Possible toxicological effects of nanocellulose – An updated literature study, No 2

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Summary

This literature review covers open publications and reports on the subject of nanocellulose and its possible toxicological effects. There is currently a rather low number of peer reviewed articles on the subject. However, from the articles reviewed, caution of inhalation of nanocellulose would be recommended since *in vivo* tests have shown immunotoxicity effect on lungs even though residues of other production chemicals, biocides and endotoxins from bacterial contamination might affect the results.

1 Introduction

This report is an updated version of the Innventia report No 648 from 2015. Some more studies have been published since then and in this report the topic of regulation is also mentioned.

Nanotechnology is an emerging technology that has potential to increase the development of new industrial applications with more nano-enabled products that reach the market. The scale of nano and engineering to nanomaterial gives the “old” material various new and attractive functions. However, these functions and the fact that nanomaterials are similar in size to intra- and extracellular biological components may also lead to unexpected toxicity of the nanomaterial

Cellulose is the most abundant polymer on earth and due to its biodegradable, recyclable and renewable nature it has gained increased interest in the recent past. To attain potential novel applications and broader applicability, cellulose can be defibrillated to obtain nanocellulose. Nanocellulose has a wide field of application areas such as paper and board products, cosmetics, medicine, food, composite and construction materials.

The nanocellulose family includes cellulose nanocrystals (CNC) and cellulose nanofibrils (CNF) obtained from plant fibers such as wood or cotton, for example (Klemm et al. 2011). Nanocellulose can also be produced by organisms such as bacterial nanocellulose (BNC) which is built-up in biotechnology processes by the genus Gluconoacetobacter (Klemm et al. 2011) and nanocellulose from the filamentous green algae Cladophorales and Siphonocladales (Mihranyan 2011).

Nanocrystalline cellulose CNC has a diameter of about 5-7 nm and a length of 100 to 250 nm (plant cellulose). CNC is often produced by hydrolysis of cellulose under strongly acidic conditions with either HCl or H_2SO_4. CNC is rod-like and highly crystalline, since the amorphous domains have been degraded during the hydrolysis. CNC are also frequently called whiskers (i.e. cellulose nanowhiskers).

Cellulose nanofibrils (CNF) have a diameter of about 3 to 60 nm, and a length of 1 – 2 microns, which is determined by the type of employed manufacturing process (Isogai et al. 2011; Lindström et al. 2014). CNF has high-aspect-ratio fibrils that form strong and entangled networks. The CNF production often involves a physical or chemical pre-treatment step, followed by a mechanical step at which the nanofibrils are liberated by shearing of the fibres through, for example, homogenizers, fluidization or refiners. Two

of the pre-treatment processes that have attained considerable interest in the scientific community are the TEMPO-oxidation (Isogai et al. 2011) route and enzymatic pre-treatment (Pääkko et al. 2007). TEMPO-oxidation introduces high amount of negative charges into the fibre (≈ 0.5-3 mmol/g), which lead to an effective delamination of the fibres due to electrostatic repulsion between the fibre charges, which in turn swell the fibre wall (Isogai et al. 2011). Addition of enzyme gives a mild hydrolysis of amorphous cellulose that also promote cell wall delamination (Pääkko et al. 2007). Fractionation of CNF with different kinds of screens (Tanaka et al. 2012) claimed that TEMPO pre-treatment gave a narrower size distribution (> 80 % was found at 1-0.1 μm) as compared to the enzymatic pre-treatment process which had a broader span.

Toxicity of a substance describes its capacity to harm a living organism and is an essential part when doing hazards assessments and evaluating the effect a substance has on humans or the environment. The toxicity depends on dose and response; types and severity of adverse effects; mechanism of action, time period of exposure (Shatkin 2013). For fibres, if the length is too long for a macrophage to phagocytize, i.e. too long for the macrophage cell to completely devour it, it could stimulate inflammatory factors which in turn could lead to a potential carcinogenic effect or fibrosis (Endes et al. 2016).

The general types of testing toxicity include studies *in vitro* or *in vivo*. *In vitro* means that biological experiments are performed in an artificial environment, for example in a test tube. *In vivo* means that the studies or biological experiments are conducted in its natural environment, for example in whole animal systems.

The focus in this report has been on CNF, but some reports on CNC are also included.

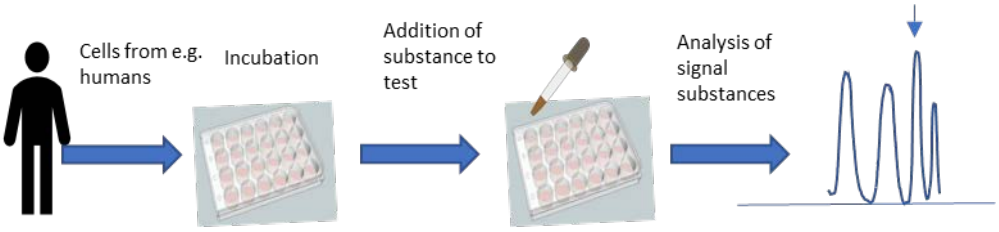

Figure 1. *Schematic illustration of an* in vitro *laboratory test.*

2 Cytotoxicity

Cytotoxicity is the degree to which an agent has specific destructive action on specific cells. Treating cells with a cytotoxic compound can result in a variety of cell fates. The cells may undergo necrosis, in which they lose membrane integrity and die rapidly as a result of cell lysis, i.e. the cell breaks down and the cell content is leaked to the surrounding. The cells can stop actively growing and dividing (a decrease in cell viability), or the cells can activate a genetic program of controlled cell death. Methods to determine the cytotoxicity involves activity measurements from enzymes that are leaked from the damaged cells. Stains that only penetrate damaged cells can also be

used as indicator for damaged cells in microscopic examinations. Studies on exposure of CNF on various human and mouse cells (human keratinocyte cells, mouse hepatoma cells, human macrophages, mouse macrophages, human cervical cell line, mouse fibroblasts and human lung and dermal fibroblasts) did not show any signs of cytotoxicity (Pitkänen et al. 2010; Vartiainen et al. 2011; Ferraz et al. 2012; Väänänen et al. 2012; Alexandrescu et al. 2013; Čolić et al. 2015; Lopes et al. 2017). However, when crosslinking CNF with cetyl trimethylammonium bromide (CTAB) or polyethyleneimine (PEI) Alexandrescu et al. (2013) found some reduction in cell viability of the fibroblasts and Kangas (2013) found some indication of cytotoxicity from the finest fractions of fractionated CNF when using the total protein content method. When discussing the result in a later article (Pitkänen et al. 2014a) the authors speculate that the biocide concentration in the samples was near its toxic values and may have impacted the results.

Using human fibroblasts Hua et al. (2014) showed in an indirect test that extracts of CNF films from Innventia was not cytotoxic and when testing the cytocompatibility (cells able to adhere to CNF films), the cationic nanocellulose (generation 5, i.e. trimethylammonium modified) presented a more compatible profile (more cells adhered) than both unmodified nanocellulose (generation 1) and anionic nanocellulose (generation 2) where there was a poorer adhesion of cells. Nordli et al. investigated in 2016 *in vitro* the possible cytotoxic effect from a CNF very low in levels of endotoxin (so-called ultrapure CNF) on human epidermal and dermal cells. Endotoxins are products form bacterial cell walls that is known to trigger inflammation. CNF dispersions of 50 μg/ml did not indicate any cell-death. However, a small decrease in metabolic activity could be detected in some cells.

Various size fractions of CNC prepared from microcrystalline cellulose (MCC) showed no cytotoxicity in the range of 10 – 250 μg/ml on human fibroblasts and colon adenocarcinoma cells for any of the fractions, but concentrations above induced a significant cytotoxic effect for all fractions, since the viability of the cells decreased to under 70 % of the non-toxic reference (Hanif et al. 2014). When comparing cytotoxicity from fibrillar and crystalline nanocellulose on pulmonary epithelial cell responses Menas et al. (2017) found that CNF was more toxic compared to CNC with respect to cytotoxicity and oxidative stress responses. However, CNC caused a significantly elevated inflammatory response compared to CNF.

The CNF used in the various studies are produced with different kinds of methods (enzymatic or TEMPO pre-treatment and different homogenisation techniques) so comparison between the methods are difficult. However, combining and taking into account the variety of different kinds of cells and kinds of CNF that has been used in these studies for cytotoxicity and cytocompatibility shows that CNF generally does not have cytotoxic properties i.e. CNF does not directly interfere with cellular functions. However, the concentration of CNF may influence the cytotoxic effect.

3 Microscopical examination

One way to analyse if cells are influenced by a toxic substrate is to investigate with microscopy and study the shape of the cells (their morphology) and compare them with a negative, non-toxic control.

Since there are no enzymes present in lungs that can break down cellulose, it is considered to be biodurable with long persistence and this has been identified as a key factor for biological response (Endes et al. 2016). Mechanical clearance would be the primary and the most important mechanism for lung clearance of cellulose materials. Investigating the fate of nanocellulose in cells by microscopy is one way to assess the possible harm they can cause. One way to visualize CNF is to use a biotinylated carbohydrate binding module of β-1,4-glycanase (Knudsen et al. 2015). Four different kinds of CNF (e.g. enzymatic pre-treatment and carboxymethylated, fibre width ranging between 2 and 7 nm) and one bulk sized cellulose were used to test this new method. *In vivo* trials (pharyngeal aspiration in mice), one single dose (10, 40, 80 and 200 μg in 50μl phosphate-buffer saline) showed that deposited nanocellulose in lung tissue at all concentrations and for all five materials could be visualised.

Endes et al. (2015) investigated the fate of CNC aerosols deposited on lung cells *in vitro* with confocal laser scanning microscopy. They did not see any signs of alterations in cell morphology. They compared CNC from cotton (aspect ratio 9 ± 5) and from tunicate (aspect ratio 81 ± 68) and saw that the nanofiber length and concentration had a significant influence on the interaction with the cells. The shorter CNC from cotton was internalized and cleared away, thus the longer CNC from tunicate had a lower clearance than the shorter CNC. Menas et al. (2017) tested both CNF and CNC *in vitro* on human lung epithelial cells and staining the cells for presence of cellulose indicated that CNC particles were taken up by the cells while CNF particles were not. Lopes et al. (2017) used light microscopy to evaluate *in vitro* cell morphology to assess the three types of nonionic, anionic and cationic CNF and did not see any change in cell morphology. Transmission electron microscopy (TEM) also showed that none of the CNF were taken up by the cells after exposure. On the other hand, *in vivo* test on mice (pharyngeal aspiration, single dose of either 10, 40, 80, 200 μg/mouse) with TEMPO produced CNF Catalán et al. (2017) revealed a dose-related accumulation of cellulose material in the bronchi, alveoli and in the cytoplasm of macrophages, when the samples were collected after 24 h.

Nanocellulose does not seem to change the morphology of cells indicating that it is not toxic, but *in vivo* tests show an accumulation of nanocellulose and since there are no enzymes that can break down cellulose in humans more long-term tests need to be performed to be able to evaluate the possible detrimental impact of nanocellulose in lungs.

4 Genotoxicity

Genotoxic responses are associated with DNA damage and mutations. Mutations may lead to cancer, but not all mutations cause cancer. Common test methods include Ames test (bacterial based), the Comet assay (DNA damage), the micronucleous assay (chromosome damage) and the chromosome aberration assay (measures the deviation

from the normal). Pitkänen et al. (2010) tested the genotoxicity of both CNF (produced by grinding in a laboratory scale super mass collider) and Abrocel MF 40 (CNC) by Ames test and found no toxic effect on any of the cellulose types. Fractionation of CNF and testing the smallest fractions, Pitkänen et al. (2014) did not show any toxic effect for fractions. Väänänen et al. (2012) tested several kinds of CNF *in vitro* on human bronchial epithelial cells and found that almost all the material induced a slight DNA damage, but only some of the materials gave oxidative DNA damage. However, they did not see any chromosome damage with the micronucleus test for any of the CNF tested and the micronucleus test is generally regarded as one of the most reliable assays for genotoxic carcinogens. Another factor to be remembered is that the Comet assay, which was utilized in this case, measures damages that may be repaired by the cells.

De Lima et al. (2012) tested CNC from different kinds of cotton and curaura on both plant cells (*Allium cepa*) and human lymphocytes cells from blood *in vitro.* The results showed that plant cells appeared to be more liable to genetic alterations than human cells. One explanation could be that the human cells probably possessed more effective repair mechanisms. Various kinds of cotton gave different result showing that the starting material could influence the toxicity.

Väänänen et al. (2012) also tested a TEMPO produced CNF *in vivo* as a single exposure (pharyngeal aspiration 10, 40, 80, 200 μg/mouse) on mice and did not detect any DNA damage or oxidative DNA damage when using comet assay on lung cells.

In contrast, when Catalán et al. (2017) used TEMPO produced CNF *in vivo* as pharyngeal aspiration exposure on mice they found a significant induction of DNA damage for the two lowest doses (10 and 40 μg/mouse), while the higher doses (80 and 200 μg/mouse) did not give a statistical significant induction of DNA damage using the comet assay. The results could be due to the fact that CNF formed larger aggregates at the higher doses, and the results from the higher doses are therefore not entirely comparable with those of the lower doses. They did not find systematic genotoxic effect in the bone marrow.

From deliverable 4.16 from the EU project NanoReg, *in vivo* studies in mice investigated both acute and subacute responses for four different CNF (origin or characterisation not described at the time of this report), bulk-sized pulp and multiwalled carbon nanotubes. All the materials, including CNF, caused DNA damage in lung or BAL cells as determined by the Comet assay, both after 24 hours and 28 days after exposure. However, none of the material showed systematic genotoxic properties in bone marrow.

The number of studies is still small, and the *in vitro* test is not quite in agreement with the *in vivo* test. The *in vitro* and *in vivo* tests show that CNF and CNC have the potential, as a nanomaterial, to damage DNA. Since our last survey there have been additional *in vivo* tests which in Comet assays show that CNF has the tendency to damage DNA.

5 Immunotoxicity

A compound that induces an immune dysfunction upon exposure to the organism is called immunotoxic. The immune dysfunction may take the form of immunosuppression or, alternatively, allergy, autoimmunity or any number of inflammatory-based diseases or pathologies. The main concern with nanocellulose is an increased risk for pulmonary fibrosis, as has been shown for cellulose fibres (Adamis et al. 1997). Vartiainen et al. (2011) performed *in vitro* test of CNF and microcrystalline cellulose (MCC) on both mouse and human macrophages to detect any effects on the macrophages inflammatory systems. The tests did not show any increased amounts of the inflammatory signal substances (cytokines) for CNF. For MCC, however, there was an increase for some of the cytokines when human macrophages were used. In the study of Väänänen et al. (2012), five different CNF (grinding with and without enzymatic pre-treatment; three TEMPO-treated with grinding, lab-scale high pressure homogenization, pilot scale high pressure homogenization) were tested *in vitro* and they did not detect any response in inflammatory signal substances from human macrophages except from the one that was TEMPO treated and homogenised in pilot scale. This batch of CNF also contained bacteria so one explanation for the positive results could be that either the bacteria themselves or endotoxins from the bacteria combined with CNF induced the positive reaction. Menas et al. (2017), when comparing CNF with CNC *in vitro* on pulmonary epithelial cells, showed that CNF did not yield a high release of cytokines in contrast to CNC where the cytokine response was elevated. Lopes et al. (2017) tested CNF from Innventia (unmodified generation 1, anionic generation 2 and cationic generation 5). They saw that unmodified CNF presented a pro-inflammatory response as increased cytokine secretion from THP-1 monocytes. This increase was not found for the two other CNF samples indicating that the inflammatory response might be driven by the surface chemistry and that it is possible to design safe nanocellulose materials. *In vitro* test with ultrapure CNF (low content of endotoxins) on epidermal and dermal cells by Nordli et al. (2016) did not give any increased cytokine secretion from the cells indicating no inflammatory response.

In the *in vivo* test with CNF that Väänänen et al. (2012) performed on mice (pharyngeal aspiration exposure), immunotoxicity was also tested. The results showed a clear dose dependent induction of pulmonary inflammation. When CNC (whiskers) was evaluated *in vivo* as pharyngeal aspiration exposure test, a clear dose dependent response in oxidative stress, tissue damage and robust inflammatory response in the lungs on mice were also found (Yanamala et al. 2014). This was also corroborated by Shvedova et al. (2016) who investigated the pulmonary outcome induced by *in vivo* repeated exposure (pharyngeal aspiration, 40 μg/mouse, two times a week for three weeks) to CNC in female and male mice. They found that the exposure caused impaired pulmonary functions including pulmonary inflammation and oxidative stress. The effects were more pronounced in females compared to male mice, showing a gender difference. The authors indicated that sex hormones may play an important role in inflammatory airway conditions. *In vivo* test with CNF (Catalán et al. 2017) also showed an acute pulmonary inflammation with a dose dependent increase in mRNA expression of the four cytokines analysed. However, the short post-exposure (24 h) time did not allow investigating into whether or not the response was transient or persistent. In the EU project NanoReg, *in vivo* studies in mice investigated both acute (24 h) and subacute (28 days) responses for

four different CNF (origin or characterisation not described), bulk-sized pulp and multiwalled carbon nanotubes (MWCNT). All tested materials were able to induce inflammatory responses in 24 h. However, the mice exposed to CNF showed signs of recovery after 28 days contrary to the mice exposed to MWCNT. Only a minor induction of mRNA expression for cytokines was detected in response of CNF treatment.

There seems to be a lack in coherence between *in vitro* experiments and *in vivo* experiments. When using *in vitro* experiments, generally no effect of CNF is recorded, but a possible detrimental effect on cells can be seen in the *in vivo* experiments.

6 Ecotoxicity

Ecotoxicology has been defined as: "the branch of toxicology concerned with the study of toxic effects, caused by natural or synthetic pollutants, to the constituents of ecosystems, animal (including human), vegetable and microbial, in an integral context" (Truhaut 1977). Since nanocellulose is a rather new material, there have been only a few studies on the fate of the material after production and its possible effect on the environment.

Vartiainen et al. (2011) used standard ecotoxicity tests (*Vibrio fischeri* and *Daphna magna*) for evaluation of CNF and CNC. In the *V. fischeri* test they detected acute toxicity of CNF only at very high level (300 mg/l). In the *D. ma*gna test the authors detected decrease in flea movement, but one reason for this was probably mechanical obstacles and traps that could be produced by the cellulose.

Väänänen et al. (2012) used the same kind of CNF for *in vivo* experiments on the nematode (*Caenorhabditis elegans*) as were used for the *in vivo* immunotoxicity tests. The habitat of the nematode is in soil or compost where it feeds on bacteria so the authors assumed that since CNF is in the same size as bacteria, the nematode would probably also engulf CNF. The CNF addition had no effect on movement, did not cause mortality or interfered with the reproduction.

An ecotoxical evaluation of CNC has also been investigated by Kovacs et al. (2010). The CNC was produced from bleached kraft pulp with sulphuric acid and toxicity tests were performed on rainbow trout hepatocyte cells and nine aquatic species. The conclusion was that CNC did not reveal serious environmental concerns. Even though CNC affected the reproduction of feathered minnow at 0.29 g/l no other effects on endpoints such as survival and growth occurred in the other species at a concentration below 1 g/l. The authors calculated that the concentration of 0.29 g/l is still higher than the concentration would be in receiving water in a worst-case scenario. No genotoxicity could be detected in the *in vitro* rainbow trout hepatocytes test.

Harper et al. (2016) tested the toxicity of diverse nanocellulose materials (eight different CNC and three CNF) on developing zebrafish. Neither the aspect ratio (between 9-21) of CNC nor the surface chemistry or charge had any significant difference from control on the mortality of the zebra fish at exposure concentration between 0.2 and 200 mg/l. One of the CNF (mechanical homogenisation of Kraft pulp) showed a significant

increase in mortality at the highest dose. The authored reasoned that one explanation for this could be higher aspect ratio (not measured) or the starting material.

In the few studies performed, no alarming toxicity at concentrations that probably could be found in ecosystems was discovered.

7 Degradation

An important property of a sustainable material, as CNF is claimed to be, is that it is biodegradable and non-toxic in compost, waste water treatment plants or in an anaerobic digestion plant. Effective degradation and disintegration are for example required for certification of compostability.

The fate of CNC in wastewater treatment was investigated by O'Connor et al. (2013) by electron microscopy. When spiking a laboratory system, they saw that 40 % of the CNC was removed in the primary clarifyers and in the settling basin the concentration was below the detection limit (0.1 mg/l).

In the SUNPAP EU-project Sadocco et al. (2012) reported on some of the test included in evaluation of compostability, i.e. biodegradability and disintegration. They found that paper products containing TEMPO oxidized CNF in coatings did not have a negative effect on the biodegradability of the board and that it did not influence the disintegration. Another EU-project (NanoSustain) corroborate these findings for composting since they state in their final newsletter that “nanocellulose is non-toxic and do not harm the environment”. They have used the bacteria *Vibrio fischeri* for assessment of acute toxicity. However, they found that nanocellulose does not degrade efficiently when measuring evolved carbon dioxide from aerobic biodegradation in a closed bottle test (Nanosustain, case study 4). In short, bottles with mineral medium and the organic matter that shall be tested are inoculated with a mixed population of bacteria and the produced carbon dioxide is measured under a period of time. On the contrary, Kümmerer et al. (2011) showed that CNC produced from cotton wool (pure cellulose) are better and faster degradable than their macroscopic counterpart when using a water another variant of closed bottle test, where they instead monitor the oxygen consumption.

Production procedures may also influence the degradability. Homma et al. (2013b) investigated degradation of TEMPO treated cellulose pulp before and after homogenisation (CNF) with a crude cellulose preparation that possibly contained small amount of other active and crucial enzymes such as hydrolases and lyases. It was showed that untreated pulp degraded more easily than TEMPO treated pulp. However, when the TEMPO treated pulp was processed to CNF the degradability rate increased. In a further study, they continued with a crude enzyme to investigate the effect of counter ions on degradation behaviour (Homma et al. 2013a) and saw that the kind of counter ion had large effect on degradation rate. Na^+, K^+, NH_4^+ and Cs^+ ions had higher degradation than Ca^{2+}, H^+ and Cu^{2+}. Degradation of CNF films after soil burial showed the same pattern as degradation with the enzyme.

Vikman et al. (2015) investigated the biodegradability and compostability of six CNF based products (CNF films and paper containing CNF) in controlled biodegradation and

composting tests (EN 13432, EN 14046 and EN 14045). The films degraded completely after three weeks and the CNF did not influence the degradation of paper products containing CNF. The compost was also tested toward *V. fischeri* after 7 and 65 days and none of the samples showed any inhibition of the bacteria i.e. produced any toxic degradation products.

CNF seems in most cases be rather easily degraded in both compostability tests and in wastewater tests. The smaller size of the raw material provides a larger area for the bacteria to attack the substrate and hence degrade it a bit faster.

8 Regulations

There is, as of today, no common regulation for nanomaterials in the EU. The nanomaterials present in different industrial sectors are addressed in the corresponding sector. For example, there is an EU regulation for cosmetics that among other things states that nanomaterials must be included in the table of contents. Nanomaterials in contact with foodstuff are on the other hand evaluated on a case by case basis. Different countries have also various kinds of system to gather information and register nanomaterial. Among the countries that has initiated/legislated the information of usage of nanomaterial are: France, Denmark, Norway, Belgium, Great Britain, USA, Canada. In Sweden, there is a proposal from the Swedish Chemicals Authority that those who report chemical products to the products register shall, in addition to the information that is currently reported, also provide further information regarding any nanomaterials contained in the product. The proposal covers nanomaterials which have been intentionally added to the product, regardless of concentration. It is proposed that it will enter into force 1 of January 2018 and hence the first reporting will be in February 2019. An overview is reported in (Björkgren et al. 2015) (Swedish).

Rauscher et al. (2017) has compiled information around the regulation of nanomaterials in the EU. An overview is presented in the article with the selected EU regulations with relevance for nanomaterials, which includes nine different regulatory frameworks.

There are several committees working around health aspects and nanoparticles and Figure 1 is an attempt to compile some of the information from Rauscher et al. (2017)

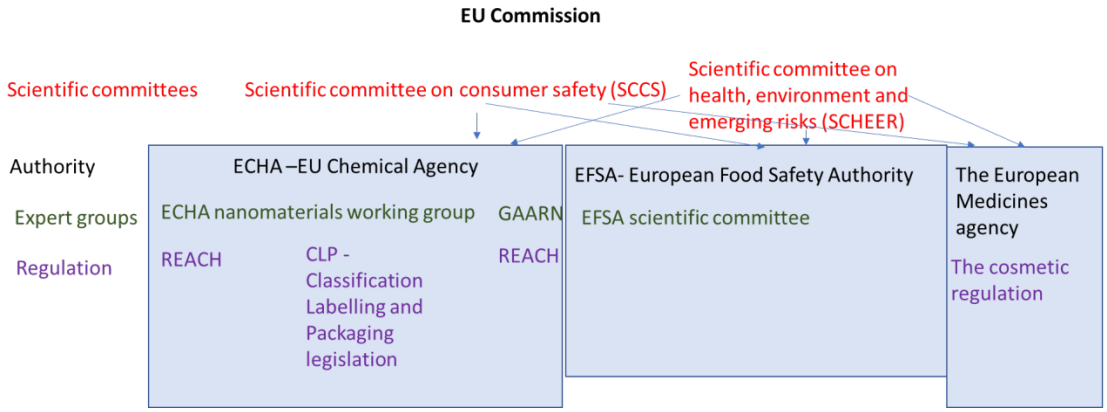

Figure 2. *Different committees working with nanomaterials and health aspects. Acronyms not explained in the Figure: GAARN: Group assessing already registered nanomaterials; REACH: Registration, Evaluation, Authorisation and restriction of Chemicals.*

Worldwide, the OECD (the organisation for economic cooperation and development) has a programme “OECD’s working Party on Manufactured Nanomaterials” to ensure that the approaches for hazard, exposure and risk assessment for manufactured nanomaterials are internationally harmonised and of a high and science-based quality. They have compiled data for several materials (available at their homepage), but nanocellulose is not included.

Several projects financed by EU around nanotoxicity and how to assess the possible toxic effect for nanoparticles have started in recent years. To maximises the synergies between these different European projects, the EU NanoSafety Cluster has been formed on an initiative of the European Commission Directorate-General for Research and Innovation (DG RTD). The studied aspects include toxicology, ecotoxicology, exposure assessment, mechanisms of interaction, risk assessment and standardisation. The cluster is supposed to work as an open platform for dialogue and discussion (www.nanosafetycluster.eu). When searching for nanocellulose in ”Compendium of Projects in the European NanoSafety Cluster (2017)” only one hit was found in 215 pages and that was the project that RISE Innventia participated in (GUIDEnano).

9 Conclusion

This survey shows that there only exist a few published peer reviewed articles into the topic of the toxicological effects of nanocellulose, however; some more *in vivo* studies had been published recently. There have been several EU-projects dealing with nanoparticles, but there have only been a few EU-projects where nanocellulose’s potential toxicity has been investigated. Other factors that make it difficult to draw assertive conclusions on this topic are that different kinds of nanocellulose have been used and they have been produced in various ways. The analytical methods have also been varying. The NanoReg project has now produced, tested and published several

general methods so that future research can be comparable. In most *in vitro* tests CNF did not have any toxic effects, but in the EU-project NanoReg they concluded that the same material tested both with *in vitro* and *in vivo* gave contradicting results and they observed that the effect observed for *in vivo* was not fully present in the *in vitro* cell systems used.

Many of the studies performed has also been short term. More investigations on low exposure during long time are needed.

Caution of inhalation of CNF would be recommended since several *in vivo* tests have shown some immunotoxic and genotoxic effects on lungs. Contaminations of production chemicals might have affected the results and remaining endotoxins from bacteria, which has been discussed by Li et al. (2016), or biocides in the material might contribute to the effect CNF has on humans.

In the few studies mentioned in the review of Pitkänen et al. (2014b) bacterial cellulose did not seem to have any toxic effects either *in vitro* or *in vivo.* In a review from Endes et al. (2016) they concluded that the data seems to suggest that in realistic doses and exposure scenarios nanocellulose has limited toxic potential, but that it also depends on their specific physical characteristics.

Concentration and aggregation of the nanocellulose will probably play a part in how the toxicity of the material will behave. De Lima et al. (2012) noticed that the highest chromosomal aberration index value (high deviation from normal) was found at the lowest concentration of tested cotton CNCs, which they explained by aggregation at higher concentration which in turn reduced the degree of interaction with the cells and hence reduced cellular DNA breaks.

Shatkin et al. (2015) used NANO LCRA (built on traditional risk assessment and characterize the potential risks from raw material through the end-of-life or disposal/reuse) for screening pre-commercial nanocelluloses for identifying occupational, consumer and environmental risks. The analysis revealed that occupational exposure and handling of nanocellulose as a dry powder was the highest priority data gap including the challenge of quantitative measurement for exposure. The authors have also compiled toxicity data from literature and gap analysis in a comprehensive Table.

The fate of nanocellulose after production and usage is also important. Nanocellulose seems in most cases be rather easily degraded since CNC is removed in wastewater plants and CNF without modifications is easily broken down in various degradation tests.

10 References

Adamis, Z., Tátrai, E., Honma, K. and Ungváry, G. (1997).

In vitro and in vivo assessment of the pulmonary toxicity of cellulose.

Journal of Applied Toxicology 17(2): 137-141.

Alexandrescu, L., Syverud, K., Gatti, A. and Chinga-Carrasco, G. (2013).

Cytotoxicity tests of cellulose nanofibril-based structures.

Cellulose 20(4): 1765-1775.

Björkgren, V., Hellmér, L., Ifverberg, M., Lagovardos, E., Lestander D., Lindh, J. and E., S. (2015)**.**

Förslag om utökad anmälningsplikt för

nanomaterial - Rapport från ett regeringsuppdrag.

RAPPORT 10/15.

Stockholm, Kemikalieinspektionen.

Catalán, J., Rydman, E., Aimonen, K., Hannukainen, K.-S., Suhonen, S., Vanhala, E., Moreno, C., Meyer, V., da Silva Perez, D., Sneck, A., Forsström, U., Højgaard, C., Willemoes, M., Winther, J. R., Vogel, U., Wolff, H., Alenius, H., Savolainen, K. M. and Norppa, H. (2017).

Genotoxic and inflammatory effects of nanofibrillated cellulose in murine lungs.

Mutagenesis 32(1): 23-31.

Čolić, M., Mihajlović, D., Mathew, A., Naseri, N. and Kokol, V. (2015).

Cytocompatibility and immunomodulatory properties of wood based nanofibrillated cellulose.

Cellulose 22(1): 763-778.

de Lima, R., Feitosa, L. O., Maruyama, C. R., Barga, M. A., Yamawaki, P. C., Vieira, I. J., Teixeira, E. M., Corrêa, A. C., Caparelli Mattoso, L. H. and Fraceto, L. F. (2012).

Evaluation of the genotoxicity of cellulose nanofibers.

International Journal of Nanomedicine 7: 3555-3565.

Endes, C., Camarero-Espinosa, S., Mueller, S., Foster, E. J., Petri-Fink, A., Rothen-Rutishauser, B., Weder, C. and Clift, M. J. D. (2016).

A critical review of the current knowledge regarding the biological impact of nanocellulose.

Journal of Nanobiotechnology 14(1).

Endes, C., Mueller, S., Kinnear, C., Vanhecke, D., Foster, E. J., Petri-Fink, A., Weder, C., Clift, M. J. D. and Rothen-Rutishauser, B. (2015).

Fate of Cellulose Nanocrystal Aerosols Deposited on the Lung Cell Surface In Vitro.

Biomacromolecules 16(4): 1267-1275.

Ferraz, N., Straømme, M., Fellström, B., Pradhan, S., Nyholm, L. and Mihranyan, A. (2012).

In vitro and in vivo toxicity of rinsed and aged nanocellulose-polypyrrole composites.

Journal of Biomedical Materials Research - Part A 100 A(8): 2128-2138.

Hanif, Z., Ahmed, F. R., Shin, S. W., Kim, Y. K. and Um, S. H. (2014).

Size- and dose-dependent toxicity of cellulose nanocrystals (CNC) on human fibroblasts and colon adenocarcinoma.

Colloids and Surfaces B: Biointerfaces 119: 162-165.

Harper, B. J., Clendaniel, A., Sinche, F., Way, D., Hughes, M., Schardt, J., Simonsen, J., Stefaniak, A. B. and Harper, S. L. (2016).

Impacts of chemical modification on the toxicity of diverse nanocellulose materials to developing zebrafish.

Cellulose 23(3): 1763-1775.

Homma, I., Fukuzumi, H., Saito, T. and Isogai, A. (2013a).

Effects of carboxyl-group counter-ions on biodegradation behaviors of TEMPO-oxidized cellulose fibers and nanofibril films.

Cellulose 20(5): 2505-2515.

Homma, I., Isogai, T., Saito, T. and Isogai, A. (2013b).

Degradation of TEMPO-oxidized cellulose fibers and nanofibrils by crude cellulase.

Cellulose 20(2): 795-805.

Hua, K., Carlsson, D. O., Ålander, E., Lindström, T., Strømme, M., Mihranyan, A. and Ferraz, N. (2014).

Translational study between structure and biological response of nanocellulose from wood and green algae.

RSC Advances 4(6): 2892-2903.

Isogai, A., Saito, T. and Fukuzumi, H. (2011).

TEMPO-oxidized cellulose nanofibers.

Nanoscale 3(1): 71-85.

Kangas, H. (2013).

Amount, characteristcs and safety of nano-scale cellulose fibres.

Ed: 2013 TAPPI International Conference on nanotechnology for renewable materials, KTH Royal Institute of technology, Stockholm, Sweden.

Klemm, D., Kramer, F., Moritz, S., Lindström, T., Ankerfors, M., Gray, D. and Dorris, A. (2011).

Nanocelluloses: A new family of nature-based materials.

Angewandte Chemie - International Edition 50(24): 5438-5466.

Knudsen, K. B., Kofoed, C., Espersen, R., Højgaard, C., Winther, J. R., Willemoës, M., Wedin, I., Nuopponen, M., Vilske, S., Aimonen, K., Weydahl, I. E. K., Alenius, H., Norppa, H., Wolff, H., Wallin, H. and Vogel, U. (2015).

Visualization of Nanofibrillar Cellulose in Biological Tissues Using a Biotinylated Carbohydrate Binding Module of β-1,4-Glycanase.

Chemical Research in Toxicology 28(8): 1627-1635.

Kovacs, T., Naish, V., O'Connor, B., Blaise, C., Gagné, F., Hall, L., Trudeau, V. and Martel, P. (2010).

An ecotoxicological characterization of nanocrystalline cellulose (NCC).

Nanotoxicology 4(3): 255-270.

Kümmerer, K., Menz, J., Schubert, T. and Thielemans, W. (2011).

Biodegradability of organic nanoparticles in the aqueous environment.

Chemosphere 82(10): 1387-1392.

Li, Y. and Boraschi, D. (2016).

Endotoxin contamination: A key element in the interpretation of nanosafety studies.

Nanomedicine 11(3): 269-287.

Lindström, T., Aulin, C., Naderi, A. and Ankerfors, M. (2014).

Microfibrillated cellulose.

Encyclopia of Polymer Science and Technology, John Wiley & Sons.

Lopes, V. R., Sanchez-Martinez, C., Strømme, M. and Ferraz, N. (2017).

In vitro biological responses to nanofibrillated cellulose by human dermal, lung and immune cells: Surface chemistry aspect.

Particle and Fibre Toxicology 14(1).

Menas, A. L., Yanamala, N., Farcas, M. T., Russo, M., Friend, S., Fournier, P. M., Star, A., Iavicoli, I., Shurin, G. V., Vogel, U. B., Fadeel, B., Beezhold, D., Kisin, E. R. and Shvedova, A. A. (2017).

Fibrillar vs crystalline nanocellulose pulmonary epithelial cell responses: Cytotoxicity or inflammation?

Chemosphere 171: 671-680.

Mihranyan, A. (2011).

Cellulose from cladophorales green algae: From environmental problem to high-tech composite materials.

Journal of Applied Polymer Science 119(4): 2449-2460.

Nordli, H. R., Chinga-Carrasco, G., Rokstad, A. M. and Pukstad, B. (2016).

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

Carbohydrate Polymers 150: 65-73.

O'Connor, B., Goguen, R., Berry, R., Genest, S. and Ellis, S. (2013).

EH&S studies associated with the production of NCC at CelluForce.

Ed: 2013 TAPPI International Conference on nanotechnology for renewable materials, KTH Royal Institute of technology, Stockholm, Sweden.

Pitkänen, M., Honkalampi, U., Von Wright, A., Sneck, A., Hentze, H. P., Sievänen, J., Hiltunen, J. and Hellén, E. K. O. (2010).

Nanofibrillar cellulose - In vitro study of cytotoxic and genotoxic properties.

International Conference on Nanotechnology for the Forest Products Industry 2010, 246-261.

Pitkänen, M., Kangas, H., Laitinen, O., Sneck, A., Lahtinen, P., Peresin, M. S. and Niinimäki, J. (2014a).

Characteristics and safety of nano-sized cellulose fibrils.

Cellulose 21(6): 3871-3886.

Pitkänen, M., Kangas, H. and Vartiainen, J. (2014b).

Toxicity and Health Issues.

Handbook of nanomaterials. Oksman, K., Mathew, A. P., Bismarck, A., Rojas, O. and Sain, M. (Eds.)

1**:** 181-205. New Jersey, World Sicentific.

Pääkko, M., Ankerfors, M., Kosonen, H., Nykänen, A., Ahola, S., Österberg, M., Ruokolainen, J., Laine, J., Larsson, P. T., Ikkala, O. and Lindström, T. (2007).

Enzymatic hydrolysis combined with mechanical shearing and high-pressure homogenization for nanoscale cellulose fibrils and strong gels.

Biomacromolecules 8(6): 1934-1941.

Rauscher, H., Rasmussen, K. and Sokull-Klüttgen, B. (2017).

Regulatory Aspects of Nanomaterials in the EU.

Chemie-Ingenieur-Technik 89(3): 224-231.

Sadocco, P. and Dania, S. (2012).

Organic recovery:an efficient end of life option for innovbative Sunpap paper products.

Ed: SUNPAP final conference, Milan, Italy.

Shatkin, J. A. (2013).

Nanotechnology Healt and Environmental Risks.

CRC Press, Boca Raton.

Shatkin, J. A. and Baram, K. (2105).

Cellulose nanomaterials: life cycle risk assessment, and environmental health and safety roadmap.

Environmental Science Nano 2: 477-499

Shvedova, A. A., Kisin, E. R., Yanamala, N., Farcas, M. T., Menas, A. L., Williams, A., Fournier, P. M., Reynolds, J. S., Gutkin, D. W., Star, A., Reiner, R. S., Halappanavar, S. and Kagan, V. E. (2016).

Gender differences in murine pulmonary responses elicited by cellulose nanocrystals.

Particle and Fibre Toxicology 13(1).

Tanaka, A., Seppänen, V., Houni, J., Sneck, A. and Pirkonen, P. (2012).

Nanocellulose characterization with mechanical fractionation.

Nordic Pulp and Paper Research Journal 27(4): 689-694.

Truhaut, R. (1977).

Ecotoxicology: objectives, principles and perspectives.

Ecotoxicology and Environmental Safety 1(2): 151-173.

Vartiainen, J., Pöhler, T., Sirola, K., Pylkkänen, L., Alenius, H., Hokkinen, J., Tapper, U., Lahtinen, P., Kapanen, A., Putkisto, K., Hiekkataipale, P., Eronen, P., Ruokolainen, J. and Laukkanen, A. (2011).

Health and environmental safety aspects of friction grinding and spray drying of microfibrillated cellulose.

Cellulose 18(3): 775-786.

Vikman, M., Vartiainen, J., Tsitko, I. and Korhonen, P. (2015).

Biodegradability and Compostability of Nanofibrillar Cellulose-Based Products.

Journal of Polymers and the Environment 23(2): 206-215.

Väänänen, V., Rydman, E., Ilves, M., Hannukainen, K., Norppa, H., von Wright, A., Honkalampi, U., Tsitko, I. and Rouhiainen, J. (2012).

Evaluation of the suitability of the developed methodology for nanoparticle health and safety studies.

Report from EU project SUNPAP.

Yanamala, N., Farcas, M. T., Hatfield, M. K., Kisin, E. R., Kagan, V. E., Geraci, C. L. and Shvedova, A. A. (2014).

In vivo evaluation of the pulmonary toxicity of cellulose nanocrystals: A renewable and sustainable nanomaterial of the future.

ACS Sustainable Chemistry and Engineering 2(7): 1691-1698.

Innventia Database information

Title

Possible toxicological effects of nanocellulose – An updated literature study, No 2

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Abstract

This literature review covers open publications and reports on the subject of nanocellulose and its possible toxicological effects. There is currently a rather low number of peer reviewed articles on the subject. However, from the articles reviewed, caution of inhalation of nanocellulose would be recommended since in vivo tests have shown immunotoxicity effect on lungs even though residues of other production chemicals, biocides and endotoxins from bacterial contamination might affect the results.

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