce ROS formation at any of the time points. The formation of ROS by NFC samples with the same surface functionalization synthetized by Aalto University and provided by Uppsala University are shown in Figures 7 and 8, respectively.

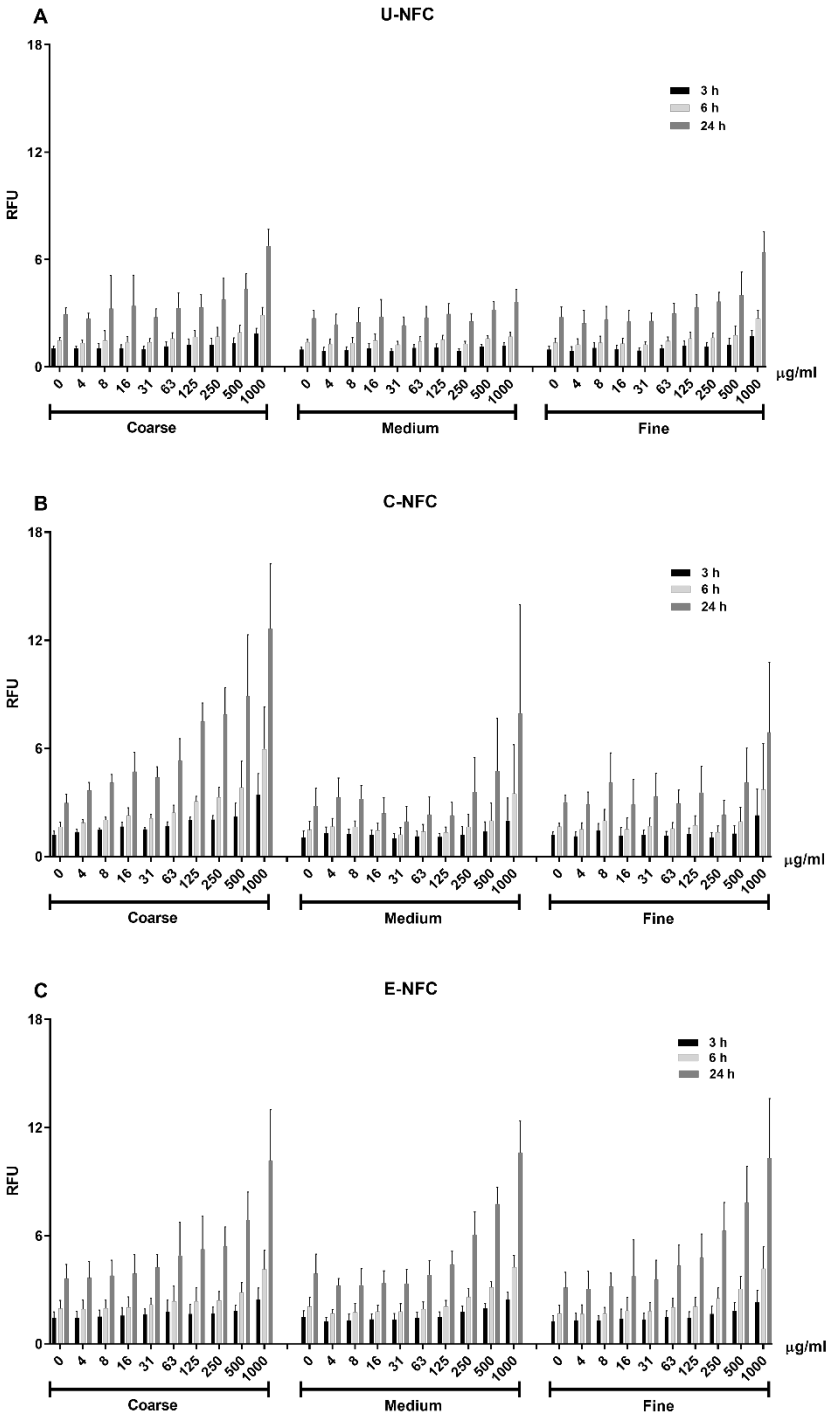
A
U-NFC
RFU
18
12
6
0
3 h
6 h
24 h
0 4 8 16 31 63 125 250 500 1000
µg/ml
Coarse
Medium
Fine
B
C-NFC
C
E-NFC

Figure 7. Induction of intracellular reactive oxygen species by NFC materials synthetized by Aalto University. The production of ROS was assessed at 3, 6 and 24 h exposure to (**A**) U-NFC, (**B**) C-NFC and (**C**) E-NFC. Data are expressed as relative fluorescence units (RFU) and presented as the mean ± standard error of the mean. Linear dose-response (linear regression) was statistically significant for all materials ($p < 0.01$) expect the coarse fraction of C-NFC at 3 h, and the medium fraction of U-NFC at all the time points.

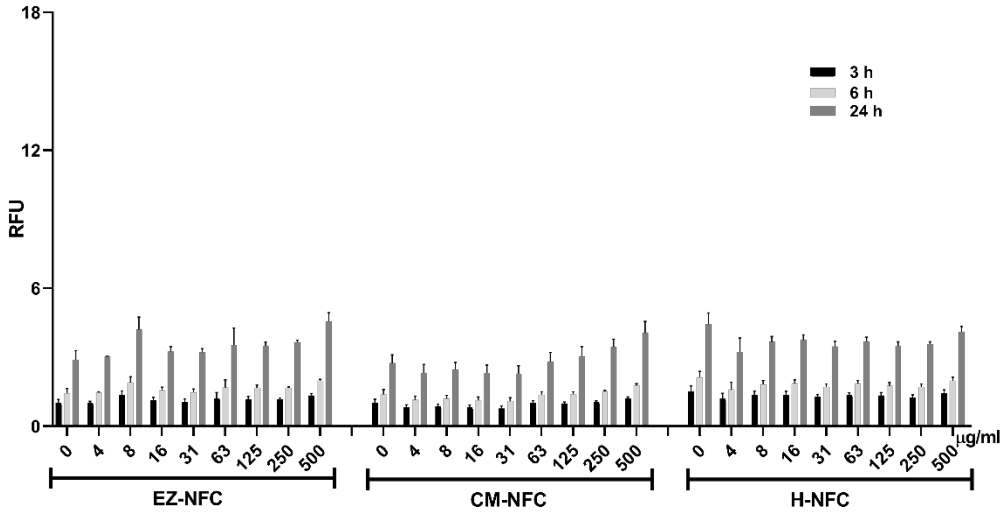

Figure 8. Induction of intracellular reactive oxygen species by NFC materials provided by Uppsala University. The production of ROS was assessed at 3, 6 and 24 h exposure to EZ-NFC, CM-NFC and H-NFC. Data are expressed as relative fluorescence units (RFU) and presented as the mean ± standard error of the mean. Linear dose-response (linear regression) were statistically significant for EZ-NFC at 24 h ($p < 0.01$), and CM-NFC at 3 h ($p < 0.01$), 6 h ($p < 0.001$) and 24 h ($p < 0.0001$).

Surface chemistry and size did not always affect the induction of ROS in the same way. Instead, they influenced one another. All NFCs synthetized in this project significantly increased the formation of ROS at the three time points analyzed, except the coarse fraction of C-NFC at 3 h, and the medium fraction of U-NFC at all the time points. However, the materials differed in the effectivity of the induction, E-NFC and C-NFC showing the most pronounced effects. The original pulp also induced a significant increase in ROS at the three time points, but the average levels were lower than with the coarse fraction of U-NFC, which it was compared to (data not shown).

The positive control, H_2O_2 (2 mM), induced a statistically significant increase in ROS production over the negative control values in all the experiments performed (7.91 ± 0.44-fold increase; $p < 0.003$), confirming the validity of the experiments (data not shown).

4.1.2 Genotoxicity

Surface chemistry and size also influenced one another in the generation of DNA and chromosome damage by the NFCs synthetized by Aalto University.

Figure 9 shows the induction of DNA damage by the pulp and NFCs of different surface chemistries and size fractions.

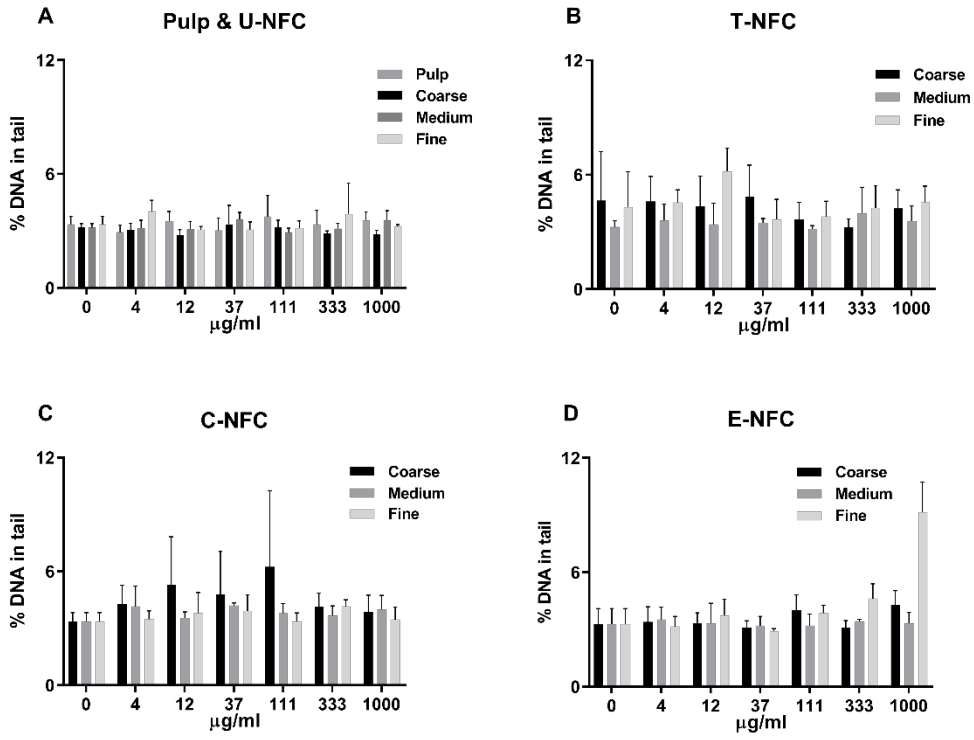

Figure 9. DNA strand breaks assessed by the comet assay in BEAS-2B cells after 24-h exposure to pulp and U-NFC (**A**), T-NFC (**B**), C-NFC (**C**) and E-NFC (**D**). Data are expressed as percentage of DNA in tail and presented as the mean ± standard error of the mean (SEM). The fine fraction of E-NFC induced a significant dose-dependent increase ($p < 0.0001$, linear regression) in DNA damage. The positive control, H_2O_2 (20

mM), induced a statistically significant increase in the percentage of DNA in tail over the negative control values in all the experiments performed (15.2 ± 1.1-fold increase; $p < 0.02$), confirming the validity of the experiments.

The original pulp did not significantly affect the level of DNA damage. Among the NFCs synthetized in this project, only the fine fraction of E-NFC induced a significant dose-dependent increase ($p < 0.0001$) in DNA damage, the other size fractions and surface functionalized types showing not significant effect. On the other hand, none of the NFCs provided by Uppsala University were able to induce DNA damage compared with the negative control at any of the tested doses (data not shown).

The induction of micronuclei by the pulp and the NFCs synthetized by Aalto University are shown in Figure 10.

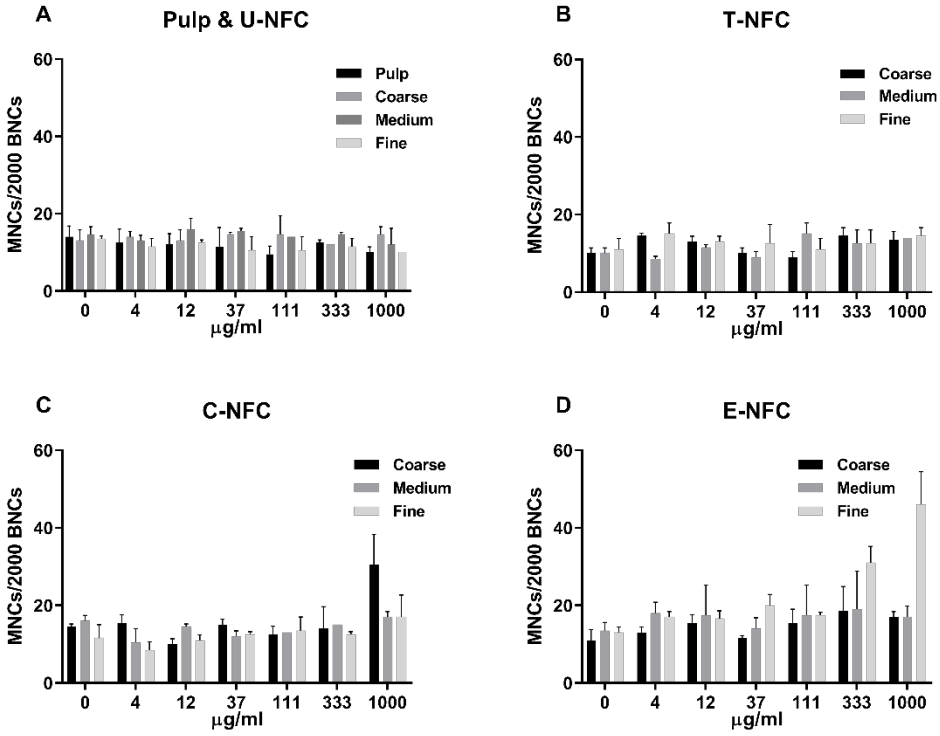

Figure 10. Chromosome damage assessed by the micronucleus assay in BEAS-2B cells after 48-h exposure to pulp and U-NFC (A), T-NFC (B), C-NFC (C) and E-NFC (D). Data are expressed as frequency of micronucleated cells in 2000 binucleated cells (MNCs/2000 BNCs) and presented as the mean ± standard error of the mean (SEM). Significant dose-

responses (p<0.0001, linear regression) were induced by the coarse fraction of C-NFC and the fine fraction of E-NFC. The positive control, MMC (150 ng/ml), induced a statistically significant increase in the frequency of micronucleated cells over the negative control values in all the experiments performed (21.6 ± 1.8-fold increase; $p < 0.01$; 72.4 ± 3.5 % cytostasis) confirming the validity of the experiments.

Significant dose-responses ($p < 0.0001$) only existed for C-NFC in the case of the coarse fraction, and for E-NFC in the case of the fine fraction. No significant increase in micronuclei was either induced by the birch pulp. On the other hand, none of the NFCs provided by Uppsala University were able to induce an increase in chromosome damage compared with the negative control at any of the tested doses (data not shown).

4.1.3 Cellular uptake of NFCs

Figure 11 shows an example of visualizing NFCs using the calcofluor staining. Calcofluor staining was performed on cellulase pre-treated slides, therefore non-internalized NFC was not expected to be present in these preparations. The stained NFC that appears to be associated with some cells (Figures 11a, 11b) may reflect cellular internalization. However, it may also be NFC material attached to the cell membrane that was not efficiently eliminated by the cellulase treatment. Most cells showed no calcofluor-stained material, suggesting that the possible NFC internalization concerned a minority of the cells. No stained material could be found in the untreated cultures (Figure 11c).

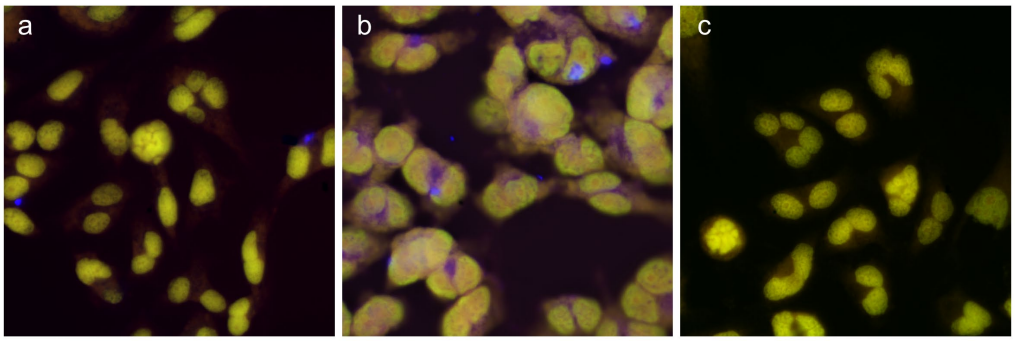

Figure 11. Examples of calcofluor staining (blue) in cells treated with U-NFC (111 µg/ml) (**a**), EZ-NFC (167 µg/ml) (**b**) and untreated cells (**c**). Counterstaining by acridine orange.

Results from the hyperspectral imaging analyses showed that spectra reflected by the NCF were nonuniform and very similar to the spectra reflected from the cellular structures. Hence, the rate of recognition of NFC was very low, making the analysis of NFC internalization difficult. Results from BEAS-2B cells exposed to 12 µg/ml of the fine

fraction of E-NCF are shown in Figure 12. The distribution of the matching pixels implies localization of the NFC on the cell membrane. Most matches are located on large bundles of fibers, which appear to be attached to, but not internalized by the cells. However, the low rate of recognition precluded clear conclusions.

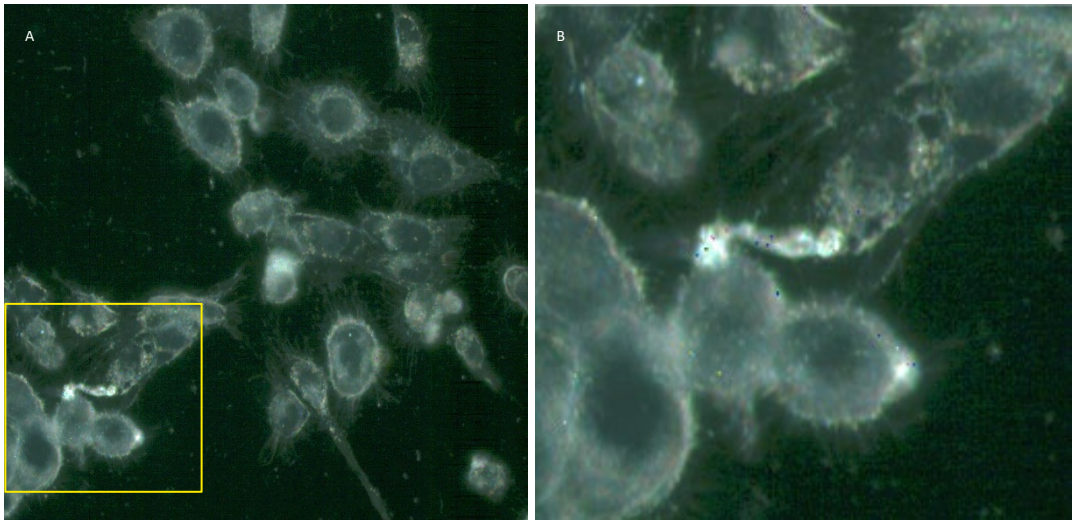

Figure 12. Hyperspectral images (400x) of cells exposed to 12 µg/mL of the fine fraction of E-NCF. The hyperspectral images were matched with the material's spectral library. The matching pixels are shown in false colors that overlaid on top of the hyperspectral image (**A**). A magnification of the area is indicated by the square (**B**).

TEM analysis confirmed that NFC materials could be found inside the cytoplasm of some BEAS-2B cells (Figure 13). However, most of the cells showed no NFC internalization.

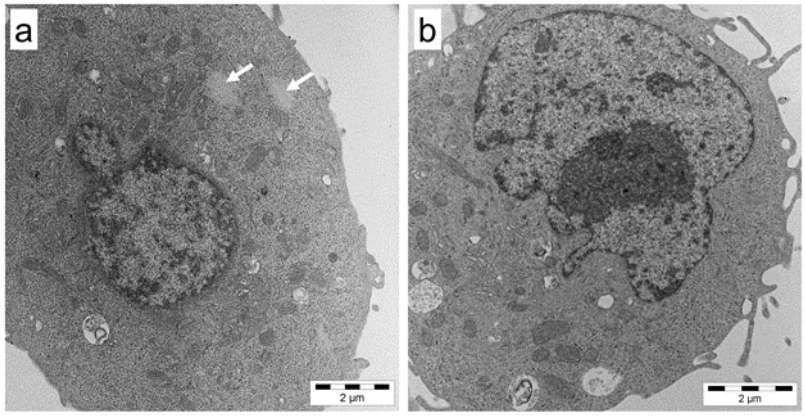

Figure 13 Transmission electron microscopy (TEM) pictures of cells exposed for 6 h to 111 µg of the fine fraction of E-NFC (**a**) and untreated cells (**b**). Intracellular NFC indicated with arrows.

4.2 Influence of surface chemistry on the pulmonary toxicity induced by NFCs

A summary of the toxicological results induced by NFCs with different surface functionalization in the treated mice is provided in Table 4.

Table 4. Summary of the *in vivo* toxicological responses displayed by NFCs with different surface functionalization.

	Toxicological endpoint (tissue)													
	Immunotoxicity (BAL & lung)			DNA damage						Chromosome damage (bone marrow)		Biopersistence (lung)		
				BAL		Lung		Liver						
	Exposure time (d)													
	1	28	90	28	90	28	90	28	90	28	90	1	28	90
U-NFC	+++	++	+	-	-	-	-	-	-	-	-	+	+	+
T-NFC	+++	++	+	-	+	-	-	-	-	-	-	+	+	+
C-NFC	+++	++	-	-	-	-	-	-	+	-	-	+	+	+

BAL, bronchoalveolar lavage

4.2.1 Clinical signs and and body weight

No clinical signs of toxicity were observed during the study period. Decreases in body weight, compared to the untreated group, were only found for the highest dose of U-NFC at 1 d post-administration.

4.2.2 Inflammatory reaction

Based on bronchoalveolar lavage (BAL) fluid cellularity (Figure 14) all NFCs induced an acute neutrophil influx in the lungs, accompanied by eosinophilia. The reaction gradually subsided within the following 3 months.

Figure 14. Percentage of different inflammatory cell types in the bronchoalveolar lavage (BAL) fluid of mice treated by pharyngeal aspiration with different doses of U-NFC, T-NFC and C-NFC, at 1, 28 and 90-d post-administration. Animals in the positive control

groups were treated with Mitsui-7 (28 µg/mouse/aspiration) and Mitomycin C (40 µg/mouse, intraperitoneal injection).

The histopathological examination of the H&E stained lung tissue (Figure 15) showed an acute inflammatory reaction surrounding bronchia and in the alveolar region around material aggregates. Pulmonary inflammation was mostly resolved after 3 months, but some macrophages were still present around material aggregates.

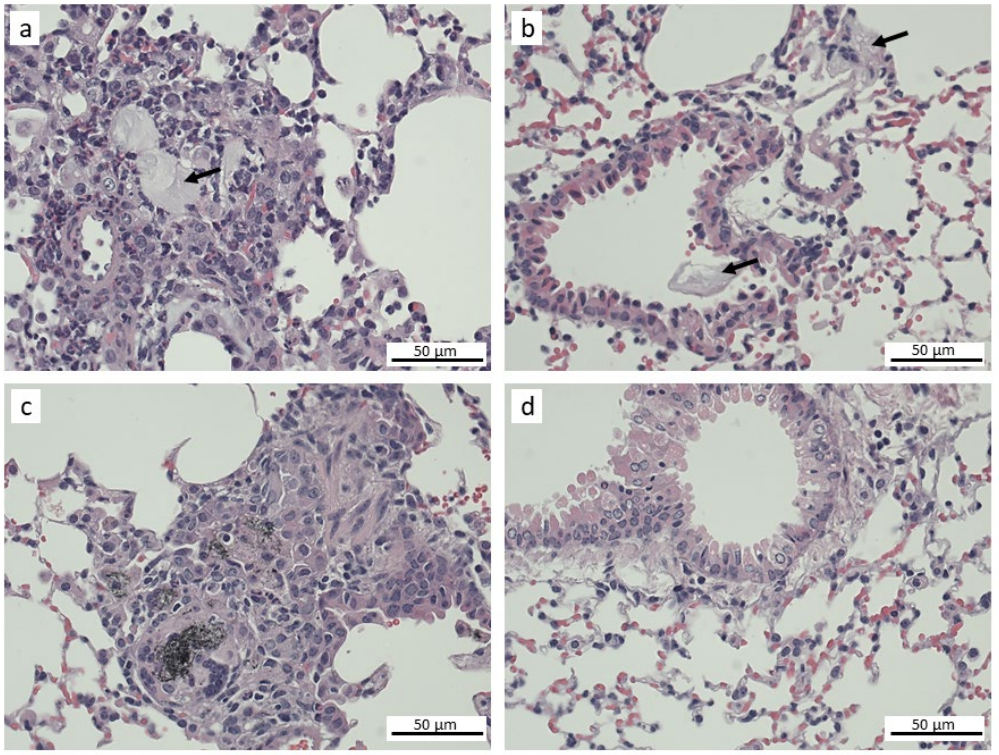

Figure 15. Hematoxylin and Eosin stained mouse lung tissue sections showing an acute inflammatory reaction 1 day after exposure to 28 µg/mouse/aspiration of T-NFC (**a**) that has resolved after 90 days (**b**), recruitment of macrophages induced by MWCNTs at 90 d post-administration (**c**) and normal lung tissue in water-treated mice at 90 d post-administration (**d**). NFC material indicated with arrows.

NFC induced a lower pulmonary inflammation than long, straight multi walled carbon nanotubes (Mitsui-7, 28 µg/mouse/administration) which produced a stronger acute neutrophilic reaction in BAL fluid and in lung tissue than any of the NFC materials. Mitsui-7 also showed signs of granuloma formation in the mouse lung tissue 90 days after the pulmonary exposure (Figure 15c).

4.2.3 Genotoxicity

Pulmonary exposure to the anionic NFCs was associated with local or systemic genotoxic effects 3 months after the administration. A statistically significant increase in DNA damage was observed 90 days after the last exposure to all doses of T-NFC in BAL cells ($p < 0.004$), and of C-NFC in liver cells ($p < 0.007$) (Figure 16). None of the tested materials was able to induce the formation of MNi in blood erythrocytes at any of the doses or timepoints studied (data not shown).

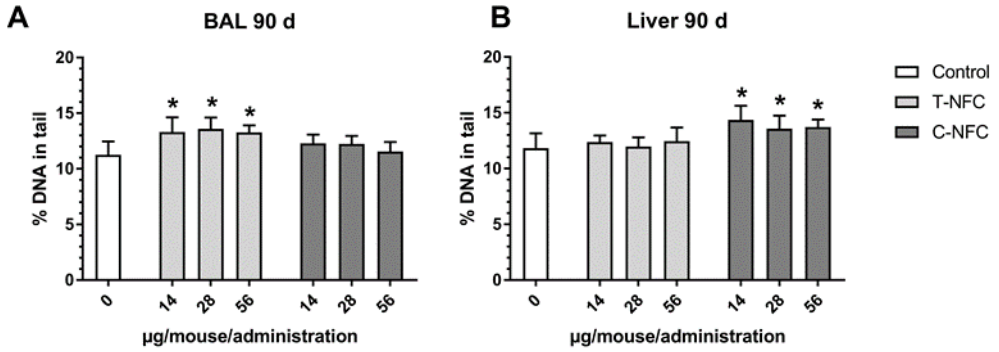

Figure 16. Mean (+SEM) percentage of DNA in tail (comet assay) in bronchoalveolar lavage (BAL) cells (**A**) and liver cells (**B**) of mice exposed to T-NFC and C-NFC, respectively. Asterisks indicate a significant difference ($p < 0.05$) in comparison with the negative control group.

4.2.4 Biopersistence

NFC material was clearly present in the H&E stained lung tissue samples at all studied timepoints (Figure 15). Material aggregates were seen both inside bronchia and in the alveolar tissue. Enlarged macrophages, suspected to contain NFC material, were also detected at all timepoints. As all tested materials were still present in the lung tissue at large quantities after 3 months, the NFC materials showed a high biopersistence after the pulmonary exposure.

4.3 The influence of endotoxin contamination

4.3.1 Clinical signs and and body weight

No clinical signs of toxicity were observed during the study period. No differences in body weight and body weight gain were found between the different treatments and the vehicle at any post-administration time (data not shown).

4.3.2 Inflammatory reaction

Based on bronchoalveolar lavage (BAL) fluid cellularity (Figure 17) and histopathological examination of the lung tissue (Figure 18), all LPS-contaminated T-NFC samples induced an acute pulmonary inflammation. The highest effect was observed with the lowest LPS concentration (0.02 ng/mouse). The two higher LPS concentrations (1 and 50 ng/mouse) showed neutrophilic and eosinophilic reactions similar to or lower than the uncontaminated T-NFC. Similar to the finding observed with the other cellulosic materials, the inflammatory reaction generated by the LPS contamination subsided within the 90-day follow-up period.

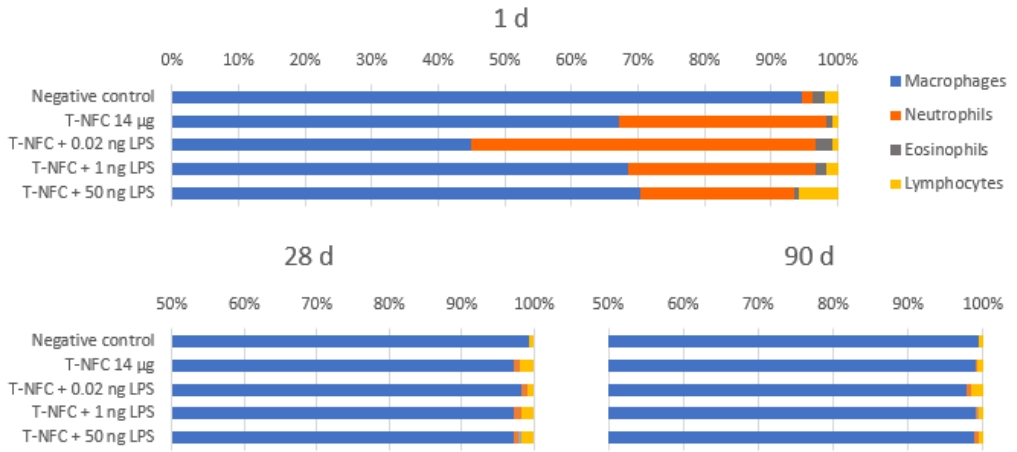

Figure 17. Percentage of different inflammatory cell types in the bronchoalveolar lavage (BAL) fluid of mice treated by pharyngeal aspiration with different doses of contaminated T-NFC (0.02, 1 and 50 ng/mouse/administration) at 1, 28 and 90-d post-administration.

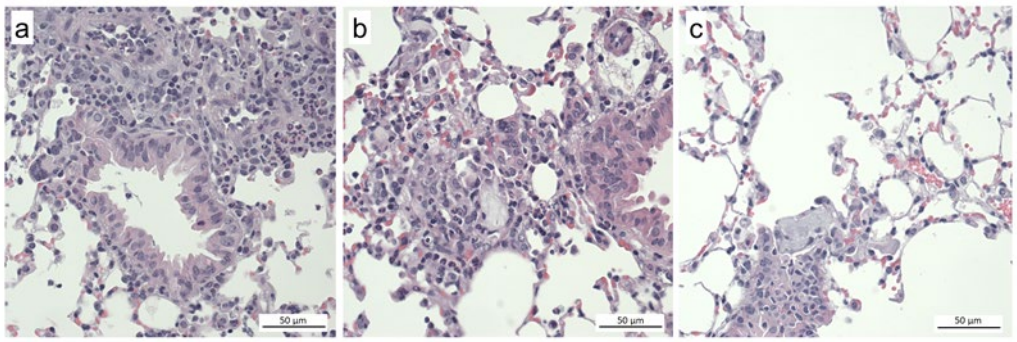

Figure 18. Hematoxylin and Eosin (H&E) stained mouse lung tissue sections 1 day after exposure to 14 µg/mouse/aspiration of T-NFC (a), and after exposure to the same dose doped with 0,02 ng of Lipopolysaccharides (LPS) for 1 day (b) and 90 days (c).

4.3.3 Genotoxicity

No higher levels of DNA damage, as compared with the non-contaminated T-NFC sample, were observed in the BAL and lung tissue of mice treated with LPS-contaminated samples of T-NFC at any of the post-administration times (data not shown). LPS-contamination neither increased hepatic DNA damage 28-day post-administration. Unfortunately, several liver samples from mice followed up to 90-d could not be analyzed due to inadequate number of cells, precluding conclusions at this time point.

None of the contaminated T-NFC samples induced an increase in MNi in the bone marrow of the treated mice at any of the LPS doses or exposure times.

5 DISCUSSION AND CONCLUSIONS

5.1 Role of size and surface chemistry on the toxicological effects induced by NFCs

None of the cellulosic materials - birch pulp, NFCs synthetized in the present project or provided by Uppsala University - affected cell viability as compared with the untreated cultures. The birch pulp, the medium fraction of U-NFC, and all the NFCs provided by Uppsala University, except P-NFC, did not induce ROS formation. Conversely, all the other NFCs and P-NFC were able to induce a dose-dependent intracellular formation of ROS. However, the increase in ROS did not result in an increased DNA or chromosome damage, except for the fine fraction of E-NFC and the coarse fraction of C-NFC.

Although some of the NFC synthetized by Aalto University and provided by Uppsala University had similar surface chemistry (e.g. C-NFC and CM-NFC were both carboxymethylated nanocelluloses, E-NFC and H-NFC were both obtained by EPTMAC pre-treatment), they showed different capacity in the formation of ROS and inducing genotoxic effects. As concerns genotoxicity, comparison between materials from both sources was difficult, as the effect was size-dependent (e.g. only the fine fraction of E-NFC was genotoxic, whereas the largest two fractions were not). However, an increase in ROS was induced by all the size fractions of C-NFC and E-NFC but not by CM-NFC and H-NFC. One reason for the discrepancy in the behavior of the materials may have been the different dispersion methods applied. The NFCs synthetized by Aalto University were only diluted and mixed by vortexing, whereas the NFCs from Uppsala University were sonicated to obtain the dispersions that were then sterilized (Aimonen et al., 2021). Sonication may affect the structural properties of the material (DeLoid et al., 2019), which may alter the way the material interacts with cells. In fact, EZ-NFC, CM-NFC and H-NFC, dispersed and sterilized exactly in the same way, neither showed an increase in the formation of ROS in THP-1 macrophages treated for up to 120 min with the same doses (Lopes et al., 2017).

Previous studies conducted with different NFCs in various *in vitro* models reported conflicting results (Ventura et al., 2020). No signs of cytotoxicity, evaluated by different assays, were observed in human intestinal Caco-2 cells (Lopes et al., 2020), human dermal fibroblasts, lung cells, and macrophages (Lopes et al., 2017) exposed to NFCs similar to the ones provided by Uppsala University. Similarly, no cytotoxicity was observed in Caco-2 cells exposed to unmodified NFC (Chen et al., 2020) or when pre-digested NFCs were assessed in a triculture model including Caco-2 cells (DeLoid et al., 2019). Furthermore, neither cytotoxic nor genotoxic effects were observed in BEAS-2B

cells treated with four different NFCs (carboxylated, carboxymethylated, and two NFCs without chemical pre-treatment) with the same dose range and exposure times as used in the present study (Lindberg et al., 2017). On the other hand, two types of unmodified NFC (freeze-dried powder and gel) tested in A549 cells, another human lung cell line, caused a significant decrease in cell viability together with an induction of oxidative stress (Menas et al., 2017). However, no effect on cell viability or ROS formation was seen in the same cell line after treatment with freeze-dried powder of hydrophilic and hydrophobic forms of NFCs (Yanamala et al., 2016), although both NFCs induced dose-dependent cytotoxic and inflammatory responses in THP-1 cells.

The reported conflicting *in vitro* results may partly be due to differences in the cellular uptake of NFCs, which may vary depending on both the material properties and the cell type. Few studies have assessed the cellular uptake of nanocelluloses, probably because it is difficult to identify these materials in biological samples (DeLoid et al., 2019; Foster et al., 2018). Overall, the uptake of nanocelluloses by cells is considered to be quite low (Ventura et al., 2020). In the present study, most BEAS-2B cells showed no material, suggesting that NFC internalization only concerned a minority of the cells; even though BEAS-2B cells have been reported to be capable of nanomaterial endocytosis (Nymark et al., 2015; Siivola et al., 2020; Vales et al., 2020). In fact, intracellular uptake of NFCs seemed to be confirmed by the TEM analyses we did. However, this technique did not allow a quantitative comparison among the different types of NFCs.

A small number of *in vivo* studies have been performed by administrating NFCs through the respiratory tract, either by intratracheal instillation (Hadrup et al., 2019) or (oro)pharyngeal aspiration (Catalán, Rydman, et al., 2017; Ilves et al., 2018; Lindberg et al., 2017; E. J. Park et al., 2018). In all these studies, the maximum post-treatment period analyzed was up to one month. Hadrup et al. (2019) concluded that the carboxylation of NFC was associated with reduced pulmonary and systemic toxicity, as compared with an unmodified, enzymatically pre-treated NFC. Similar results were found in other studies where the same materials, as well as another unmodified NFC, were administrated to mice by (oro)pharyngeal aspiration (Ilves et al., 2018; Lindberg et al., 2017). Unmodified NFCs (with or without enzymatical pre-treatment) were more prone to trigger inflammation (Ilves et al., 2018) and to induce DNA strand breaks in the lungs (Lindberg et al., 2017) than NFCs modified by carboxymethylation. In the present study, C-NFC triggered a higher inflammatory response (at the highest dose) than the unmodified NFC and induced systemic DNA damage in the liver. In agreement with the previous studies, we observed a progressive resolution of the inflammatory response with time, and a high biopersistence of all the cellulosic materials in the lungs. T-NFC was the only material able to induce local (BAL cells) genetic damage. We have previously observed a significant increase in lung cell DNA damage 24 h after administrating another TEMPO

oxidized NFC (Catalán, Rydman, et al., 2017). The genotoxic responses observed by the anionic NFCs included in our study raise concerns about their possible carcinogenicity.

5.2 Influence of endotoxin contamination

All the NFCs provided by Uppsala University, except H-NFC, showed a high endotoxin level above the upper detection limit (Table 2). Hence, the *in vitro* ROS induction by EZ-NFC and CM-NFC detected in our study may have been triggered by the endotoxins present in the NFC samples. As the endotoxin content of these four NFCs could not be determined accurately, it remains unclear whether the ROS induction could have reflected a substantially higher amount of endotoxin in CM-NFC and EZ-NFC compared with the other NFCs (Aimonen et al., 2021). On the other hand, most of the NFCs synthetized in this project, as well as the original birch pulp, also triggered the *in vitro* formation of intracellular ROS. The only exception was the medium fraction of U-NFC. However, no association between the ROS response and the endotoxin contamination could be established with these materials. The most pronounced ROS induction was induced by the different size fractions of C-NFC and E-NFC which, however, showed lower endotoxin levels than the other NFCs, all of them below the limit accepted by FDA for inhalation studies. Hence, the endotoxin contamination does not seem to influence the capacity of the cellulosic materials to trigger intracellular ROS formation in BEAS-2B cells. Our results agree with previous observation about nanomaterials. Although the involvement of endotoxins in inducing oxidative stress in different cell types cannot be excluded (Fernández-Cruz et al., 2018), evidence in favor of endotoxin affecting *in vitro* toxicity endpoints other than immunological responses is limited (Pagani et al., 1988).

Endotoxins could play an important role in the observed *in vivo* toxicological responses induced by some of the functionalized NFCs, as endotoxins have shown to trigger the inflammatory response (Giannakou et al., 2019; Li & Boraschi, 2016). Therefore, we ran a parallel experiment exposing mice to T-NFC samples with increasing concentrations of LPS, as a way of mimicking increasing endotoxin contamination. As expected, an acute inflammatory response was induced by all the administrated samples, the highest effect being observed at the lowest level of LPS contamination. As the inflammatory response is triggered within a few hours after exposing the animals, higher concentrations of LPS may have induced an earlier response than the lowest concentration, which could have been partly resolved at the time of the evaluation.

Although the inflammatory response progressively subsided at later post-administration times, the acute reaction might have been enough to initiate the cascade of cellular events giving raise to genotoxic effects. However, no pulmonary DNA damage was observed at any concentration of LPS contamination after 28 and 90 days post-administration. Systemic genotoxic effects were neither observed in the bone marrow at

these time points or in the liver at 28 days post-administration. At 90 days, the lack of enough samples from several animals precluded clear conclusions.

Our findings seem to suggest that endotoxin contamination is not the cause of the local genotoxic effects induced by the TEMPO oxidized NFC. Althought, it is still unclear whether endotoxins could play a role in hepatic genotoxicity, the levels detected in the carboxymethylated NFC sample were too low to explain the genotoxic effects that this material induced in the liver.

5.3 Mechanisms involved in the genotoxic response to NFC

All the *in vitro* experiments performed in the present project were done with monolayer cultures of the human bronchial epithelial BEAS-2B cells. Tests performed in monolayer cultures of non-inflammatory cells can only detect genotoxic effects mediated by primary mechanisms of action (Evans et al., 2019). The coarse fraction of C-NFC and the fine fraction of E-NFC were the only materials able to induce a significant dose-dependent increase in the frequency of micronuclei in the BEAS-2B cells. E-NFC was also able to induce a significant dose-dependent increase in DNA damage. Although NFC was seen in the cytoplasm of some cells, it could not be established whether NFCs could enter the nucleus and interact with the DNA. On the other hand, direct interaction could also be possible during the mitosis when the nucleus membrane disappears (Gonzalez & Kirsch-Volders, 2016). However, direct interaction with DNA has not been reported for nanomaterials. Genotoxic nanomaterials are mostly assumed to act through an indirect mechanism, mainly by triggering the formation of ROS (Gonzalez & Kirsch-Volders, 2016; Kohl et al., 2020). This could also be the mechanism involved in the present study, as the coarse fraction of C-NFC and the fine fraction of E-NFC, which showed the most pronounced induction of ROS in their respective size fractions, were genotoxic. However, the formation of ROS induced by the other NFCs did not result in genotoxic effect.

Several in *in vivo* studies conducted with nanomaterials have pointed out that the permanent changes induced in DNA are often the result of secondary genotoxicity associated with inflammation (Kohl et al., 2020). In the present study, treatment of mice with TEMPO oxidized NFC was still associated with a neutrophilic reaction at 90 d post-administration, similarly as observed in mice treated with multiwall carbon nanotubes. The same material also induced DNA damage in bronchoalveolar lavage cells at this time point. On the other hand, mice treated with carboxymethylated NFC showed an elevated level of hepatic DNA damage at 90 days, although the neutrophilic reaction was totally resolved by that time. One possibility is that C-NFC operates through a primary mechanism of action, as suggested by the *in vitro* finding. If so, the lack of hepatic DNA damage at 28 days post-administration could be because the material has not yet reached the liver. In fact, the previously reported reduction of pulmonary and systemic

toxic response associated with NFC carboxylation (Hadrup et al., 2019; Ilves et al., 2018) might reflect the transposition of this functionalized material from the lung to internal organs during the time period assessed (up to 28 days post-administration).

In conclusion, our findings suggest that the surface chemistry of nanofibrillated celluloses is a central property with respect to their harmful effects. Both cationic and anionic functionalization are able to induce genetic damage. EPTMAC and carboxymethylated NFC appear to be able to operate by a primary mechanism of action, probably through the generation of ROS. An inflammation-mediated mechanism seems to be involved in the genotoxic response induced by the TEMPO oxidized NFC. The size of the nanofibrils modulates the genotoxic effects of NFCs, although its influence seems to depend on surface chemistry. On the other hand, contamination with bacterial endotoxin does not seem to play a role in the observed genotoxic effects. However, our results cannot be generalized to all types of NFCs, as the synthesis process and the dispersion method used for testing them may affect their physico-chemical properties and, hence, their toxicological effects. Therefore, further investigations are needed to better understand the mechanisms involved in the toxicity of NFC and the possible connections between the properties of the materials and their toxic effects.

6 DISSEMINATION OF KNOWLEDGE

Besides the present report, the results of the project will be published in the international and domestic scientific literature. The following research results have so far been published or are in preparation:

- Catalán J, Norppa H. Safety aspects of bio-based nanomaterials (2017). *Bioengineering* 4, pii: E94.
- Aimonen K, Suhonen S, Hartikainen M, Lopes VR, Norppa H, Ferraz N, Catalán J. Role of surface chemistry in the *in vitro* lung response to nanofibrillated cellulose (2021). *Nanomaterials*, 11(2), 389.
- Chinga-Carrasco G, Rosendahl J, Catalán J. Nanocelluloses – Nanotoxicology, safety aspects and 3D bioprinting (2021). *Adv Exp Med Biol* (in press).
- Aimonen K, Imani M, Hartikainen M, Suhonen S, Vanhala E, Moreno C, Rojas OJ, Norppa H, Catalán J. Size and surface composition affect the toxicity of cellulose nanofibrils (in preparation)

The findings will also be presented in international and domestic scientific conferences, symposia, workshops, committees and working groups dealing with the safety of nanomaterials. So far, the project and its results have been presented, as oral presentations, in the following fora (main presenter underlined):

- Aimonen K. Materiaaliominaisuuksien vaikutus nanoselluloosan toksisuuteen. Kohti turvallista nanoteknologian tulevaisuutta -webinaari. National Seminar on Nanosafety, on-line event organized by the Finnish Ministry of Social Affairs and Health, and the Finnish Institute of Occupational Health, 16.9.2020.
- Aimonen K, Suhonen S, Siivola K, Vales G, Norppa H, Catalán J. *In vitro* approaches for the genotoxicity testing of nanomaterials. NanoScience Days 2020, International on-line conference organized by the University of Jyväskylä, 6-7.10.2020.
- Aimonen K, Imani M, Hartikainen M, Suhonen S, Siivola K, Vales G, Moreno C, Rojas O, Norppa H, Catalán J. Effects of size and surface composition on the genotoxic and oxidative stress potential of cellulose nanofibrils. NanoSAFE' 20, International digital conference organized by the French Alternative Energies and Atomic Energy Commission (CEA), 16-20.11.2020.

A couple of abstracts summarizing the *in vitro* results were also accepted as oral presentations at the European Environmental Mutagenesis and Genomics Society meeting 2020 and at the TAPPI (Technical Association of the Pulp and Paper Industry)

conference 2020. However, both meetings were cancelled because of the Covid-19 pandemic.

Two abstracts summarizing the *in vitro* and *in vivo* results have been submitted to the TAPPI conference 2021, to be held on June 2021 (Helsinki, Finland). Accepted abstracts will be notified after February 2021. A third abstract has also been submitted to the NanoTox 2021, 10th International Conference on Nanotoxicology, which will be held as a virtual conference on April 2021. The abstract as been accepted as oral presentation.

In addition, the research results are to be utilized in the following ongoing projects:

- EU H2020 SAbyNA (*Simple, robust and cost-effective approaches to guide industry in the development of safer nanomaterials and nano-enabled products*), where our results will support identifying which physico-chemical properties of nanomaterials can modulate their toxicological responses.
- EU H2020 NanoInformaTIX (*Development and Implementation of a Sustainable Modelling Platform for NanoInformatics*), where primary and secondary mechanisms of genotoxicity will more thoroughly be assessed.
- A new EU H2020 funded project within the call SC1-BHC-36-2020 (*Micro- and nano-plastics in our environment: Understanding exposures and impacts on human health*), which will start in April 2021, where similar *in vivo* experiments as the ones carried out in the present study will be performed to compare the behavior and effects of micro- and nano-plastics with non-synthetic nanofibers.

7 ACKNOWLEDGEMENTS

The authors would like to thank UPM Kymmene Oy for supplying the bleached sulfite birch dissolving pulp. Dr Natalia Ferraz (Uppsala University) is acknowledged for providing NFC samples, together with the characterization data. The animal facilities for performing the *in vivo* experiments were outsourced to the Laboratory Animal Center of the University of Helsinki. Processing of TEM samples was conducted at the Electron Microscopy Core Unit of the University of Helsinki.

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Wood-derived nanofibrillated cellulose (NFC) is a renewable material that has been the subject of increased industrial interest. However, some of the nanoscale features of NFC may endow novel properties and biological effects, raising concerns about possible harmful effects on human health.

Previous studies have suggested that pulmonary exposure to some NFC types is able to induce genotoxic effects and acute inflammation, which might result on malignancy and fibrosis. However, the observed effects of NFC may partly depend on its physico-chemical properties. This report shows that surface chemistry is a central property in determining the toxic effects of NFC. Both cationic and anionic functionalization are associated with genotoxic effects. The size of the nanofibrils modulates the genotoxic effect of NFCs, although the effect varies depending on surface chemistry.

However, the results cannot be generalized to all types of NFCs, as the synthesis process and the dispersion method used for testing them may influence their toxic effects. The present findings can aid in developing safer-by-design principles for NFC and our understanding of predictive toxicological outcomes.

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