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Brand, W.; van Kesteren, P.C.E.; Swart, E.; Oomen, A.G.

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Overview of potential adverse health effects of oral exposure to nanocellulose

Walter Brand, Petra C. E. van Kesteren, Elmer Swart and Agnes G. Oomen

National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands

ABSTRACT

Nanocellulose is an emerging material for which several food-related applications are foreseen, for example, novel food, functional food, food additive or in food contact materials. Nanocellulose materials can display a range of possible shapes (fibers, crystals), sizes and surface modifications. For food-related applications in the EU, information on the safety of substances must be assessed. The present review summarizes the current knowledge on (possible) adverse health effects of nanocellulose upon oral exposure, keeping EU regulatory aspects in mind. The overview indicates that toxicity data, especially from *in vivo* studies, are limited and outcomes are not unambiguous. The hazard assessment is further complicated by: the diversity in morphologies and surface modifications, lack of standard reference materials, limited knowledge about intestinal fate and absorption, analytical difficulties in biological matrices, dispersion issues, the possible presence of impurities and interferences within biological assays. Two subchronic *in vivo* toxicity studies show no indications of toxicity for two specific nanocellulose materials, even at high doses. However, these studies may have missed certain early or nano-specific toxic effects, such as inflammation potential, for which other, subacute studies provide some indications. Most *in vitro* studies show no cytotoxicity; however, several indicate that effects on oxidative stress and inflammatory responses depend on differences in size or surface treatments. Further, too few studies assessed genotoxicity of nanocelluloses. Therefore, immunotoxicity, oxidative stress and genotoxicity require further attention, as do absorption and effects on nutrient uptake. Recommendations for future research facilitating the safety assessment and safe-by-design of nanocellulose in food-related applications are provided.

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Introduction

Cellulose is a polysaccharide consisting of a linear chain of several hundred to many thousands of linked D-glucose units. As an important structural component of cell walls, for instance in plants, algae and bacteria, it can be regarded as the most abundant organic polymer on earth (Klemm et al. 2005), and an important source of dietary fiber in human and animal diets (Coffey et al. 2006). Conventional cellulose molecules are tenths of micrometers in length. When their size is reduced to the nanoscale, cellulose is referred to as nanocellulose. Several types of nanocellulose can be distinguished: cellulose nanocrystals (CNCs), cellulose nanofibers (CNFs) and bacterial nanocellulose (BNC) (Foster et al. 2018; Klemm et al. 2011). CNCs and CNFs are usually made out of wood, cotton or other cellulose fibers by physical and/or chemical processes, while BNC is biosynthesized (*de novo*) by bacteria. The different production processes of nanocellulose subsequently result in different types with different sizes, shapes, purities and other properties. Table 1 provides an overview of typical characteristics of these different types of nanocellulose and their production methods. In addition, these nanocellulose types can be altered (functionalized) by modification of the surface with, for instance, sulfate, carboxyl, phosphate or acetyl groups, resulting in altered specific properties (Frank et al. 2021; Peng et al. 2021). Table 2 provides a brief overview

CONTACT Walter Brand walter.brand@rivm.nl Centre for Safety of Substances and Products, National Institute for Public Health and the Environment (RIVM), Antonie van Leeuwenhoeklaan 9, P.O. Box 1, 3720 BA, Bilthoven, the Netherlands

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Table 1. Typical characteristics of different types of nanocellulose and their production methods.

Properties	CNCs (cellulose nanocrystals)	CNFs (cellulose nanofibers)	BNC (bacterial nanocellulose)
Shape	Rod, spherical	Fibrils/fibers	Fibrils/fibers
Diameter	5–70 nm [1,2]	5–100 nm [1,2]	10–100 nm [2,3,4,5]
Length	50–350 nm [1,2]	0.1–2 μm [1,2]	Network of nanofibers. Individual fibers several to tens of μm [2]
Aspect ratio (ratio of length to width)	5–30 [1]	50–100 [1,2]	≥100 [2]
Alternative names	Nanocrystalline cellulose, cellulose nanowhiskers	Nanofibrillated cellulose, cellulose nanofibrils	Bacterial cellulose, biotech cellulose, biocellulose, microbial cellulose
Source material	Predominantly (various) plant sources. Alternative sources incl. tunicates, algae, bacteria	Predominantly (various) plant sources. Alternative sources incl. tunicates, algae, bacteria	*De novo* bacterial synthesis
Production method	Delamination of cellulose through chemical treatments (predominantly acid hydrolysis)	Delamination of cellulose through mechanical and optional chemical pretreatments	Biosynthesis using low molecular weight carbon sources (e.g. D-glucose)

[1]Foster et al. 2018; [2]Klemm et al. 2018; [3]Jozala et al. 2016; [4]Klemm et al. 2011; [5]de Amorim et al. 2020.

Table 2. Nonexhaustive list of common modifications of the hydroxyl groups on the surface of nanocellulose and their function or application. Abbreviations are listed in a footnote.

Surface group (Chemical formula)	Cation/ anion	Typical treatment	Function/application
Carboxyl (Cel-$COOH^-$)	Anion	Carboxylation of hydroxyl groups via TEMPO oxidization of cellulose surfaces during pretreatment	Enhanced surface charge to facilitate the delamination process, dispersibility and solubility in water and reduced irreversible aggregation of the fibrils (hornification) [1,5,10]
Carboxymethyl (Cel-CH_2-$COOH^-$)	Anion	Carboxymethylation of hydroxyl groups using e.g. sodium monochloroacetate	
Sulfate half ester (Cel-O-SO_3^{2-})	Anion	Hydrolysis of cellulose using sulfuric acid resulting in grafting of sulfate half ester groups	
Phosphate half ester (Cel-O-$H_2PO_3^{2-}$)	Anion	Phosphorylation of hydroxyl groups using phosphoric acid/salt [2]	Improved flame retardancy [4], enhanced viscosity [2] and dispersibility [3]
Acetyl (Cel-O-CH_3CO)	Anion	Addition of acetic anhydride and acetic acid in combination with a catalyst such as sulfuric acid [5]	Enhanced hydrophobicity [5]
EPTMAC (Cel- $C_6H_{14}NOCl$)	Cation	Etherification of hydroxyl groups	Introduction of cationic groups to the surface [5]
poly(APMA)	Cation	surface-initiated single-electron transfer living radical polymerization method	Controlled drug release through delivery carriers [8, 9]
poly(NIPAAm)	Anion	surface-initiated single-electron transfer living radical polymerization method	Controlled drug release through delivery carriers [8, 9]
Metals (e.g. Ag)	Cation	Addition of metals to growth medium resulting in precipitation of metals on surface of BNC	Ag: production of nanocelluloses with antibacterial coating
Other polymers/ molecules such as fluorophores	Polymer/molecule dependent	Various methods including grafting of polymers using ring opening polymerization [5] and atom transfer radical polymerization [6]	Modification of various properties such as fluorescence, dispersibility in different mediums and nanocomposite strength
Biomolecule conjugation		Conjugation of peptide motifs (e.g RGD) [7]	Improved adhesion of cells for biomedical application (e.g. wound dressing)

[1]Klemm et al. 2011; [2]Noguchi et al. 2017; [3]Camarero Espinosa et al. 2013; [4]Ghanadpour et al. 2015; [5]Habibi 2014; [6]Eyley and Thielemans, 2014; [7]Klemm et al. 2018; [8]Jimenez et al. 2017; [9]Zhang et al. 2016; [10]Lopez et al. 2015.
BNC: bacterial nanocellulose; EPTMAC: epoxypropyltrimethylammonium chloride; poly(APMA): poly(N-3-aminopropylmethacrylamide); poly(NIPAAm): poly(N-isopropylacrylamide); RGD: arginine-glycine aspartic acid; TEMPO: 2,2,6,6-tetramethylpiperidin-1-oxyl.

of such possible surface modifications and their function or application.

Nanocellulose receives increasing attention because of its special properties - e.g. its high surface to mass ratio, strength, modification possibilities, and bio-based origin - while its methods of production have become more economical (Endes et al. 2016; Gómez et al. 2016; Khan et al. 2018; Klemm et al. 2011; Li et al. 2021). Many applications of nanocellulose in food, pharmaceutics, cosmetics, and agricultural formulations are under development, or already present, for instance as stabilizer in emulsions, in biodegradable packaging, or as wound dressing material (Li et al. 2021; Mu et al. 2019; Portela da Gama and Dourado 2018). Increased use of nanocellulose will subsequently

lead to an increased human exposure. Especially its (foreseen) use in foods and food-related products such as food contact materials can lead to oral exposure. The potential exposure from food contact materials will depend on the migration ability of nanocellulose from such products. There are (obviously) already many applications of conventional cellulose, including modified celluloses, for example as food additive. Conventional cellulose is not absorbed from the gastrointestinal tract and is unlikely to exert adverse effects (EFSA ANS Panel et al. 2018). However, this could be different for nanocellulose because of its different physicochemical properties such as its smaller size and possible specific surface modifications.

So far, a comprehensive overview of the potential adverse health effects of nanocellulose after oral exposure is missing. Therefore, the current publication focusses on the plausible food-related applications of nanocellulose, and the possible adverse effects as a result of oral exposure as a consequence of those applications. Up to date, a substantial number of *in vitro* studies, and some *in vivo* studies has been performed on the toxicity of different nanocellulose materials. The current publication summarizes the current state of knowledge regarding toxicity as a result of oral exposure to different forms of nanocellulose. The overview aims to aid the safety and risk assessment of nanocellulose in food and food-related applications, from a EU perspective. The following sections summarize: the potential application of nanocellulose in food and regulatory aspects, and what is currently known in the public domain about the various aspects of nanocellulose upon oral exposure. The latter includes the gastrointestinal fate and absorption of nanocellulose, and the results from toxicity studies, both *in vivo* as well as *in vitro*. Lastly, the available information is discussed, conclusions are drawn and recommendations are provided for future research, in order to facilitate safety and risk assessment of nanocellulose. The information may also be useful for the safe-by-design development of nanocellulose (Dhali et al. 2021; Shatkin and Kim 2015).

Potential application of nanocellulose in food and regulatory aspects

Based on their use, different potential food-related types of nanocellulose applications can be distinguished. From a legislative perspective, these types of applications can be categorized as: functional and/or novel foods, food additives or as food contact material (Brand, van Kesteren, and Oomen 2020). These types are briefly explained below with some examples and current status, including the legislative aspects from the EU perspective. Recently, the European Food Safety Authority (EFSA) updated the guidance to aid the risk assessment of the application of food-related nanomaterials, better addressing the nano-specific properties (EFSA Scientific Committee et al. 2021a, b). Such information will be critical to ensure the safety of nanomaterials, including nanocellulose, in food related applications. This guidance will aid the assessment of nanomaterials in the respective categories.

Functional foods

So-called functional foods aim at improving the nutritional value of food or exerting a certain beneficial health effect, sometimes with a health claim. They also include foods (i.e. fortified foods) with an increased amount of an existing component (e.g. dietary fiber). In food, nanocellulose can be used as fat mimetic or fat replacer in low calory nutritional foods (i.e. to reduce the caloric value) or as a dietary fiber to increase gastro-intestinal health (DeLoid et al. 2018; Liu and Kong 2021). Nanocellulose could also be used as a delivery system, that is, as a carrier for other ingredients in hydrogels or by microencapsulation (Khan et al. 2018). For such products, the Regulation on the addition of vitamins and minerals and of certain other substances to foods (Reg. (EC) No. 1925/2006), and in case of a health claim the Regulation on nutrition and health claims made on foods (Reg. (EC) No. 1924/2006) apply. A health claim needs to be substantiated, irrespective whether nanomaterials are present or not. In addition to the European legislation, European member states can have additional national legislation for functional foods. However, the addition of a new type of cellulose (i.e. nanocellulose) would likely make the ingredient a 'novel food' (see below).

Novel foods

Novel foods includes foods not consumed 'significantly' prior to May 15th 1997. According to

the Novel Food Regulation (Reg. (EU) No. 2015/2283), it also includes vitamins, minerals and other substances with a changed composition, structure, or metabolism or foods containing a nanomaterial (EC 2015). This means that many applications of nanocellulose (including many of those mentioned above as functional food) are to be considered a novel food and have to be assessed by the European Food Safety Authority (EFSA) NDA Panel (the Panel on Nutrition, Novel Foods and Food Allergens), before it can be placed on the market. The above mentioned EFSA guidances provide recommendations for the assessment of food-related nanomaterials, addressing the nano-specific properties of novel foods (EFSA Scientific Committee et al. 2021a, b). At present, to our knowledge, there is no publicly available information on the official assessment of nanocellulose materials, e.g. by the NDA Panel. However, the cross-cutting working group on nanotechnologies of the EFSA Scientific Committee and Emerging Risk Unit was consulted recently for their opinion whether the mentioned EFSA guidance is applicable on gellable substances, specifically (conventional) sodium carboxy methyl cellulose E 466 (https://www.efsa.europa.eu/sites/default/files/wgs/cross-cutting-science/wg-nanotechnologies.pdf)

Food additives

Food additives are added to food to fulfill a certain technological function. Several celluloses are already being used in the EU as food additive. These include conventional celluloses which are partially depolymerized (microcrystalline cellulose (E 460 (i)), mechanically disintegrated powdered cellulose (E 460(ii)), or chemically modified celluloses, i.e. methyl cellulose (E 461), ethyl cellulose (E 462), hydroxypropyl cellulose (E 463), hydroxypropyl methyl cellulose (E 464), ethyl methyl cellulose (E 465), sodium carboxy methyl cellulose (E 466), enzymatically hydrolyzed carboxy methyl cellulose (E 469) and cross-linked carboxy methyl cellulose (E 468). Different cellulose types can have different size ranges. The above mentioned microcrystalline cellulose (E 460(i)), for example, is relatively small with a degree of polymerization (the number of monomeric cellulose units of the polymer) of typically <400 whereas the degree of polymerization of powdered cellulose (E 460(ii)) is >1000 (EFSA ANS Panel et al. 2018). For both of these two cellulose types, Regulation (EC) No. 231/2012 specifies that no more than 10% of the particles can be smaller than 5 μm. For the other types, no such specification exist (EFSA ANS Panel et al. 2018). As food additives, conventional celluloses are usually applied as stabilizer, emulsifier, thickener, humectant, anticaking, foaming, bulking, gelling or glazing agents (EFSA ANS Panel et al. 2018). Celluloses currently authorized as food additives are not nano-sized. However, owing to its unique properties, nanocellulose may be put forward as a food additive (Khan et al. 2018; Portela da Gama and Dourado 2018). Food additive use needs to be approved by the European Commission and assessed by the EFSA FAF Panel (the Panel on Food Additives and Flavorings), following the Regulation on food additives (Reg. (EC) No. 1333/2008). Like with the assessments of novel foods, nano-specific aspects need to be taken into account according to the above mentioned EFSA guidances.

The predecessor of the EFSA FAF Panel, the ANS Panel (the Panel on Food Additives and Nutrient Sources added to Food), reevaluated conventionally sized celluloses in 2017 (EFSA ANS Panel et al. 2018). The assessment included the microcrystalline cellulose (purified, partially depolymerized), powdered cellulose (purified, mechanically disintegrated) and the chemically modified (with methyl-, ethyl- and/or hydroxypropyl-groups) celluloses mentioned above. Nanocellulose was not considered or specifically taken into account. The reevaluation concluded there was no need for deriving an acceptable daily intake (ADI) and the exposure was considered not of safety concern. The conventionally sized celluloses are not absorbed and usually only fermented to a very small extent in the human colon. Microcrystalline cellulose and powdered cellulose are also not absorbed, but could be fermented to a larger extent during their passage through the large intestine by bacteria found in the human colon (EFSA ANS Panel et al. 2018). At present, no nanocellulose has been assessed as food additive in the EU.

Food contact materials

Nanocellulose can also be applied in food contact materials, for instance as fillers or films on food

packaging, or as bio-based nanocomposites (Silva et al. 2020; Souza, Gottschalk, and Freitas-Silva 2020). For example, transparent nano-paper with UV-blocking functionality as a biobased alternative for plastic (Hayden et al., 2019), and other antibacterial, biodegradable, edible and active/intelligent food packaging applications materials with nanocellulose have been developed (Ahankari et al. 2021; Ludwicka, Kaczmarek, and Białkowska 2020; Pal et al. 2021; Zhang et al. 2021). Nanocellulose materials could be suitable as an alternative for per- and polyfluoroalkyl substances (PFAS) in moisture or grease resistant layers of food contact paper and board (Glenn et al. 2021). Food contact materials must meet the requirements of the Regulation on materials and articles intended to come into contact with food (Reg. (EC) No. 1935/2004). The materials contained must not migrate to food in quantities which could, among others, endanger human health (EC, 2004). Although the legislation itself does not address specific provisions for nanomaterials, the above-mentioned EFSA guidances provide some recommendations for the assessment of food contact materials (EFSA Scientific Committee et al. 2021a, b). The regulation applicable to plastic materials (Reg. (EU) No. 10/2011) as well as the Regulation on active and intelligent packaging materials (Reg. (EC) No. 450/2009) indicate that a specific evaluation is required for substances in nanoform (EC 2009; EC 2011). Their authorization should be specifically mentioned in the positive lists of allowed substances in these regulations. An authorization based on the risk assessment of a substance with conventional particle size does not cover the use of the same substance in nanoscale. European member states can have additional national legislation (i.e. restrictions) for substances in plastics as well as with other materials.

Taken together, for new types of nanocellulose to be used in foods, their function determines whether it concerns a novel food or a food additive and the risk should be assessed by EFSA according to the relevant regulations. For new types of nanocellulose to be used in food contact materials, their safety needs at least to be guaranteed and fulfill the respective European and possible national requirements.

As an exception, certain types of nanocellulose do not have to be regarded as 'new' such as 'nata de coco', a traditional fermented coconut gel from the Pacific region, typically consumed as dessert, that can be regarded as product containing BNC (Azeredo et al. 2019; Portela da Gama and Dourado 2018; Zhong 2020). As a low-calory, fiber-rich food product, nata de coco is also marketed to assist weight loss, and to exert other beneficial health effects. Nata de coco is produced from coconut water by *Komagataeibacter xylinus* or *Acetobacter xylinum*, which synthesize BNC fibers. According to the EU Novel food catalogue, nata de coco is not subjected to the Novel Food Regulation as it was on the market and consumed to a significant degree before 15 May 1997 (https://ec.europa.eu/food/safety/novel_food/catalogue/search/public/?event=home&seqfce=1044&ascii=F).

Gastrointestinal fate and absorption

Conventional cellulose is not digested in the human small intestine (EFSA ANS Panel et al. 2018). It is not absorbed and usually only fermented to a very small extent by bacteria in the human colon. For smaller cellulose particles, i.e. nanocellulose, these properties can be different. Therefore, it is essential to understand the behavior of nanocellulose in the gastrointestinal tract (GIT) for the assessment of the impact of nanocellulose on human health upon oral exposure. The behavior of nanocellulose along the GIT depends on both its intrinsic properties as well as the extrinsic environment, and their interactions, but is not fully understood (Liu and Kong 2021). The intrinsic properties of nanocellulose are determined by its physicochemical characteristics, including particle size, morphology, surface charge, surface chemistry and rheological properties (Liu and Kong 2021). The extrinsic environments include differences in pH and ionic composition (i.e. ionic strength), digestive enzymes, other substances present in GIT content including biopolymers and surface active components such as bile salts, gastrointestinal movement, different biological surfaces of the GIT wall, and finally microbiota (McClements et al. 2016). Nanocellulose can also affect food digestion and nutrient absorption (DeLoid et al. 2018; Guo et al. 2021; Liu and Kong 2021).

Digestion and absorption

Although many types of nanocellulose exist, in general it seems that the interaction with the GIT components (such as digestive enzymes, bile salts, mineral ions and phospholipids) increases with a decrease in particle size (i.e. an increase in surface area), which is generally similar to other nanoparticles. This is reflected by a better dispersibility or digestibility that is generally attributed to relatively smaller particles. An important driver for the fate of nanocellulose in the GIT appears to be the agglomeration status, as larger particles may lose nano-specific properties. Extrinsic factors, such as pH and ionic strength, strongly influence the agglomeration state, as well as the surface chemistry and surface charge of nanocellulose. Although nanocellulose is an insoluble fiber, it exhibits gelling behavior like soluble fibers under certain conditions, affecting its structure/morphology.

As mentioned above, in humans cellulose is normally resistant to digestion and cannot be absorbed. Also nanocellulose is believed not to be digested, i.e. broken down in the human small intestine. However, because of its smaller size and other physicochemical properties, nanocellulose may be able to cross the intestinal barrier. A first hurdle for nanocellulose in this process would be passing the mucus layer. This layer covers the surface of the intestinal barrier and may prevent the passage over the intestinal barrier due to mucoadhesion. However, smaller and negatively charged nanocellulose may easier pass this mucus layer. Once passed the mucus, substances can be absorbed by the intestinal epithelium. Uptake by the intestinal epithelium can occur through endocytosis by e.g. phagocytosis, which seems to be the most likely mechanism of transfer of nanocellulose particles across the intestine (Koshani and Madadlou 2018; Powell et al. 2010). However, because of agglomeration of nanocellulose in the intestinal lumen, mucoadhesion and the limited transport capacities, it is believed that *in vivo* the absorption of nanocellulose in general is limited or negligible (Liu and Kong 2021).

To our knowledge, there are at present no (bio)-distribution studies available in which the absorption of nanocellulose upon oral exposure was investigated *in vivo*. Detecting and quantifying nanocellulose is analytically and technically challenging as it cannot be distinguished from the biological matrix as easily as other substances such as metal nanomaterials. In order to detect nanocellulose *in vivo* (or *ex vivo*), it can be labeled with a fluorescent or radioactive marker which in turn, especially with fluorescent markers, could affect the intrinsic physicochemical properties or detach from the nanocellulose (Foster et al. 2018; Patel et al., 2021). For example, a specific technique applying a biotinylated carbohydrate binding module of β-1,4-glycanase to visualize CNFs in biological matrixes has been developed (Catalán et al. 2017; Knudsen et al. 2015; Virkkunen et al. 2017). There have been some efforts to study the gastrointestinal passage of nanocellulose *in vitro*. Lin et al. (2021), for example, used fluorescently-labeled CNCs to investigate whether nanocellulose can cross a Caco-2 cell monolayer with an additional mucus layer. The results suggested CNCs were not capable to penetrate the Caco-2 cell monolayer with mucus layer (Lin et al. 2021), although it should be taken into account that typical Caco-2 monolayer transport experiments may not be able or suitable to detect passage by substances with very limited intestinal transport characteristics *in vivo* (Kucki et al. 2017), and also the permeability of membranes on which such monolayers are cultured could limit the transport of particles such as nanocellulose (Chung et al. 2018).

Interactions with food components

The interaction of nanocellulose with its environment during its passage through the GIT may also concern the food matrix present in the GIT. These interactions can be non-covalent as well as hydrogen binding with other substances such as proteins or polysaccharides. In addition, the presence of substances from food can also influence extrinsic factors, e.g. pH, ionic strength, digestive enzyme activity and transition time, which in turn affect the fate of the nanocellulose. Further, the possible influence of nanocellulose on food digestion and nutrient absorption has been reported (DeLoid et al. 2018; Guo et al. 2021). By binding to enzymes or substrates, nanocellulose could for instance affect specific digestive enzyme activities, influence starch, lipid and protein digestion, as well as mineral

absorption. This ability has been suggested as a way to assist in weight loss and the management of obesity (DeLoid et al. 2018). On the other hand, nanocellulose has also been reported to enhance glucose absorption (Guo et al. 2021).

Interactions with microbiota

Finally, effects on the microbiota of the large intestine are also possible. Nanocellulose can affect the composition and activity of the colonic microbiome, as was shown for other nanoparticles (Siemer et al. 2018). The intestinal microbiota ferment soluble fibers, but conventional cellulose is virtually unfermentable by the human colonic microbiota (EFSA ANS Panel et al. 2018). It is not likely that nanocellulose can be fermented to a large extend in the human colon, although it cannot be excluded that nanocellulose is fermented (slightly) better than conventional cellulose because of its larger surface area (Liu and Kong 2021). It is important to consider that in rats the relatively larger cecum and colon play a more pronounced role in their GIT, which helps them to ferment feed such as grains and seeds through the help of the bacteria contained (Hatton et al. 2015; Karasov and Douglas 2013). This characteristic also enables rats to break down cellulose to some extent into organic acids, at least more easily than humans. In addition, another interspecies behavioral difference between humans and rats in behavior, namely coprophagy (feces eating), also attributes to the fact that rats are better at digesting cellulose than humans (Williams and Senior 1985).

Altogether, the behavior and effects of nanocellulose along the GIT are not fully understood. With regard to absorption, it is believed that *in vivo* the absorption of nanocellulose in general is limited or negligible (Liu and Kong 2021). However, specific physicochemical properties of nanocelluloses could affect this process and (limited) absorption cannot be excluded. The gastrointestinal fate and absorption of nanocellulose is further summarized in the Conclusion and discussion section.

Toxicity studies *in vivo*

The review of *in vivo* toxicity studies with nanocellulose was limited to subchronic as well as subacute oral toxicity studies. No chronic oral toxicity studies with nanocellulose were available. Studies were included if they were performed with nanocellulose as a test material, for which the nano-size of the material was characterized. Studies with insufficient physicochemical characterization of the material, for example self-made nanocellulose without information on particle size and impurities, were excluded from the evaluation. Remarks related to the relevance and reliability of the studies are presented, and taken into consideration in the overall assessment. Furthermore, with human food consumption in mind, data from the subchronic studies are regarded as more important than from subacute studies. The overview of *in vivo* studies on nanocellulose and their details is presented in Table 3, and further described below.

Subchronic oral toxicity studies

Two subchronic oral toxicity studies were performed in rats according to OECD guideline 408, one with sulfated CNCs and one with CNFs. In the study by Ede et al. (2020), rats were exposed for 90 days to 0, 2, 3 or 4% sulfated CNCs via diet, equal to 1056, 1584 and 2085 mg/kg bw/day for males and 1278, 1930 and 2683 mg/kg bw/day for females in the exposed animals, respectively (Table 3). An additional control group with conventional cellulose (food-grade Solka Floc) at the same percentages was included in the study. The rats received a standard diet that contained fibers as such, but no additional fibers other than CNCs, as the test material under investigation, were added. Comparable fat, protein, and carbohydrate contents in the diet were maintained across dose groups and between CNC and conventional cellulose groups (Ede et al. 2020). Default parameters examined included clinical pathology, hematology, serum chemistry, urinalysis, anatomic pathology and histopathology. No toxicity was observed in both the sulfated CNCs and conventional cellulose groups, with the exception of the presence of vacuolation (with cytoplasmic vacuoles) of periportal hepatocytes in both the 4% dose groups. The incidence and severity of the vacuolation were similar in the sulfated CNCs and conventional cellulose groups. No altered liver function, hepatic degeneration or

Table 3. Overview in vivo oral toxicity studies performed with nanocellulose.

Reference	Study design (type of study, dose, duration)	Test material	Effects observed	Remarks regarding quality of study
Subchronic studies				
Ede et al., 2020	Repeated dose 90 day oral toxicity study (OECD 408). *Animals*: Sprague-Dawley CD IGS rats; males and females; n = 10/sex/group (total: n = 120). *Exposure*: 90 days *Route:* via diet *Diet:* OpenStandard Diet with no added fiber (D11112219N; Research Diets Inc.) *Dose*: 0, 2, 3 and 4% CNC in diet, equal to 1056, 1584 and 2085 mg/kg bw/day (males) and 1278, 1930 and 2683 mg/kg bw/day (females). *Control:* conventional cellulose (CC); 2, 3 and 4%. *Dispersion*: sample dispersion by using a Vortex for 10 min prior to analyses. *Parameters*: body weight, food consumption, ophthalmology, clinical pathology, hematology, serum chemistry, urinalysis, anatomic pathology, histopathology.	CNC: - Impurities: low levels of metal impurities (0.01-0.1 ppb range) - Endotoxins: no data - Appearance: spray-dried powder (100% wt.) - Particle size: average HDD in water 893 ± 251 nm (DLS); average DI 0.51 ± 0.02. Light and electron microscopy: widths <10 nm; lengths 25-250 nm - Surface treatment: sulfate groups - Shape: rod-shaped - Surface area: no data - Zeta potential: −50.8 ± 6 mV - Analytical method: DLS, light microscopy, TEM, SEM, EDXS - Production: produced through sulfuric acid hydrolysis of wood pulp, purification, and subsequent neutralization with sodium hydroxide	Early effects: Vacuolation of periportal hepatocytes was observed at 4% CNC. Incidence and severity was the same as in the CC group. Adverse effects: No pathology findings or other treatment-related adverse effects were observed. NOAEL = 2085.3 (m) and 2682.8 (f) mg/kg bw/day. No internal concentrations measured. CNC versus CC: Food consumption and body weight were higher in the 4% CNC group (females) compared with the 4% CC group (females).	Unclear if dispersion by Vortex is adequate. HDD is determined.
		CC: - Food-grade Solka Floc - Impurities: no data - Appearance: no data - Particle size: average HDD 26.7 ± 6 μm; average DI 0.65 ± 0.07; Microscopy: length and width in microns - Surface treatment: none - Shape: amorphous - Surface area: no data - Zeta potential: −2.1 ± 0.9 mV - Analytical method: DLS, light microscopy, TEM, SEM, EDXS - Production: no data	Early effects: Vacuolation of periportal hepatocytes was observed at 4% CC. Incidence and severity was the same as in the CNC group. Adverse effects No pathology findings or other treatment-related adverse effects were observed. No internal concentrations measured.	No data provided on impurities.
Ong et al., 2020	Repeated dose 90 day oral toxicity study (OECD 408). *Animals*: Sprague-Dawley rats; males and females; 6-7 weeks old; n = 10/group. *Exposure*: 90 days *Route:* via diet. *Diet:* OpenStandard Diet with no added fiber (D11112219N; Research Diets Inc.) *Dose groups*: 6 - CNF: 2%, 3% and 4%, equal to 1044, 1550, and 2194 mg/kg bw/day for males and 1302, 1886, and 2667 mg/kg bw/day for females. - Cellulose: 2%, 3% and 4%,	CNF - Impurities: assessed, but no data - Endotoxins: no data - Appearance: cellulose in distilled water to 2 % wt. - Particle size: average width of the finest fractions: 25.06 nm; aggregates, average size: 227.7 μm; hydrodynamic diameter: 3330 nm (DLS) - Density: no data - Surface treatment: none - Shape: entangled network of fibers and fibrils of varying widths - Surface area: no data - Zeta potential: −37.5 mV - Analytical method: light	Early effects: not measured. Adverse effects: Vacuolation of periportal hepatocytes was present in the 4% CNF group, but was also observed in the group treated with 4% conventional cellulose. No adverse observations were noted in relation to the administration of fibrillated cellulose.	Unclear if dispersion by Vortex is adequate. HDD is determined. No data provided on impurities. No negative control group without treatment was included.

(*continued*)

Table 3. Continued.

Reference	Study design (type of study, dose, duration)	Test material	Effects observed	Remarks regarding quality of study
	equal to 1070, 1536, and 2119 mg/kg bw/day for males and 1311.7, 1920.2, and 2597.5 mg/kg bw/day for females. *Dispersion*: a Disruptor Genie (60 kHz; 240 W; 3000 rpm) was used to vortex the solution for 10 min during dilution; no other data. *Parameters*: Survival, clinical observations, body weight, food consumption, ophthalmologic evaluations, hematology, serum chemistry, urinalysis, postmortem anatomic pathology, and histopathology.	microscopy, TEM, SEM, DLS, EDXS - Production: produced through mechanical homogenization of a wood pulp starting material CC - Food-grade Solka Floc - Impurities: provided by manufacturer, no data - Appearance: cellulose in distilled water to 2% wt. - Particle size: average width: 3.72 μm; aggregates, average size: 58.6 μm; hydrodynamic diameter: 625 nm (DLS) - Density: no data - Surface treatment: none - Shape: amorphous morphology, not an entangled network of fibers, lower aspect ratio than fibrillated cellulose - Surface area: no data - Zeta potential: −24.3 mV - Analytical method: light microscopy, TEM, SEM, DLS, EDXS - Production: no data	Early effects: not measured. Adverse effects: Vacuolation of periportal hepatocytes was present in the 4% CC group.	No data were provided on impurities.
Subacute studies				
Adewuyi et al., 2018	14-day oral toxicity study. *Animals*: Wistar rats; males; n = 20/group. *Route*: oral, no details provided. *Diet:* commercial pelleted diet (Ladokun Feeds, Ibadan, Nigeria); no data on fibers. *Exposure*: 14 days, once, daily. *Dose groups*: controls (olive oil), 50, 75 and 100 mg/kg bw/day CNC. *Dispersion*: ultrasonication in a Cole Parmer sonicator (model CV334) for 15 min to disperse the nanocrystals and break any agglomerates formed. *Parameters*: biomarkers of renal oxidative damage, inflammation, immunohistochemical expressions of selected genes, histopathology.	CNC - Impurities: no data - Endotoxins: no data - Appearance: suspension in deionized water - Particle size: mean distribution size of 0.0149 μm - Density: no data - Surface treatment: sulfated (in the publication named as 'sulfonated' by the authors) - Shape: monomodal, flaky surface with agglomerations - Surface area: no data - Zeta potential: ∼ −30 mV at pH 1.5; ∼ −5 mV at pH 7; ∼ 5 at pH 12 - Analytical method: SEM, EDX, FTIR, XRD, TG, PSD - Production: Sulfonated nanocellulose was obtained by acid hydrolysis with 65% sulfuric acid solution (v/v) at 50 °C	Early effects: Body weight was not affected. CNC had no effect on markers of kidney function. Calcium and potassium levels were significantly decreased at 50 mg/kg bw/day (calcium and potassium) and 75 mg/kg bw/day (potassium), but not at the other dose levels. Sodium was increased at the low and high dose level, but without a dose-response relationship. Several antioxidant enzymes were changed in kidney tissue upon treatment: - SOD was increased in the low and mid dose group ($P < 0.01$). - GPx was increased in the mid dose group ($P < 0.01$). - GSH was increased in the low dose group ($P < 0.05$) and decreased in the high dose group ($P < 0.05$). - MPO was decreased in all dose groups ($P < 0.01$). CAT, GST, MDA, hydrogen peroxide and nitric oxide	The study was limited to kidney; effects on other organs were not examined. No data were provided on impurities. The description of the methods lacks details on the treatment of the animals.

(*continued*)

Table 3. Continued.

Reference	Study design (type of study, dose, duration)	Test material	Effects observed	Remarks regarding quality of study
			were not significantly changed. Immunohistochemical staining showed more intense expression of iNOS and COX-2 upon treatment, indicative of inflammation. Adverse effects: Histopathology of the kidney demonstrated moderate cortical congestion at 50 mg/kg bw/day and interstitial hemorrhage and presentation of protein casts in the tubules of rats at 75 and 100 mg/kg bw/day.	
DeLoid et al., 2019	Five-week oral toxicity study. *Animals*: Wistar-Han rats; males; n = 13/group. *Route:* via oral gavage (10 ml/kg). *Diet*: PicoLab Rodent Diet 5053; contains 4.7% fiber (cellulose, hemi-cellulose and lignin). *Exposure*: 5 weeks, twice a week gavage. Suspension in water or cream (20% fat) *Dose groups*: water, cream, 1% CNF, or cream + CNF-50. *Dispersion*: by using a disintegrator, which consists of an agitator with a variable rotation setting. The disintegrator was set for 10,000 revolutions. *Parameters*: analysis of whole blood and serum markers, histology of lung, liver, kidney and small intestine.	CNF-50 - Impurities: no data - Endotoxins: free of endotoxins (all below LOD of 0.5 EU/mg) - Appearance: 2.5% w/w cellulose, in water (stock) - Particle size: mean diameter 64 ± 29 nm; length 6.71 ± 5.61 μm - Density: 1.312 ± 0.016 g/cm^3 - Surface treatment: not mentioned - Shape: not specified, only microscopy data - Surface area: 34 m^2/g - Zeta potential: no data - Analytical method: SEM - Production: mechanical grinding of dried sheets of softwood bleached kraft fiber	Early effects: blood differential counts, hematological parameters and serum markers showed no significant effects. Adverse effects: Histopathology, showed no significant adverse effects. A moderate but statistically insignificant reduction in weight gain was observed between rats receiving CNF-50 alone and all other groups. Rats receiving CNF-50 alone gained on average 30–40% less weight than other groups during the five weeks of treatment. No internal concentrations measured.	The description of analytical methods is missing in the publication by error. More methods than SEM may have been used. Unclear if dispersion by a disintegrator is adequate. No data were provided on impurities. Only one dose tested.
Khare et al., 2020	Five-week oral toxicity study. *Animals*: Wistar-Han rats; males; 12 weeks old; n = 4-5/group (controls: n = 3). *Route:* oral gavage (10 ml/kg). *Diet*: PicoLab Rodent Diet 5053; contains 4.7% fiber (cellulose, hemi-cellulose and lignin). *Exposure*: 5 weeks, twice a week gavage. *Dose groups*: control (water), 1% CNF, 20% cream, or 1% CNF + 20% cream *Dispersion*: by using a disintegrator, which consists of an agitator with a	CNF 50: - Cellulose nanofibers - Impurities: no data - Endotoxins: endotoxin level determined, data not provided - Appearance: 2.5% w/w cellulose in sterile deionized water - Particle size: length 6710 ± 5611 nm (SEM); diameter 64 ± 29 nm (SEM); aspect ratio 107.6 ± 54.5 - Density: 1.312 ± 0.0185 g/cm^3 - Surface treatment: no - Shape: fibrils in a web-like structure - Surface area: no data - Zeta potential: no data	Early effects: - CNF altered microbial diversity. The population *Bificobacterium* was 38% decreased compared to the control group ($p < 0.05$). - CNF diminished specific species that produce short chain fatty acids, and that are associated with increased serum insulin and IgA production. - CNF had few effects on the fecal metabolome, with significant changes in only ten metabolites of 366 measured. - Exposure to CNF also	Unclear if dispersion by a disintegrator is adequate. No data were provided on impurities. Only one dose tested.

(*continued*)

Table 3. Continued.

Reference	Study design (type of study, dose, duration)	Test material	Effects observed	Remarks regarding quality of study
	variable rotation setting. The disintegrator was set for 10,000 revolutions. *Parameters*: fecal microbiome and metabolome, intestinal epithelial expression of cell junction genes, and ileal cytokine production.	- Analytical methods: TEM, SEM - Production: mechanical grinding of dried sheets of softwood bleached kraft fiber	altered expression of genes involved in intestinal epithelial cell junction integrity and permeability, and increased production of cytokines that modulate proliferation of CD8 T cells. Adverse effects: not measured.	
Chen et al., 2020	4-6 week oral toxicity study. *Animals*: C57BL/6 mice; males and females; 8-10 weeks old; n = 4-6/group. *Route:* via oral gavage (0.01 ml/g body weight). *Diet*: PicoLab Rodent Diet 5053; contains 4.7% fiber (cellulose, hemi-cellulose and lignin). *Western diet:* Rodent Western diet (D12079B, Research Diets Inc.); contains 5% fiber (Solka Floc, FCC 200). *Exposure*: 4-6 weeks. *Dose groups*: - normal diet + water; - Western diet + water; - Western diet + CNF; 30 mg/kg bw/day - Western diet + cellulose; 30 mg/kg bw/day (Western diet: high in fat and sugars). *Dispersion*: solutions were mixed well immediately before use. No additional data. *Parameters*: body weight, food consumption, body composition, microscopy liver and GI-tract, serum triglyceride, glucose levels, glucose and insulin tolerance test, D-xylose absorption test.	CNF - Impurities: no data - Endotoxins: no data - Appearance: white, odorless, slurry form, 3% w/w. 98 w/% dry powder, 3.0 w/% aqueous gel - Particle size: nominal fiber width 50 nm; lengths up to several hundred microns - Density: 1.5 g/cm^3 dry powder - Surface treatment: none - Shape: no data - Surface area: 31-33 m^2/g - Zeta potential: −48 to −5 mV - Analytical method: no data, characterization was done by manufacturer - Production: produced by an ultrafine grinder to reduce the fiber bundles of softwood kraft pulp to nanoscale	Early effects: Summarized, CNF decreased the intestinal absorption compared with the control group and caused disturbance of glucose homeostasis. - Less severity of fatty liver in the CNF group, compared with the Western diet control group. Fat globules were seldomly seen in the CNF group. This is contrary to what is observed in the cellulose group, see below. - Significant increase of body fat and a decrease in lean body mass in the CNF group, compared to the normal diet control group. Slight increase was also observed in the control and cellulose Western diet groups, but not significant. - Disturbance of glucose homeostasis in the CNF group, including a decreased glucose metabolism and increased insulin resistance. - Decreased GI tract weight in the CNF group. No overt toxicity of the GI tract. - The D-xylose absorption test to examine the small bowel mucosal function showed a decrease in D-xylose concentrations compared to the control, indicative of a lower intestinal absorption. Late adverse effects: not examined.	It is noted that most characteristics are obtained from a manufacturers report. No data were provided on impurities. Only one dose tested. Dispersion by mixing well only, no further information. Unclear if dispersion was adequate; no characterization performed after dispersion.
		Cellulose No data reported.	Early effects: Similar of more fatty liver was observed as compared to the Western diet control group. More fat globules were found in the jejunum than in the Western diet control group.	No data on characterization provided. Only one dose tested. Dispersion by mixing well only.

(*continued*)

Table 3. Continued.

Reference	Study design (type of study, dose, duration)	Test material	Effects observed	Remarks regarding quality of study
			The body fat was slightly increased when compared to the normal diet control group, but comparable to the Western diet control group. Late adverse effects: not examined.	

Additional studies without sufficient information on characterization are not included in the table but mentioned in the text. Abbreviations are listed below the table.
CC: conventional cellulose; CNC: cellulose nanocrystal; CNF: cellulose nanofiber; DI: dispersity index; DLS: Dynamic Light Scattering; EDXS: Energy Dispersive X-ray Spectroscopy; FTIR: Fourier transformed infrared; HDD: Hydrodynamic Diameter; PSD: particle size distribution; SEM: scanning electron microscope; TEM: Transmission Electron Microscopy; TG: thermogravimetric analysis; XRD: X-ray diffraction.

pathological findings in the liver were seen and further examination was not performed.

In the other study, Ong et al. (2020) treated rats with 2, 3 or 4% CNFs for 90 days via the diet, equal to 1044, 1550, and 2194 mg/kg bw/day for males and 1302, 1886, and 2667 mg/kg bw/day for females, respectively (Table 3). The CNFs were produced through mechanical homogenization of wood pulp and were not further surface modified. In this study, conventional cellulose (food-grade Solka Floc) was included as a control at the same concentrations as the nanocellulose treatments. OECD test guideline 408 describes that an untreated group shall be used (OECD, 2018), however, such a negative control group without exposure to cellulose was not included. The animals were given feed that contains fibers as such, but no additional fibers other than CNFs, as the test material under investigation, were added. Comparable fat, protein, and carbohydrate contents were maintained across dose groups (Ong et al. 2020). Default parameters examined included clinical pathology, hematology, serum chemistry, urinalysis, anatomic pathology and histopathology. No adverse effects of both CNFs or conventional cellulose exposure on any of the measured endpoints were observed at all tested doses, with the exception of vacuolation of periportal hepatocytes in the 4% CNFs as well as the 4% conventional cellulose group. This finding is the same as was observed by Ede et al. (2020) upon treatment with 4% CNCs or conventional cellulose.

Subacute oral toxicity studies

Four subacute studies were available, with each measuring a range of endpoints (Table 3). One subacute study was performed with sulfated CNCs and focused on renal effects (Adewuyi et al. 2018). Characterization of the material included particle size, information on surface treatment and zeta potential (surface charge), however, no information was provided on the presence of impurities. Male rats were treated by oral gavage with 0, 50, 75 or 100 mg/kg bw/day CNCs for 14 days ($n = 20$ per group). No details on the fiber content of the feed were available. Histopathology of the kidney was performed, and also biomarkers of oxidative damage and inflammation in kidney tissue were examined. The results showed no effect of CNCs on kidney function, but showed changes in antioxidant enzyme activity and results from immunohistochemical analysis, which were indicative of oxidative stress and inflammation. Further, moderate cortical congestion (i.e. congestion of veins in the renal cortex) was observed, but only in the low dose group, while interstitial hemorrhage and presentation of protein casts were recorded in the kidney tubules in the mid and high dose groups.

Two subacute studies were performed in rats or mice with unmodified CNFs. DeLoid et al. (2019) treated male rats with 1% CNFs for 5 weeks via gavage (twice a week), with or without cream (20% fat) to investigate the effects in relation to high fat diet. Also a dose group with only cream and a control group treated with water were included. The rat diet contained 5% dietary fiber (cellulose, hemi-cellulose and lignin). Characterization of the material included particle size, surface area and density, but information on impurities was not provided. Blood and serum were analyzed and histopathology was performed on lung, liver, kidney and small intestinal

tissue. Further, the effect of CNFs on intestinal microbiota, intestinal permeability and cytokine production was examined and published in a companion paper by Khare et al. (2020). No CNF-induced changes were seen in blood differential counts, hematological parameters and serum markers. Histopathology revealed no significant findings in the tissues examined. Rats receiving CNFs alone gained on average 30–40% less weight than the control animals that received water. This effect was not observed in the group receiving CNFs and cream, compared with its control group receiving cream only. The reduction was not regarded as statistically significant (DeLoid et al. 2019). However, this effect seems consistent over time and might indicate a relevant effect and also the authors acknowledge that this requires further investigation (DeLoid et al. 2019). CNFs altered the microbial diversity and diminished specific species that are capable of producing short chain fatty acids and that are associated with increased serum insulin and IgA production. However, these parameters were not measured in the study. Changes inexpression of genes involved in intestinal epithelial cell junction integrity and permeability were indicative of impaired intestinal barrier function. Further, cytokine production of IL-7 and IL-18, involved in promotion of CD8 T-cell proliferation, was statistically significantly increased by CNFs but not by cream only or by CNFs with cream (Khare et al. 2020). It is noted that only one dose was tested, thereby it is not possible to examine any dose-response relationship.

In another subacute study with unmodified CNF, mice were treated with 30 mg/kg bw/day CNFs or conventional cellulose via oral gavage for 4-6 weeks, combined with a Western diet (high in fat and sugars) (Chen et al. 2020). Also control groups exposed to water with or without a Western diet were included. Both the normal diet and the Western diet contained about 5% fibers, consisting mainly of cellulose. Characteristics of the material were obtained from a manufacturers report, which did not include data on impurities. Next to body weight and food consumption, also triglyceride levels, glucose homeostasis and intestinal absorption of nutrients was examined. The liver and gastrointestinal tract were examined by microscopy. Compared with the Western diet control group, CNFs disturbed the glucose homeostasis and nonspecifically decreased the intestinal absorption as indicated by a decrease of the nutrient D-xylose in serum and a decreased lean body mass. Fatty liver was less severe in the CNFs treated mice as compared with the control groups and fat globules were seldomly seen. It is noted that also in this study only one dose was tested. Any dose-response relationship can therefore not be examined.

Two studies were performed with 0, 5 or 10% BNC, which is considered to be composed of nanofibrils with a width $<$100 nm (Zhong 2020). Both studies did not include any information on the characteristics of the material tested, such as fibril size, presence of impurities, modifications, surface area, shape or appearance. As a result, the reliability is insufficient to include the data in further hazard assessment. However, since these are the only *in vivo* subacute and subchronic studies with BNC available, a short summary on the results is provided here.

A 13-week study was performed in Sprague-Dawley rats, which were treated with 0, 5 or 10% BNC in their diet (calculated to be 0, 3200 and 7000 mg/kg bw/day, respectively). Body weight, food consumption, hematology, clinical chemistry and organ weights were analyzed and microscopical analyses were done. As a control, microcrystalline cellulose was included with the same dose levels. No characteristics of the material were provided. The results show that food consumption increased in rats that were fed food with BNC or microcrystalline cellulose compared to the negative control. This was considered to be a result of the relatively high test article concentration in the feed, causing a lower nutritional value of the diet for which the animals adjusted. No other treatment-related effects were observed (Schmitt et al. 1991).

Hagiwara et al. (2010) treated rats with 0, 1.25, 2.5 or 5% BNC for 28 days via diet. Also this study provided no characteristics of the material. Body weight and food consumption were measured, and ophthalmologic examinations, urinalysis, hematology analyses, blood biochemistry analyses, gross observations at necropsy were performed. Organ weights were measured and histopathological examination was done. Some treatment-related effects were observed on hematology parameters, including an increase in cholesterol levels in males

and a decrease in phospholipid and calcium concentration in females. However, a clear dose-response was lacking and data were within the historical control range. Further, an increase in filled and empty cecum weight was observed, as well as an increase in weight of the salivary gland (males and females), relative kidney weight (females) and adrenal weight. The increase in cecum weights is considered to be an adaptation of the tissue to the poorly absorbed cellulose.

Overall, histopathological examination in the subacute and subchronic studies demonstrated (1) vacuolation in hepatocytes (Ede et al. 2020; Ong et al. 2020), considered to be an early response to (nano)-cellulose which may eventually progress to liver damage, and (2) cortical congestion and protein casts in the kidneys upon exposure to sulfated CNCs (Adewuyi et al. 2018). The effects in the kidney were accompanied by signs of oxidative stress and inflammation, measured by antioxidant enzyme activity and immunohistochemical staining. Other signs of immunotoxicity were limited to an increased production of cytokines upon treatment with CNFs (Khare et al. 2020). Local effects on the microbiome included an altered microbial diversity and reduced the relative abundance of specific bacterial species. The available studies also demonstrated a reduced weight gain (DeLoid et al. 2019), decreased lean mass, less severity of fatty liver, decreased intestinal absorption and a disturbance of glucose homeostasis (Chen et al. 2020), indicative of a disturbed nutritional balance.

Further interpretation of the *in vivo* studies is presented in the Discussion and conclusion section.

Toxicity studies *in vitro*

The review of *in vitro* toxicity studies was limited to 1) review studies supplemented with recent studies to gain general insight in effects of nanocellulose *in vitro*, and 2) studies that used different nanocellulose materials, and/or conventional cellulose in the same study in order to compare the impact of structural differences on toxicity. The summaries of *in vitro* studies are presented in Table 4.

In vitro effects of nanocellulose

The *in vitro* toxicity studies on different types of nanocellulose has been reviewed before (Endes et al. 2016; Stoudmann et al. 2020; Ventura et al. 2020) and is usually directed at cytotoxicity, oxidative stress, inflammation potential and genotoxicity. A substantial number of various *in vitro* studies has been performed, but their results are often not in line with each other. According to Stoudmann et al. (2020) the detected adverse effects cannot be directly attributed to the size of nanocellulose, surface modifications, tested concentrations, specific assays or cell lines. However, this is partly caused by the varying quality of the studies performed (Stoudmann et al. 2020). For the hazard or risk assessment of nanocellulose, it is critical that certain quality criteria are met. For instance the presence of chemical or biological impurities, originating from (previous) processing (Liu et al. 2017) or the potential interferences of nanocellulose with assay systems could seriously affect the outcome of *in vitro* studies (Endes et al. 2016; Stoudmann et al. 2020). Many studies do not report on such issues and therefore the usefulness of these studies for hazard assessments purposes is limited.

Some studies with *in vitro* results were published recently and were not taken into account in the abovementioned reviews (Chen et al. 2020; Ede et al. 2020; Lopes et al. 2020; Weiss et al. 2021). The results of those studies, in addition to the conclusions from previous review papers regarding the different endpoints, are discussed briefly below.

Cytotoxicity and oxidative stress

Cytotoxic effects and oxidative stress induced by nanocellulose exposure in *in vitro* assays are generally insignificant or at most relatively mild, especially compared to other nanostructures such as carbon nanotubes (Endes et al. 2016; Stoudmann et al. 2020; Ventura et al. 2020). However, little is known about the potential of nanocellulose to form radicals in cell-free and cellular environments (Endes et al. 2016). Oxidative stress caused by the increased presence of free radicals is related to other toxicological endpoints and is therefore measured in several studies in which other endpoints such as cytotoxicity were investigated. With regard to cytotoxicity, also the recently published studies do not show that nanocellulose causes such effect. Chen et al. (2020), who exposed six different cell types to unmodified CNFs or to conventional

Table 4. Overview of in vitro studies that tested for effects of different types (shape, size or surface modification) of nanocellulose.

Study	Cellulose types tested and key experimental procedures	Are there differences in effects between different nanocellulose types?				Summary of findings and comments relevant for the risk assessment
		Cyto-toxicity	Geno-toxicity	ROS	Immuno-toxicity	
Alexandrescu et al., 2013	Eight types of CNFs from various plant sources and different production methods and surface modifications. Size method: not measured Dispersion: NA (exposure as film) Endotoxins: not measured Impurities: no removal procedure, not measured	Yes	–	–	–	- Decreased cell viability only for CTAB modified CNFs - CTAB is a antimicrobial substance which explains the toxic effects of these CNCs - Exposure was done as films instead of dispersed particles. This may affect the actual exposure of cells to individual particles compared to studies that used dispersed fibers. - Sizes of individual fibers not reported and no information about impurities
Bhattacharya et al., 2017	Four types of CNFs and cellulose of different sizes and zeta-potentials, but ranges were large and medium dependent. Size method: DLS in exposure medium Dispersion: sonication Endotoxins: most below LOD Impurities: no removal procedure, not measured	No	–	–	Yes	- CNFs induced pro-inflammatory responses - Cytokine production was CNF dependent - Effects could not be explained by endotoxins - One CNF type did not induce cytokine production but this was not further explained in the publication - Dispersion well described, endotoxin measured, other impurities not reported. - Size of materials measured with DLS in exposure medium
Catalán et al., 2015	Two crystals: CNCs and CMCs (crystalline micro crystals). Size method: AFM, TEM Dispersion: sonication Endotoxins: not measured Impurities: removal procedure, not measured	No	No	–	Yes	- CMC induced cytokines, CNC did not - No reported impurities removal procedure for CMCs which could explain immunotoxicological effects - Clear dispersion and impurities removal procedure for CNCs - Endotoxins not measured
Chen et al., 2020	One type of CNFs of commercial source and cellulose. Size method: not measured Dispersion: mixing, no further details Endotoxins: not measured Impurities: no removal procedure, not measured	No	–	–	–	- Effects reported only at the highest CNF dose, likely caused by covering of cells by CNFs - CNF size was not measured, only (minimal) data from supplier is provided - No details provided on dispersion, endotoxins or other impurities
DeLoid et al., 2019	One CNC type , one CNF type and cellulose (MC). Size method: TEM Dispersion: vortexing, no further details Endotoxins: Below LOD or low Impurities: <1% trace metals. CNCs: 0.08% w/w sulfur[a]	No	–	Yes	–	- Marginal effect (1.1 fold increase) at the highest CNC dose, but ROS production was higher in cellulose and TiO_2 controls - (Nano)celluloses were digested in food matrix to mimic passage through stomach. Cells were exposed to digesta (up to 1.5 % w/w nanocellulose) which may impact exposure - Few details on dispersion/mixing procedure reported

(*continued*)

Table 4. Continued.

Study	Cellulose types tested and key experimental procedures	Are there differences in effects between different nanocellulose types?				Summary of findings and comments relevant for the risk assessment
		Cyto-toxicity	Geno-toxicity	ROS	Immuno-toxicity	
Despres et al., 2019	Two types of CNCs (anionic and cationic). Size method: measured but not available Dispersion: not reported Endotoxins: not measured Impurities: removal procedure in place	–	–	Yes	Yes	- Surface modification had an effect on mitochondrial function and mitochondria derived ROS formation - Cationic CNCs induced stronger immune responses than anionic CNCs - Incomplete size characterization (no data provided) - Details dispersion procedure missing - CNCs were modified with two polymers which may be used in drug delivery - Not clear if these surface modifications are expected for food applications
Ede et al., 2020	One CNC type and cellulose. Size method: DLS Dispersion: vortex Endotoxins: not measured, but no microbiological contamination Impurities: according to international standard (FCC)	No	–	No	No	- No differences in cytotoxicity, ROS formation and immunotoxicity between cellulose and CNC - Dispersion of materials through vortexing only - Detailed particle characterization and impurities assessment
Hanif et al., 2014	Four types of CNCs of different size and each a different chemical treatment. Size method: TEM Dispersion: no details Endotoxins: not measured Impurities: removal procedure, not measured	Yes	–	–	–	- Effects on cytotoxicity only at high concentrations (500 or 1000 μg/ml) - At higher concentrations, different CNCs showed slightly different cytotoxic effects, but overall dose-response trends were similar for all types - Details dispersion procedure missing
Hua et al., 2015	Three types of CNFs, either unmodified or cationic of anionic surface treatment. Size method: NA (exposure as film) Dispersion: NA (exposure as film) Endotoxins: not measured, but UV treated or autoclaved Impurities: not measured, HCl and NaOH removed through washing	–	–	–	Yes	- Cationic CNFs promoted inflammation response more than anionic CNFs and activity monocytes dependent on surface treatment - Exposure using films instead of dispersed fibers - Endotoxins not measured and limited impurities removal step. Hence, it cannot be ruled out that immunological differences are due to different levels of impurities
Jimenez et al, 2017	Four types of CNCs, with either anionic (3 types) or cationic (1 type) surface treatment.	No	–	–	–	- Two cell lines tested using MTT and LDH assays at 24h and 48h - Different effects of different CNCs on cell viability were only reported in the 48h exposures but effects were dependent on cell line and assay - Overall, no clear link between CNC type and cytotoxic effects

(*continued*)

Table 4. Continued.

Study	Cellulose types tested and key experimental procedures	Are there differences in effects between different nanocellulose types?				Summary of findings and comments relevant for the risk assessment
		Cyto-toxicity	Geno-toxicity	ROS	Immuno-toxicity	
	Size method: DLS Dispersion: no details Endotoxins: not measured Impurities: removal procedure, not measured					- Details dispersion procedure missing
Lopes et al., 2017	Three CNFs, either unmodified or cationic of anionic surface treatment.	No	–	No	Yes	- Pro-inflammation response was reported for unmodified CNFs but not for the surface modified CNFs
	Size method: TEM Dispersion: sonication Endotoxins: not measured Impurities: biocide free, no bacterial contamination					- Addition of a cytokine suppressor reduced cytokine secretion which indicated endotoxin contamination of CNF suspensions - Endotoxins were not measured and therefore it cannot be excluded that differences between types are due to endotoxins
Lopes et al., 2020	Five types of CNFs, either unmodified, or cationic, anionic, phosphorylated, or sulfo-ethylated.	No	–	–	–	- No effects of CNFs on cell metabolic activity of Caco-2-cells
	Size method: not measured, sizes not reported Dispersion: sonication Endotoxins: not measured, CNFs were autoclaved Impurities: no procedure, not measured					- Detailed description of exposure procedure with Caco-2-cells missing - Size characterizations of CNFs are missing
Mahmoud et al., 2010	Two types of CNCs labeled with different fluorescent molecules.	No	–	–	–	- No significant cytotoxicity in human embryonic kidney cells in 24h exposures
	Size method: TEM Dispersion: sonication Endotoxins: not measured Impurities: removal procedure, not measured					- FITC labeled CNCs had negative zeta potentials, whereas RBITC labeled CNCs had positive zeta potentials
Wang et al., 2019	Two CNF and seven CNC types, each with different sizes and source materials.	No	–	Yes	Yes	- None of the tested nanocelluloses had an effect on cytotoxicity - Use of fluorescently labeled nanocellulose showed that different nanocellulose types were taken up different by THP-1 cells - Different types induced different levels of cytokine production (IL-1β) and generated different levels of ROS and glutathione
	Size method: TEM and DLS Dispersion: vortexing and sonication Endotoxins: measured, below 1 endotoxin unit per ml Impurities: no metal, alkaline or salt impurities (ICP-OES measurements)					- All assays included a positive controls and where relevant also LPS controls - Good characterization and dispersion procedure - Impurities thoroughly assessed

(*continued*)

Table 4. Continued.

Study	Cellulose types tested and key experimental procedures	Are there differences in effects between different nanocellulose types?				Summary of findings and comments relevant for the risk assessment
		Cyto-toxicity	Geno-toxicity	ROS	Immuno-toxicity	
Weiss et al., 2021	Twelve types of CNCs of different sizes, source materials, chemical treatments and surface modifications. Size method: TEM Dispersion: no clear procedure Endotoxins: measured, <5 endotoxin units per ml Impurities: removed (dialysis) and measured (NMR)	No	–	–	No	- No effects of any type on cytotoxicity - Different CNCs did not induce different immune responses - Dispersion of the particles not described in detail - Detailed characterization - Impurities and endotoxins successfully removed and measured

Hyphens (-) indicate that the respective endpoint was not tested. More details of these studies, including nanocellulose specifications and the study outcomes are reported in the Supplemental Table S1 (Supplementary information).
[a]Information on impurities taken from Pyrgiotakis et al. (2018).

cellulose, found no significant effects in an Alamar blue cell viability assay at a 500 μg/ml concentration, a concentration which can be considered as too high to be realistic and which could have caused interferences with the assay. Ede et al. (2020), using a co-culture of Caco-2 cells, HT29-MTX cells and Raji B cells, assessed the effect of their pristine sulfated CNCs, digested CNCs and conventional cellulose (food-grade Solka Floc) up to 0.02% (∼0.2 mg/ml). No significant effects on metabolic activity, oxidative stress, cell viability, and barrier integrity of cells were seen for the different materials. Lopes et al. (2020) assessed the cytotoxicity of four types of CNFs with different surface modifications (carboxymethylation, hydroxypropyltrimethylammonium substitution, phosphorylation and sulfoethylation), as well as unmodified CNFs, in a resazurin assay with Caco-2 cells up to 500 μg/ml. No cytotoxic effects of CNFs with or without modifications in Caco-2 cells were recorded (Lopes et al. 2020). Weiss et al. (2021) did also not detect cytotoxicity in RAW-Blue macrophage cells after exposure to CNCs from different origins (cotton, wood, *Miscanthus* or sea tunicate) and with different surface modifications (uncharged or modified with positive or negative charges by placing amines, carboxylates or sulfate half-ester moieties) up to 100 μg/ml.

Inflammation potential

In contrast to the mild cytotoxic effects, reviews on *in vitro* studies indicate that CNCs or CNFs can trigger a moderate to severe inflammatory reaction. Previous reviews have made a link between this reaction and different physicochemical properties and surface modifications (Čolić et al. 2020; Endes et al. 2016; Ventura et al. 2020). Although there is a lack of coherent data, it has been suggested that a reduced size (or aggregation status) or increased hydrophilicity could increase the immunotoxic potential of CNCs, or that surface modification with carboxyl or phosphonate groups could increase the immunotoxic potential of CNFs (Čolić et al. 2020; Ventura et al. 2020). However, a structure-activity relationship regarding this topic is far from being established. It should be taken into account that also impurities might be a cause of effects in toxicity studies with nanocellulose. More recently, Ede

et al. (2020) studied pro-inflammatory effects in their *in vitro* experiments, i.e. the expression of IL-6, but found no significant effects. Also Weiss et al. (2021) found no immune system activation (i.e. induction of NF-kB signaling in RAW-blue macrophage cells) by CNCs. However, low levels of TNF-α production were observed, indicating that the CNCs induced minimal immune activation, whereas no induction of IL-6, was observed either (Weiss et al. 2021).

Genotoxicity

There are only few studies that assessed the genotoxicity of nanocellulose (Endes et al. 2016; Stoudmann et al. 2020; Ventura et al. 2020), especially with regard to CNCs (Catalán et al. 2015). Some studies have reported genotoxic effects, while others report an absence of genotoxicity. Certain types of nanocellulose will be too large to allow entrance to the cell nucleus, but indirect genotoxic might still be possible, especially for nanocellulose with certain surface modifications (Endes et al. 2016). The current conclusions on the genotoxic potential of nanocellulose are ambiguous or conflicting, and this endpoint needs to be better studied (Stoudmann et al. 2020; Ventura et al. 2020).

Comparing different types and modifications of nanocellulose

Aiming at gaining insight on the effect of structural differences of nanocellulose on *in vitro* toxicity, an overview of studies using different nanocellulose materials, and/or conventional cellulose in the same study was made. Only a limited number of studies that used *in vitro* test systems compared different types of nanocellulose and/or compared nanocellulose with conventional cellulose (Alexandrescu et al. 2013; Bhattacharya et al. 2017; Catalán et al. 2015; Chen et al. 2020; DeLoid et al. 2019; Despres et al. 2019; Ede et al. 2020; Hanif et al. 2014; Hua et al. 2015; Jimenez et al. 2017; Lopes et al. 2017, 2020; Mahmoud et al. 2010; Wang et al. 2019; Weiss et al. 2021). The *in vitro* data on cytotoxicity, oxidative stress, inflammation potential and genotoxicity from these studies are presented in Table 4. Details about characterization, dispersion and impurities (with further details provided in the supplementary information) are included, critically assessed, and discussed below.

Cytotoxicity

In total we identified thirteen *in vitro* studies that compared cytotoxic effects of different types of nanocellulose (Table 4). Most of these studies reported a lack of cytotoxicity at doses up to 500 μg/ml or found no difference in effects between different (nano)cellulose types. It should be noted that interfering processes might occur, such as agglomeration and gelling, at such high concentrations (Mendoza et al. 2018; Qi et al. 2015). Only two studies in Table 4 reported that different types of nanocellulose had different cytotoxic effects. One of those was a study by Alexandrescu et al. (2013) that compared eight types of CNFs. Only one of those eight types had an cytotoxic effect: a type of CNF that was modified with cetyl trimethylammonium bromide (CTAB), an antibacterial substance, and thereby with anticipated cytotoxic effects (Alexandrescu et al. 2013). In the other study, murine embryo fibroblasts and HCT116 colon adenocarcinoma cells were exposed to very high doses (500 or 1000 μg/ml) of different sizes of CNCs prepared by different acid hydrolysis methods (Hanif et al. 2014). At these high doses, cell viability of the studied cells was significantly reduced. Though differences in responses between the different CNCs were recorded at the highest test concentration, overall, the effects were similar. Other studies with CNCs, including sulfated CNCs, found no effects on cytotoxicity (Catalán et al. 2015; DeLoid et al. 2019; Despres et al. 2019; Ede et al. 2020; Jimenez et al. 2017; Mahmoud et al. 2010; Wang et al. 2019; Weiss et al. 2021). Therefore, nanocellulose does not seem to induce cytotoxicity, and in the case that cytotoxicity was observed at very high dose, no or limited differences were observed between different types of nanocellulose (Table 4).

Oxidative stress

Some studies report no oxidative stress caused by nanocellulose, e.g. as determined for sulfated CNCs by CellROX green or SyTox Red assays by Ede et al. (2020), or only at a high dose of 1.5% (DeLoid et al.

2019). On the other hand, Despres et al. (2019) tested for differences between cationic CNCs (modified with poly(APMA)) and anionic CNCs (modified with poly(NIPAAm)) and found that different CNCs have different effects on mitochondrial function. The data suggest that cationic CNCs have a stronger impact on the mitochondrial function in lipopolysaccharide-stimulated macrophage cells, while the anionic CNCs display a greater impact on mitochondria-derived ROS, especially when cells were not stimulated by lipopolysaccharide (Despres et al. 2019). However, Lopes et al. (2017), reported no ROS formation for both anionic (carboxymethylated) and cationic (hydroxypropyl-trimethylammonium) modified CNFs. Interestingly, in a study by Wang et al. (2019), who tested a range of different CNCs and two CNFs with varying physicochemical properties, all nanocellulose types induced ROS formation at 300 μg/ml in a 2′,7′-dichlorofluorescein (DCF) assay, while some demonstrated glutathione (GSH) depletion in THP-1 cells in a GSH-Glo assay. Though studies often report negative results, others report the potential of nanocellulose materials to cause oxidative stress *in vitro*, and differences in both types of nanocellulose as well as different modifications might play a role in oxidative stress formation.

Inflammation potential

In total we identified eight *in vitro* studies that compared inflammatory responses by different types of nanocellulose (Table 4). Several of these studies showed differences in such effects between different types of nanocellulose. Some individual studies reported differences in the release of certain cytokines involved in anti-inflammatory responses as a result of exposure to different types of nanocellulose and/or conventional cellulose. Bhattacharya et al. (2017), for example, performed cytokine profiling (of 27 different cytokines) in human macrophage-differentiated THP-1 cells using four types of CNFs with different sizes and conventional cellulose. The authors found different cytokine secretion profiles for the four types of CNFs and conventional cellulose. Interestingly, one type of CNFs did not induce any cytokine expression. There are no obvious different physicochemical characteristics to which this can be attributed, although it is interesting that the endotoxin level of this nanocellulose was the lowest. Further, Catalán et al. (2015) reported an induction of a release of TNF-α and IL-1β from human monocyte-derived macrophages as a result of exposure to microcrystalline cellulose, but not by CNCs. Wang et al. (2019) used THP-1 macrophages and murine bone marrow–derived dendritic cells to test a range of CNCs, and two types of CNFs, with different physicochemical properties. They found that CNCs in the 200–300 nm length scale are more likely to induce lysosomal damage, NLRP3 inflammasome activation, and IL-1β production than CNFs. The authors showed that pro-inflammatory effects of the CNCs are correlated with higher crystallinity index, surface hydroxyl density, and reactive oxygen species generation, highlighting that different physicochemical properties of nanocellulose can have different inflammatory effects. Other studies focused on different surface modifications. For instance, Despres et al. (2019) reported that cationic CNCs (modified with poly(APMA)) induced stronger immune response (IL-1β) in LPS-stimulated mouse macrophage cells than anionic CNCs (modified with poly(NIPAAm)). The study suggest that the mechanisms by which the CNCs exert their immunomodulation depends on their surface modifications (as determined by TNF-α and NLRP3 expression) (Despres et al. 2019). Hua et al. (2015) reported that hydroxypropyl-trimethylammonium treated (cationic) CNFs promoted inflammation much more than unmodified CNFs, whereas carboxymethylated (anionic) CNFs passivated the surface of CNF films in terms of their inflammatory response. On the other hand, Lopes et al. (2017) reported production of TNF-α and IL-1-β by human THP-1 macrophages as a result of exposure to an unmodified CNF-gel, an effect that did not occur in carboxymethylated (anionic) or hydroxypropyl-trimethylammonium treated (cationic) CNF-gels. Therefore, in different assays certain types of nanocellulose (as well as conventional cellulose) are able to induce inflammatory responses, and surface modifications of nanocellulose may play a role in such effects. However, the present data do not allow to derive structural properties that in general predict the immunotoxic potential of nanocellulose.

Genotoxicity

To our knowledge, only one study compared different nanocellulose materials for genotoxic effects.

This study used four types of CNCs in a mammalian cell micronucleus test (OECD 487) but reported no significant genotoxic effects (Catalán et al., 2015).

Altogether, there is considerable variation in the available *in vitro* studies regarding the type of assays as well as the nanocellulose materials that are used. Further interpretation and summarizing of the *in vitro* studies is presented in the Discussion and conclusion section.

Discussion and conclusion

In this study, we provided an overview of the potential adverse health effects of nanocellulose upon oral exposure for foreseen applications of nanocellulose in food or food-related products. Many of such applications are being developed, varying from functional foods and food additives to food contact materials. Ultimately, these applications may potentially lead to oral exposure. Accordingly, the safety of nanocellulose needs to be assessed under the different food legislations in the EU (EC 2015; EFSA Scientific Committee et al. 2021a, b). To provide an overview of the issues which might play a role in safety assessment for human exposure, we reviewed the current state of affairs on the potential toxicity, highlighting important issues. This review may also provide starting points for safe-by-design considerations for nanocellulose applications in food or food-related applications and products.

Gastrointestinal fate and absorption

After oral ingestion, nanocellulose passes the GIT. The behavior and effects of nanocellulose along the GIT are not fully understood and further studies are needed to obtain comprehensive information, as summarized by Liu and Kong (2021). With regard to absorption (and kinetics), it is believed that *in vivo* the absorption of nanocellulose in general is limited or negligible (Liu and Kong 2021). This assumption of limited absorption is based on the interactions with other substances or agglomeration of nanocellulose in the intestinal lumen, mucoadhesion and the limited transport capacities, which all hamper nanocellulose uptake. However, these processes can be affected by the specific physicochemical properties of nanocelluloses such as size and charge or possible other surface modifications. The possible absorption of nanocellulose needs to be further studied as it is a key aspect for its risk assessment. Conventional cellulose is allowed on the EU market as a food additive because EFSA concluded that cellulose is not absorbed from the human gastrointestinal tract (EFSA ANS Panel et al. 2018). This cannot be assumed for nanocellulose given the reduced size dimensions, and possibly altered properties by surface modifications. It is acknowledged that studying the absorption and kinetics of nanocellulose is challenging because of analytical limitations and may also be complicated by gastrointestinal interspecies differences. So far, no *in vivo* absorption or biodistribution study of nanocellulose has been conducted.

In vivo toxicity studies

Few toxicological oral *in vivo* studies with nanocellulose have been published. The two available subchronic oral studies in rats, performed according to OECD TG 408, did not show adverse effects upon dietary exposure to CNFs or modified (sulfated) CNCs up to high doses of more than 2000 mg/kg bw/day (Ede et al. 2020; Ong et al. 2020). Vacuolation of hepatocytes, which occurred in the two studies at the highest dose of both types of nanocellulose (CNCs and CNFs) as well as with conventional cellulose, could be the result of cellular adaptation to stress or damage, rather than an indication of damaged cells (Nayak et al. 1996). It was not accompanied by other findings indicative of liver damage. Hence, this vacuolation can be seen as an early mode of action response to exposure to (nano)cellulose, but does not need to lead to any adverse effects. It is unclear whether this early response is a direct effect from the presence of (nano)cellulose in the liver, or an indirect effect due to changes in the nutrient status. It is noted that Ong et al. (2020) assessed the toxicity of CNFs as compared to conventional cellulose, instead of to untreated animals, so a comparison to a non-exposed group could not be made. Hence, effects such as related to nutrient uptake that are not specific to the nano size of nanocellulose may have gone unnoticed. Adverse effects of BNC, examined in a 28-day and a 90-day study, showed no treatment-related toxicity (Hagiwara et al. 2010; Schmitt

et al. 1991), however, no characteristics were available for the BNCs tested in these studies.

Subacute studies did show some adverse effects. Changes in kidney were observed upon treatment with sulfated CNCs (Adewuyi et al. 2018). These changes were observed at lower dose levels compared with the subchronic studies that showed no adverse effects (Ede et al. 2020; Ong et al. 2020). It is noted that both this subacute study and the study by Ede et al. (2020) were performed with sulfated CNCs. The role of the sulfate groups on the nanocellulose surface in the observed kidney effects remains therefore unclear. It is also noted that Adewuyi et al. (2018) did not provide information on impurities or endotoxins, hence, the effect of any impurities and endotoxins in the nanocellulose test material cannot be excluded. Further, the animals in the subacute studies received diets with about 5% fiber (mainly cellulose), while the diets with no added fiber to the standard feed were used in the subchronic studies. This leads to a standard higher background exposure to fibers, including cellulose. Any differences in background fiber content or composition between control and exposed animals should be taken into account.

Changes indicative of impaired intestinal barrier and adaptive immune response could be early effects related to CNF exposure (DeLoid et al. 2019; Khare et al. 2020). A biologically significant decrease in weight was also observed (DeLoid et al. 2019). Although these findings were not accompanied by histopathological changes, and subchronic exposure to CNFs did not show adverse effects, it is uncertain if the early changes may still lead to health implications after chronic exposure. Further, it is also noted that only one dose was tested in these subacute studies and no data on impurities were provided, thereby hampering the interpretation of the results. Effects on intestinal absorption and glucose homeostasis were observed upon CNF treatment, which can lead to adverse effect on the long term, but also could be employed to provoke health beneficial effects, e.g. weight loss in case of obesities. The studies by DeLoid et al. (2019) and Khare et al. (2020) are a good example of investigating both such early changes as well as adverse effects as measured by pathology, although the duration of these studies was relatively short.

Overall, the two OECD guideline subchronic toxicity studies were considered the most reliable and provided a good indication on the absence of adverse effects during subchronic exposure. The vacuolation in hepatocytes, observed in both studies, is likely to be an adaptation to (nano)cellulose exposure. Early effects were observed in subacute studies, but these studies had their limitations. Further investigation on the role of surface modification and the potential role of impurities is needed to clarify early effects observed in subacute studies. In addition, *in vivo* absorption data of nanocelluloses are needed for further risk assessment.

In vitro toxicity studies

Relatively many *in vitro* studies have been performed with nanocellulose. Their outcomes, however, are not always in line with each other, which can be (partly) attributed to the diversity of nanocellulose materials used as well as the assays performed. Specific experimental limitations relevant for the hazard assessment of each study are reported in Table 4. Often, the nanocellulose used is self-prepared, and sometimes lacks a proper characterization, also with regard to possible impurities. The use of reference materials, as were developed for other nanomaterials, could facilitate the comparison of study outcomes. In addition, information on the preparation of the dispersion of nanocellulose is often missing, which raises questions whether this was properly performed.

Regarding the reported *in vitro* effects, nanocellulose usually does not induce cytotoxic effects. However, studies have been reporting positive as well as negative oxidative stress responses and inflammation responses which can be indicative for immunotoxicological effects, resulting in a lack of coherent data which make it difficult to draw a conclusion on these *in vitro* endpoints. Regarding genotoxicity, there is clearly a lack of data. Nevertheless, several studies do indicate that specific physicochemical properties and surface modifications of nanocellulose are associated with differences in inflammation potential and oxidative stress effects which may be driven by different underlying mechanisms of action.

At present it is hard to predict whether *in vivo* consequences can be expected if *in vitro*

inflammation responses are found. Only the subacute *in vivo* study with sulfated CNCs by Adewuyi et al. (2018) took this endpoint into account by measuring changes in antioxidant enzyme activity and performing immunohistochemical analysis. This study showed indications of oxidative stress (by changes in antioxidant enzyme levels) and inflammation (by higher expression of iNOS and COX-2). Therefore, there is a lack of *in vivo* evidence whether (specific types or differently modified) nanocellulose could cause inflammatory effects. However, for various reasons positive effects found in *in vitro* studies do not necessarily lead to adverse effects *in vivo*. In some cases, this might be because early effects, such as ROS formation or certain interleukin expression, do not result in adverse effects. Effects found *in vitro* are not always relevant *in vivo*, e.g. systemic toxicity is unlikely to occur when *in vivo* intestinal absorption does not take place. In addition, *in vitro* effects could be artifacts due to impurities or experimental design issues (e.g. the use of very high doses, insufficient dispersion or (other) interferences with *in vitro* test systems). Still, it is recommended that future *in vivo* studies should also take immunotoxic effects into account.

The overview in Table 4 shows that different types of (nano)celluloses can have different toxicological effects *in vitro*. However, most studies only compared two or three different types of nanocellulose. Some studies compared nanocelluloses of different sizes and others compared effects of different surface modifications. In addition, different studies used different assays or used different biomarkers (e.g. different gene expression-based immune markers). This is further complicated by missing data on impurities, such as endotoxin levels, which could impact on immunotoxicological markers. Owing to the heterogeneity of materials, characterization levels and assays used, it becomes very difficult to conclude whether size, morphology and surface modifications are critical for the toxicological effects of nanocellulose *in vivo*. Nonetheless, when it comes to cytotoxicity, so far little evidence is available that different types have different cytotoxic effects. However, for ROS formation and inflammation responses there is some evidence that different types have different effects on these endpoints. But even among studies that used a range of well-characterized and well-dispersed nanocelluloses and with known and low levels of impurities (e.g. in Bhattacharya et al. (2017), Wang et al. (2019) and Weiss et al. (2021)), results of different studies are not in line with each other. Whereas the former two studies did find differences in effects between different types on immune-markers or ROS formation, the latter did not. None of the *in vivo* studies compared different types of nanocelluloses. Therefore, it remains unclear whether any differences in effects between different types as observed *in vitro* also occur *in vivo*. To resolve this issue of whether and to what extent type matters for toxicity, there is a need for more *in vitro* studies performed similar to Wang et al. (2019) and Weiss et al. (2021), that use a wide range of well-characterized and well-dispersed nanocelluloses, ideally screened for effects in a range of different assays and at multiple concentrations.

Effects on nutrition absorption an nutritional balance

The current data provide some information on the potential impact of nanocellulose on absorption of nutrients. In general, soluble dietary fibers slow the absorption of fat and decrease glucose release in the GIT, which are considered beneficial in the treatment of obesity. Although nanocellulose is an insoluble fiber, it shows gelling behavior, and may therefore show similar effects as soluble dietary fibers. It is suggested that fibers at the nanoscale are much more effective in reducing the absorption of fats and adsorption of glucose, making it an interesting application in food industry (Li et al. 2021). It has indeed been shown that nanocellulose decreases fat absorption by preventing metabolism of triglycerides into fatty acids, which are small enough to be absorbed from the small intestine into the bloodstream (DeLoid et al. 2018). Further, cellulose and nanocellulose have a high glucose adsorption capacity, resulting in a decreased glucose release (Liu and Kong 2021). However, the research by Chen et al. (2020) and Liu and Kong (2021) has shown the possible side-effects of nanocellulose, i.e. decreased absorption of various nutrients, including vitamins and minerals such as calcium, potassium and sodium. In the long-term, this may potentially lead to deficits in essential

nutrients. It is noted that nutritional imbalance is also observed at high doses of conventional cellulose, which can cause a decrease in body weight gain (EFSA ANS Panel et al. 2018). However, for conventional cellulose these effects occurred at dose levels of >1000 mg/kg bw/day, whereas effects by nanocellulose were already observed at 30 mg/kg bw/day (Chen et al. 2020; EFSA ANS Panel et al. 2018). To make it more complex, current data are conflicting, as shown by Guo et al. (2021) who demonstrated an increased uptake of glucose from digesta of starch solutions in the presence of nanocellulose, measured in an intestinal epithelium *in vitro* model. These findings demonstrate an impact of nanocellulose on uptake of nutrients. Nutrient uptake in the GIT is normally not included in toxicity studies but needs further consideration to enable assessment of the impact in the long term.

Future perspective

Apart from a specific fermented coconut gel (i.e. 'nata de coco'), which is not a Novel Food due to its long history of use, there are, to our best knowledge, no current applications (or requests for allowance with the authorized EFSA Panels) of nanocellulose as functional or novel food, food additive or food contact material. However, considering the current speed of research and development, it may be expected that nanocellulose will be applied in food or food-related products in the near future. At least in the EU, such applications of materials will have to be assessed for safety for the above-mentioned applications according to the EFSA guidance (EFSA Scientific Committee et al. 2021a, b). This would automatically imply that certain specific studies or information will be required, such as (but not limited to) physicochemical characterization, *in vitro* digestion, genotoxicity, and cell toxicity studies, *in vivo* studies directed at ADME and (sub)chronic effects. Such information will be critical to ensure the safety of new products with nanocellulose. To allow for a reliable hazard and risk assessment of nanocellulose and to be able to act on the current knowledge gaps, we formulated the following recommendations for future research.

Recommendations

As indicated above, availability of *in vivo* toxicity data on nanocellulose is limited. Further research, especially chronic studies, would be needed to fill the data gaps that hamper conclusions on the potential hazard and risks of nanocellulose for humans upon oral exposure. From future (toxicological) studies the following information is required, taking into account several practical issues.

Key knowledge gaps for safety assessment:

- Early effects. It is recommended that future *in vivo* subchronic toxicity studies with nanocellulose include markers for early effects such as inflammatory responses, oxidative stress and immunotoxicity. Such studies may help to link early and late (adverse) effects and would provide better insight in the responses to nanocellulose.
- Types and surface modifications. Specific types of nanocelluloses (e.g. CNC, CNF and BNC) are often specifically modified to achieve certain functions. Such modifications also impact the behavior and toxicity of nanocellulose. Studies that compare different types of nanocellulose, unmodified and modified nanocellulose, or different kinds of modified nanocelluloses are needed to provide more insight into the effect surface modifications on the toxicity of the different types of nanocellulose.
- Absorption. Information on the (possible) absorption of nanocellulose from the GIT is limited. Due to its smaller size or surface properties absorption of nanocellulose cannot be excluded. Absorption is a prerequisite for direct *in vivo* effects and a key aspect for the safety assessment of nanocellulose. Therefore there is a clear need for data on *in vivo* absorption of nanocellulose.
- Effects on nutrient absorption. Also few data are available on the effect of nanocellulose on absorption of other components, such as fat, glucose and vitamins.
- Genotoxicity. Given the lack of data regarding the (potential) genotoxic effects of nanocellulose, there is a clear need for further studies directed at this endpoint.

Practical issues:

- Purity and impurities. Information on the purity and impurities of nanocellulose materials is of high importance for reliable testing. Nanocellulose materials are usually produced from natural sources such as wood pulp and may include impurities, both chemical (organic as well as inorganic) and microbiological (e.g. endotoxins), that can attribute to toxic effects found (Liu et al. 2017). Hence, information on any impurities is essential for generating reliable data, especially for markers that are sensitive to such impurities (e.g. immunotoxicity).
- Reference materials. Studies on nanocellulose would benefit from the identification and testing of reference materials (as were developed for other nanomaterials). This would better facilitate the comparison of study outcomes from different studies/assays and labs. The development and use of such reference materials is highly recommended.
- Dispersion. The dispersion state of nanocellulose in exposure mediums is likely to affect the outcome of *in vivo* and *in vitro* studies. Therefore, it is key to make sure materials are properly dispersed and that the dispersion procedure is described in detail. Dispersion by applying vortex and characterization after dispersion are a minimal requirement. Recommendations on dispersion are included in the EFSA guidance (EFSA Scientific Committee et al. 2021a, b).
- Controls. In addition to testing different types of nanocellulose and comparison to conventional cellulose, also positive and negative controls are to be used in *in vitro* assays. Although this may be obvious, there are still studies that did not include positive and negative controls. In many *in vitro* assays using cell systems, such as in genotoxicity assays, it is key that nanocellulose is taken up by cells. Therefore it is important to experimentally verify that this uptake takes places. Information on the total fiber content (including cellulose) of the diet should be provided.
- Measuring in biological matrices. Detecting and quantifying nanocellulose is analytically and technically challenging as it cannot be easily distinguished from the biological matrix. Labeling nanocellulose with specific markers might help to overcome this. However, such markers might change intrinsic physicochemical properties or detach from the nanocellulose which would lead to artifacts in measurements of nanocellulose in biological matrices.
- Interferences with *in vitro* test systems. The dispersion and dose levels tested should avoid interferences with *in vitro* assay.

Conclusions

The currently available toxicity data are insufficient to draw conclusions on adverse effects of nanocellulose in humans via oral exposure. The toxicity data, especially from *in vivo* studies, are limited, and the outcomes of *in vitro* and *in vivo* studies are not always in line with each other. Also, based on early effects in the subacute toxicity studies, some indications for adverse effects in the subchronic studies might have been expected, but were not observed. The interpretation of studies is complicated by the diversity in morphologies and surface modifications of nanocellulose, the lack of standard reference materials, controls, issues related to dispersion, the limited knowledge about absorption (and kinetics), analytical difficulties in biological matrices, and the possible presence of impurities and interferences within biological assays. The available subchronic toxicity studies suggest the safety of two specific nanocellulose materials, but might have missed certain early effects. Such effects have been observed to some extent in subacute *in vivo* studies. In general, *in vitro* studies show no cytotoxicity, but several studies indicate that different nanocellulose types or surface modifications may result in different effects in oxidative stress and immunotoxicity. Regarding genotoxicty, there is a lack of data. In addition, there are indications that nanocellulose can affect the absorption of nutrients. The current overview indicates that for the safe-by-design (Dhali et al. 2021; Shatkin and Kim 2015) or the safety assessment of nanocelluloses used in food or food-related materials, attention needs to be paid to complete these information gaps taking the above mentioned recommendations into account.

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