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REVIEW

Toxicological and Safety Considerations of Nanocellulose-Containing Packaging Materials

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ABSTRACT: The global demand for renewable and sustainable non-petroleum-based resources is rapidly increasing. Lignocellulosic biomass is a valuable resource with broad potential for nanocellulose (NC) production. However, limited studies are available regarding the potential toxicological impact of NC. We provide an overview of the nanosafety implications associated mainly with nanofibrillated cellulose (CNF) and identify knowledge gaps. For this purpose, we present an analysis of the studies published from 2014 to 2025 in which the authors mention aspects related to toxicity in the context of packaging. We also analyze the main methods used for toxicity evaluations and the main studies about toxicity evaluation using different biomarkers for a broad interpretation. This comprehensive biblio-graphic review highlights the critical need for further research to elucidate the mechanisms fully underlining NC toxicity, mainly due to its nanofibrillar structure. We focus on the cellular responses across different evaluated cell types through *in vitro* evaluation, always within the context of the dose used, the type of material or its source, and the type of biomarkers used in the assessments. The importance of addressing safety considerations and key knowledge gaps for the responsible use of CNF derived from lignocellulosic biomass and its bionanocomposites in food packaging is highlighted.

KEYWORDS: Cytotoxicity studies; nanofibrillated cellulose; *in vitro*; nanosafety; packaging; toxicity

1 Introduction

1.1 NC: A Promising Green Nanomaterial

Plastic waste and its environmental repercussions have increased awareness and the development of biodegradable materials as sustainable alternatives. Global demand for renewable, non-petroleum-based products is growing due to concerns over pollution, climate change, and energy crises [1,2]. The consumer demand for natural and environmentally friendly packaging products has emphasized the need for developing materials and additives sourced from natural resources such as cellulose-based materials [3,4]. The concept of "sustainable packaging" has gained recognition in academic literature as an alternative that minimizes environmental impact and promotes circular bioeconomy principles [5,6]. A sustainable material must adhere to safety regulations, economic considerations, and quality standards throughout the product life cycle [7,8].

Lignocellulosic biomass—comprising organic plant materials like agricultural residues—offers significant potential for reuse as a high-value feedstock because of its abundance, rapid generation, and low cost [4,9]. This biomass primarily consists of varying proportions of cellulose, hemicelluloses, and lignin.

Annually produced lignocellulosic biomass totals around 1011 to 1012 t [10], providing a substantial source of cellulose recognized for its biodegradability and versatility [3]. Cellulose exhibits advantageous properties such as chemical inertness, strength, low density, and surface modification capabilities [11–13], incentivizing the development of new materials [14,15]. In this context, nanotechnology could enhance bio-based materials in the forest products industry.

Cellulose is a lineal homopolymer with a high molecular weight composed of ß-1-4-linked D-Glucose units [1]. It is a relatively low-cost material with high availability and renewability [16]. In fiber walls, the cellulose chains form elemental fibrils, the smallest structural unit in the fiber (36 cellulose chains), which form an ordered structure of larger microfibrils surrounded by hemicelluloses and lignin. The microfibrils present crystalline and amorphous regions along its axis (Fig. 1).

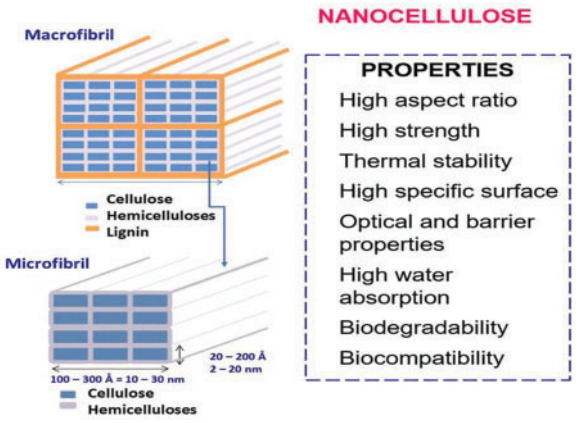

Figure 1: Structure of plant-derived NC and overview of its main properties. Adapted with permission of Area (2019) [17]

When talking about lignocellulosic biomass, the term nanocellulose (NC) typically involves nanoparticles of different sizes and shapes, like cellulose nanocrystals (CNC), cellulose nanofibrils (CNF), cellulose microfibril (CMF), and also the ligno-NC (LNC), which has a proportion (>1%) of residual lignin. NC can be extracted from various sources such as wood, seed fibers, sugarcane bagasse, and other types of fibers (e.g., flax, hemp), marine organisms, or algae. Although these nanomaterial types share the same chemical composition, variations arise due to differences in particle size, morphology, and crystallinity [18]. On the contrary, bacterial NC (BNC) is not derived from lignocellulose biomass but is bioengineered by specific fungi, bacteria, or invertebrates [19].

NC production begins with an initial stage where lignocellulosic or cellulosic fibers are obtained by mechanical (refining), chemical (alkaline or organosolv pulping, bleaching), biological (enzymatic), or combined treatments [20]. In subsequent stages, the obtained fibers, containing cellulose and a variable amount of lignin and hemicelluloses, are deconstructed to the nanoscale applying mechanical (nanofibrillation by high-pressure homogenization or grinding), biological (enzymatic), chemical (TEMPO-oxidation, carboxymethylation), or a combination of these treatments. CNF is acquired through chemical, enzymatic, and (or) mechanical disintegration processes from plant-derived cellulose, whereas CNC is isolated via chemical acid hydrolysis [21].

NC exhibits distinct attributes owing to its minute size at the nanoscale (1 nm = 10−9 m), fiber structure, and expansive surface area (Fig. 1), which give it attractive properties such as high strength, stiffness, and

surface area [22]. LNC retains some surface properties and interfacial interactions of lignin and has distinct applications [23].

1.2 Types and Applications of Cellulose Nanomaterials in Packaging Application

NC offers a versatile resource with multiple applications in various industries, e.g., biomedical, food engineering and packaging, cosmetics, electronic devices, environmental, civil engineering, and high-performance materials [13,20,24].

Paper and paperboard are commodities in the packaging market, constituting around 30% of the global market [25]. Paper is a cellulosic fiber network with a porous structure and hydrophilic surface, limiting its packaging applications due to reduced gas, water, and grease barrier properties. Currently, one or multiple coating layers are applied onto the paper substrates to improve barrier properties for food packaging using petroleum-based polymers or metallization as non-biodegradable layers. However, biodegradable bio-based polymers are abundant, renewable, and sustainable alternatives to conventional coating. The unique properties of nanocellulose materials have attracted significant attention in recent decades in the paper industry, evidenced by an increase in scientific publications, patents, and the upscaling activities of several companies.

NC has numerous applications in packaging products but is primarily used to reinforce polymeric matrices to create nanocomposites with enhanced mechanical properties because of its high tensile modulus, strength, surface area, aspect ratio, and good film-forming properties [21,26,27]. Despite its exceptional properties, there are still challenges, such as scaling up production, compatibility with other materials, and high manufacturing costs [28].

The most significant benefit of this material is that it can be obtained from renewable resources using methods that are not overly complex. However, some challenges and disadvantages still need to be addressed [29,30]. Diverse investigations have resumed these aspects, and the advantages and disadvantages in a broad context of the use of NC in this field have been published [6,31]. Nevertheless, the research on NC application in food packaging is a topic of current interest in the scientific literature. NC utilization in packaging includes films, paper, and coating. Fig. 2 presents the main preparation methods in NC-based packaging and current research on this topic.

NC films for food packaging can be enhanced by integrating composites with materials like lignin nanoparticles, polyvinyl alcohol, chitosan, and graphene oxide to improve mechanical strength and water vapor barriers. Relevant attributes of food packaging include antibacterial and antioxidant properties, often achieved by incorporating plant-derived essential oils such as cinnamon and oregano. While these oils are effective antioxidants, they do not perform well as antibacterial agents [32]. Metallic compounds like copper can enhance antibacterial efficacy without the toxicity concerns related to silver nanoparticles [33]. Nanopapers are ultra-light, flexible, strong, and thermally stable materials produced by vacuum filtration and casting. They can be impregnated with solutions to impart antibacterial properties [34].

Currently, NC-based packaging must overcome some challenges for its market application: (i) flexibility of NC films, (ii) durability and stability of the antibacterial property, (iii) adjustment and control of the barrier property, (iv) stability of the chemical structure of NC to improve its combination with other substances, (v) migration of antimicrobial compounds, (vi) nanosafety evaluations (e.g., cytotoxicity, genotoxicity, or migration risk), (vii) compression properties and resilience of aerogel, (vii) poor water resistance and poor water, oil, and gas barrier of the nanopaper.

NC can be integrated into paper packaging as a reinforcement agent in different ways, like in bulk, as a coating, or forming nanocomposites. In bio-nanocomposites, NC improves biopolymer stiffness and

tensile strength (starch, polylactic acid, polyhydroxyalkanoates, and others). Usually, an NC content of 4% of nanocomposite allows a continuous network in the biopolymer; higher content could harm the mechanical, water-vapor barrier and optical properties of the nanocomposites due to segregation (formation of OH bonds) between nanoparticles in the matrix [35].

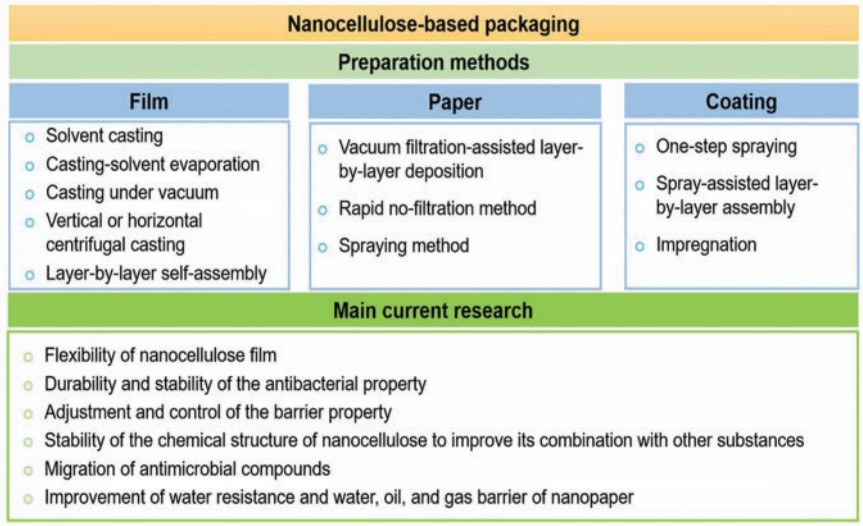

Figure 2: Main preparation methods in NC-based packaging and current research

Water vapor and oxygen barriers depend on the type of material, temperature, pressure, and relative humidity. Packaging should fulfill the moisture and oxygen transmission rate requirements for specific products. Numerous studies have shown NC as an enhancement additive of the water-vapor and oxygen barriers in paper packaging [36,37]. Other beneficial properties of adding NC to packaging have been recently developed, such as UV-barrier and antioxidant [38], active surface [39], controlled release [30], and others.

2 Scope, Objectives, and Methodology

The use of nanomaterials, their diverse applications, and the rapid advancement in nanotechnology have raised concerns about the potential effect on human and environmental health. Considering the rapid development of NC-based products and the novelty of the material, human health studies remain scarce [40]. Since new NC products are under investigation for packaging and may enter the market shortly, an extensive evaluation is necessary to ensure the human and environmental safety of the new material [41].

The increasing integration of nanotechnology into consumer products and medical applications requires a thorough understanding of their toxicity, particularly concerning direct human interactions through various exposure pathways. Fig. 3 shows a SWOT analysis (strengths, weaknesses, opportunities, and threats) highlighting the relevant aspects of NC use in packaging, emphasizing the area that aligns with the scope of the review.

This review focuses on the nanosafety aspects concerning NC use, focusing on toxicity evaluation through experimental assays. Initially, it provides a comprehensive list of representative studies based on searches conducted in the Google Scholar database using the following keywords: i) Nanocellulose-toxicity-packaging applications and, ii) Cytotoxicity-*in vitro* assay-nanofibrillated cellulose, including review articles and relevant cytotoxicity, immunotoxicity, and genotoxicity studies to contextualize findings within

a broader framework. The search was conducted for literature published between 2014 and 2025, focusing only on cellulose nanofibers (CNF) due to their potential relevance in cytotoxicity linked to fiber length [40].

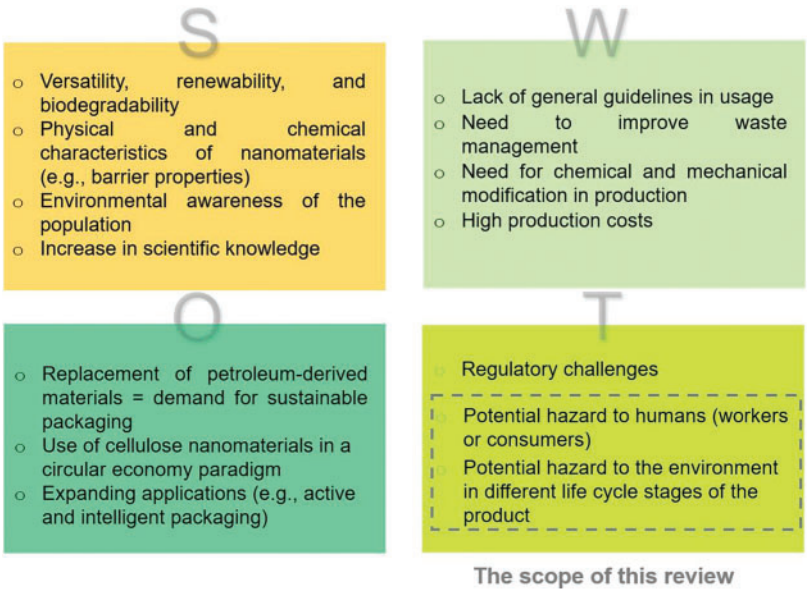

Figure 3: SWOT analysis, summarizing the main strengths, weaknesses, opportunities, and threats of NC-materials use in packaging, highlighting the area that aligns with the scope of this review

Besides, it presents an overview of the nanosafety implications regarding NC, identifying existing knowledge gaps. Then, it outlines different methodologies employed to determine toxicity while discussing pertinent issues related to packaging applications. Finally, this review synthesizes key insights from the analysis while highlighting future research directions for advancing our understanding of NC toxicity, especially in packaging applications, underscoring the relevance of prioritizing safety measures to mitigate potential risks to human or ecosystem health.

3 Results of the Search and Relevant Studies

Research on the toxicity of cellulose-derived compounds has grown significantly in recent years, driven by concerns about the impacts associated with various industrial products and processes. Researchers agree that this interest arises due to a confluence of factors, including increased environmental awareness, the development of new technologies, and the emergence of regulations.

Table 1 shows the review studies obtained following the first criteria explained in Section 2. Most articles about NC packaging applications included diverse safety aspects and implications associated with these innovative materials used in food packaging, such as biodegradability and toxicity aspects (e.g., cytotoxicity, genotoxicity). The table emphasizes the features related to nanosafety included by the authors in each study and remarks on some observations considered relevant to the main topic included in each research.

Table 2 shows a selection of relevant studies following the keywords cytotoxicity-*in vitro*-nanofibrillated cellulose. It includes the main results and the chief findings from each study. Many of them also evaluate the effects of CNC, and some results are mentioned. The studies involve the last ten years, but NC toxicity assessments date back to the past 20 years [42–44].

Table 1: Main studies obtained through the search of articles with the following keywords: Nanocellulose (NC)-toxicity-packaging applications

Safety assessments included in the paper	*Main topics*	*References*
No specific data.	Trends in NC packaging, highlighting the types of biopolymers with NC fillers used to form bio-nanocomposite materials.	Vilarinho et al. [11]
Consideration of NCs in antimicrobial food packaging materials.	Discovery and applications of the three forms of NC for use in bio-based packaging and coating for food stability.	Azeredo et al. [45]
Biodegradability, cytotoxicity, genotoxicity, and ecotoxicity references.	Various NC-based films, especially with oxygen and water vapor barrier properties.	Ferrer et al. [24]
End-of-life issues (recyclability, biodegradability).	Wide range of parameters related to the uses, general properties, barrier properties, and other attributes of the three forms of NC in packaging products.	Hubbe et al. [26]
Health-related uses: safety and biodegradability.	Methods of NC-based composite films and the effect of incorporating NC on the properties of packaging films.	Amara et al. [6]
Nanotoxicological and regulatory aspects.	NC materials in food packaging, including the principal patents involving NC in food packaging films.	Souza et al. [41]
No specific data.	Use of various forms of NC, including chemical-physical treatments for obtaining bio/polymers and their impact on the nanocomposite performance for food packaging applications.	Ahankari et al. [5]
Degradation issues.	Aspects of packaging applications (e.g., antibacterial and intelligent packaging) and cellulose-derived food package degradation.	Wang et al. [27]
No specific data.	Performance applications of different food packaging (e.g., film, coating, aerogel, nanopaper).	Wang et al. [46]
Safety of NC-based composite aspects.	Migration from NC composites and traceability of possible contaminants throughout the production chain emphasizes the need for further research.	Silva et al. [35]

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Table 1 (continued)

Safety assessments included in the paper	*Main topics*	*References*
Safety and biodegradability aspects.	Optimization of NC coating applications for packaging purposes.	Fotie et al. [47]
Toxicity and health hazards.	Sustainable packaging, e.g., NC-PLA (polylactic acid) and NC-chitosan biocomposites.	Antony et al. [48]
Cytotoxicity, genotoxicity, and ecotoxicity references.	NC application in packaging materials, cellulose resources, obtaining methods, and chemical modification.	Li et al. [31]
CNF and CNC toxicity.	Determination of the eventual adverse effects of CNF and CNC on the human body and the need for more research.	Lu et al. [49]
No specific data.	Some concerns regarding the commercial utilization of NC-based material in food packaging are due to a lack of regulatory standards and legislation.	Pradhan et al. [9]
Safety and regulatory aspects.	Application of NC in food systems, including NC in biocomposite packaging films.	Perumal et al. [50]
Security assessment of NC composite films.	NC preparation for packaging uses, focusing on methods for obtaining NC materials, properties, and related applications.	Xu et al. [21]
Biodegradability aspects.	NC-hybrid properties, including problems and advances in research. Characterize a “Package” product.	Dong et al. [51]
Safety of unmodified and modified form of NC.	Recent breakthroughs in NC and its derivates for innovative food applications. Regulatory aspects for NC-based food ingredients and food contact materials are mentioned.	Qin et al. [52]

Table 2: Relevant articles from the search with the keywords cytotoxicity-*in vitro*-nanofibrillated cellulose

Methodology	Biomarkers	Outcomes	References
Material: CNF gel (0.9 wt), CNF freeze-dried powder Concentrations: 1.5, 15 and 45 μg/cm^2 Cellular type: human epithelial alveolar cell line (A549)	Cytotoxicity (Trypan blue), oxidative stress (GSH and SH levels), and cytokine secretion	CNF caused a significant decrease in cell viability, more pronounced at 72 h of exposure. It is strongly correlated with oxidative stress response. Dose-dependent-response	Menas et al. [53]
Material: CNF from TEMPO-mediated oxidation of bleached *Eucalyptus globulus* kraft-pulp. Concentrations: 1.5, 3, 6, 12.5, and 25 μg/cm^2 Cellular type: co-culture of human alveolar epithelial (A549) and monocyte-derived macrophages (THP-1) cells.	MTT assay, LDH assay, Clonogenic assay, Comet assay, and Micronucleus assay.	Low CNF concentrations can stimulate A549 cell proliferation. Higher concentrations are moderately toxic, causing a cell viability decrease at 48 h (A549). No immunotoxicity effect was detected. CNF did not induce DNA damage in A549 cells but led to micronucleus formation at the two lowest doses.	Ventura et al. [54]
Material: four different size fractions of CNFs were obtained by taking samples after separation per time (from >50 μm to <1 μm).	Highest tolerated dose. Total protein content. RNA synthesis inhibition. AMES Test.	No change in cell viability or morphology was detected.	Pitkänen et al. [55]

(Continued)

Table 2 (continued)

Methodology	Biomarkers	Outcomes	References
		Some indications of cytotoxicity (growth inhibition and cell death after 24 h of exposure to 0.5 mg/mL of CNF). No genotoxicity effects were detected.	
Concentrations: 0.24, 0.12, 0.06,0.03, and 0.015 mg/mL. Cellular type: human cervix carcinoma (HeLa229) cell line.			
Material: CNFs of five different surface functionalizations (carboxymethylation, hydroxypropyltrimethylammonium substitution, phosphorylation, and sulfoethylation), with nonfunctionalized CNF: Concentration range: from 2.4 to 312.5 μg/cm^2 Cellular type: human bronchial epithelial (BEAS-2B) cells.	CellTiter-GloVR Luminescent Cell Viability Assay, Alkaline Comet Assay, Cytokinesis-block Micronucleus Assay, ROS formation.	All CNFs showed low cytotoxicity. Neither induces genotoxic effects. Nonfunctionalized and carboxymethylated CNFs increase ROS formation.	Aimonen et al. [56]
Material: three CMF/CNF samples from bleached *Eucalyptus globulus* kraft pulp produced by catalytic oxidation, enzymatic hydrolysis, or acid hydrolysis. Compared with two multiwalled carbon nanotubes (NM-401 and NM-402). Concentration range: from 1.5 to 50 μg/cm^2 Cellular type: human lung epithelial cells (A549).	Cell viability, ROS production, micronucleus assay.	No cytotoxic damage was detected. No genotoxic effect was detected, except CMF and NM401, which raise micronucleus frequency at the lowest and highest concentrations tested. No intracellular ROS increase.	Pinto et al. [57]

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Table 2 (continued)

Methodology	Biomarkers	Outcomes	References
Material: four CNFs (CNFs 50, 80, 30, and 25 nm) selected by NIEHS-NHIR Concentration: 50 µg/mL Cellular type: human intestinal epithelial cell line Caco-2 cells.	Cell monolayer permeability, Cytotoxicity assay, LDH assay.	50 µg/mL of CNC and CNF resulted in cytotoxic effects without affecting cell permeability. No disruption of monolayer integrity was detected.	Mortensen et al. [58]
Material: Wood-derived CNF unmodified and with different surface modifications. Concentration range: from 50 to 500 µg/mL Cellular type: Intestinal cells Caco-2 and the gut bacteria *Escherichia coli* and *Lactobacillus reuteri*.	Bacterial growth and colony forming units (CFUs) assay. Cytotoxicity assessments, cell morphology, and cell membrane integrity.	CNF samples inhibit the growth of *E. coli* but not of *L. reuteri*. The metabolic activity of cells was not affected. No signs of toxicity (membrane damage) were detected.	Lopes et al. [59]
Material: CNF from Norway spruce (*Picea abies*). Concentration range: from 31.25 µg/mL to 1 mg/mL	MTT assay, ROS detection, proliferation, and apoptosis assay.	None of the six concentrations of CNF induced cytotoxicity and oxidative stress in the L929 cells, nor induced necrosis and apoptosis of thymocytes and PBMNCs. MTT assay confirms a dose-dependent decrease in metabolic activity of L929 cells cultivated with the highest concentration.	Čolić et al. [60]

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Table 2 (continued)

Methodology	Biomarkers	Outcomes	References
Cellular type: L929 cells, rat thymocytes, or human peripheral blood mononuclear cells (PBMNCs).			
Material: bleached fibers of *Ananas erectifolius* Concentration: 5 mg/L Cellular type: Vero cells.	Direct and indirect contact assay and adhesion test.	No cytotoxicity and good initial biocompatibility for all the tested protocols (direct and indirect tests) due to the mild chemical treatments applied to the fibers.	Souza et al. [61]
Material: CNF from commercial, bleached sulfite softwood dissolved pulp, unmodified (U) and modified (M). (Carboxymethylated-CNF, hydroxypropyl trimethylammonium-CNF). Concentration range: from 50 to 500 µg/mL Cellular type: human dermal fibroblast, MRC-5 lung fibroblast, and THP-1 monocytic human cell lines.	Cytotoxicity test, Alamar Blue Assay, LDH assay, Inflammation assessment, and ROS production.	CNFs are not cytotoxic for immune, dermal, and lung cells. U-CNF promoted an inflammatory response suppressed when introducing surface charges on nanofibrils. No intracellular ROS increase was detected.	Lopes et al. [62]
Material: Two micro/NCs from *Eucalyptus globulus* kraft pulp, produced with a TEMPO oxidation pre-treatment (CNF) and enzymatic pre-treatment (CMF). Concentration range: from 5 to 165 µg/mL (MG-63) and 7.2 to 240 µg/mL (V79)	Metabolic capacity of viable cells (MTT) and clonogenic assays, cytokinesis-block micronucleus assay.	CNF and CMF showed cytotoxicity at the highest doses tested. All NC samples raise the frequency of micronucleated cells in MG-63. CNF induced micronucleus formation in V79 cells.	Ventura et al. [63]

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Table 2 (continued)

Methodology	Biomarkers	Outcomes	References
Cellular type: osteoblastic-like human cells (MG-63) and Chinese hamster lung fibroblasts (V79).			
Material: CNF from the banana rachis (BR-CNF) and Exilva (Borregaard Commercial-CNF). Concentration range: 0.025%–0.75% Cellular type: human epithelial colorectal adenocarcinoma cells Caco-2.	Erythrosine vital dye, Alamar blue, LDH enzyme activity, *in vitro* genotoxicity evaluation, or Comet assay.	No differences in cell viability were detected. The Alamar blue test indicated that BR-CNF was not cytotoxic. LDH showed little cytotoxicity at concentrations below 5% BR-CNF and 0.25% for commercial ones. No DNA damage was detected.	Mejía-Jaramillo et al. [64]
Material: CNF from *Bassia eriophora* Concentrations: 100 µg/mL and 200 µg/mL Cellular type: human mesenchymal stem cells (hMSCs).	Cell viability, cellular and morphology analysis, and JC-1 staining.	Cell viability and morphology were not affected. CNF does not cause mitochondrial membrane potential on hMSCs.	Athinarayanan et al. [65]

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Table 2 (continued)

Methodology	Biomarkers	Outcomes	References
Material: tempo-oxidized CNF (1), phosphorylated CNF (2), mechanically fibrillated CNF from bleached kraft pulp (3) and from citrus peels (4), and two fractions (coarse and ultrafine) of softwood bleached kraft pulp. Concentration: 100 µg/mL Cellular type: Rat alveolar macrophages (NR8383)	Cell viability and morphological changes, intracellular ROS, pro-inflammatory cytokines.	No effect on cell viability. No significant production of ROS or morphological changes. CNF 1, 2, and 3 increased the dehydrogenase activity and elevated proinflammatory cytokine. Cellular uptake was demonstrated.	Fujita et al. [66]

Note. AMES test: also known as the Bacterial Reverse Mutation Test, is used to detect the mutagenicity and genetic toxicity of chemical compounds; MTT assay: protocol for measuring cell viability, proliferation, and cytotoxicity; ALAMAR BLUE ASSAY: is widely used to investigate cytotoxicity, cell proliferation, and cellular metabolic activity; LDH ASSAY: is a method for determining cytotoxicity is based on measuring the activity of cytoplasmic enzymes released by damaged cells, Lactate dehydrogenase; JC-1 staining: JC-1 mitochondrial membrane potential assay is a fluorescence-based method to track the behavior of mitochondria; BAL: Bronchoalveolar lavage; CNF: nanofibrillated cellulose; ROS: reactive oxygen species, NIEHS-NHIR: National Institute of Environmental Health Sciences-Nanomaterials Health Implications Research.

4 Discussion

4.1 Overview of Nanosafety

Nanotechnology is becoming one of the most relevant tools for revolutionizing food science and industry. Many aspects related to the functionality and applicability of nanotechnology in food applications are on the rise worldwide. Cellulose-based materials are often considered biocompatible and non-toxic due to their natural origin. However, their nanoscale size may confer different properties, potentially associated with toxic characteristics. A decrease in particle size is associated with a possible penetration through biological barriers (e.g., digestive mucous membranes). For this reason, NC-containing materials have not yet been authorized as food additives or for food contact (e.g., packaging in Europe) [67]. Fig. 4 shows aspects related to the potential toxicity of nanomaterials, focusing on NC considering their application in packaging and based on human and environmental protection.

Due to the extensive range of NC applications and analyses of the existing literature throughout the life-cycle of human exposure routes, it is essential to identify the main human exposure routes for future research (Fig. 4). Inhalation (through the respiratory tract), dermal (through skin contact), and oral ingestions are the usual modes of human exposure [68,69]. Considering the type of applications, it is essential to investigate the possible major entry points into the human body to design future research.

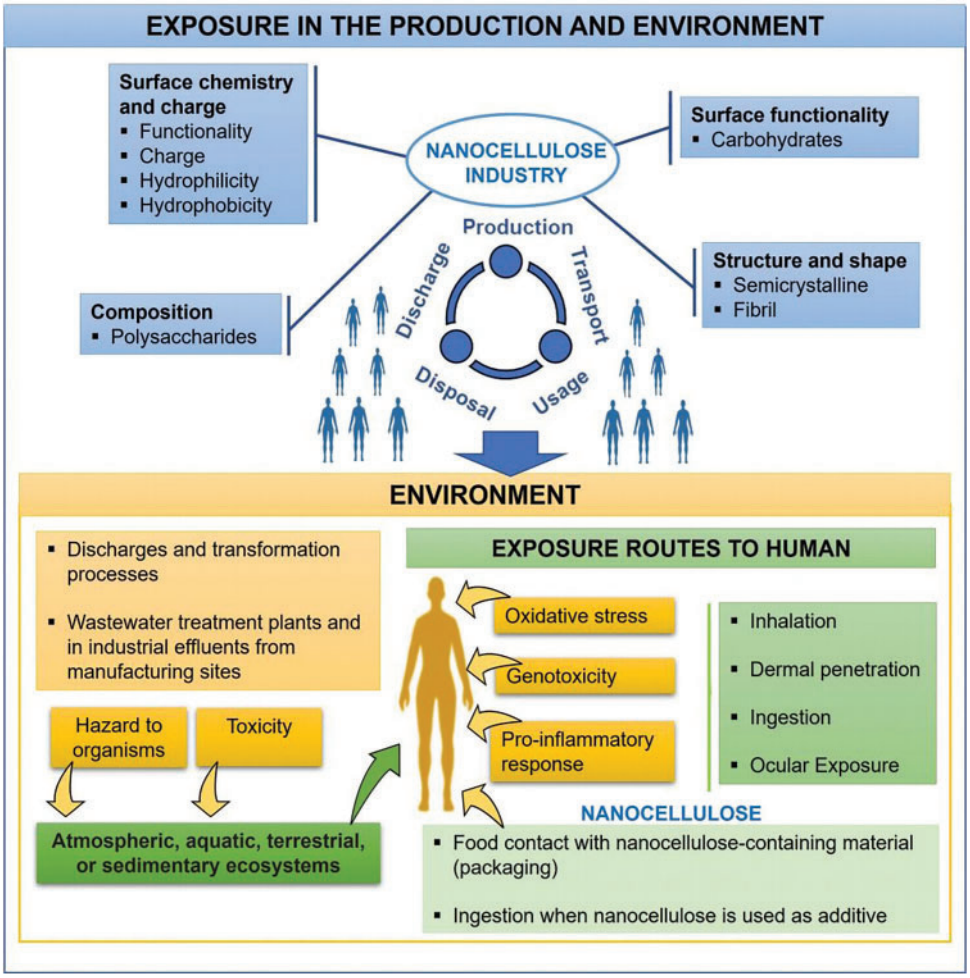

Figure 4: The potential human exposure routes in the production cycle of NC materials and main aspects of human and environmental health

Before the widespread commercialization of any product, it is crucial to detect the possible toxicological characteristics of NC to guarantee human safety. When considering its use in packaging, two aspects are relevant to nanosafety: toxicity and migration [21]. Migration refers to the transfer of substances from packaging materials into food. It plays a key role in the interaction between food products and their packaging. This interaction can occur in several ways: external migration of environmental contaminants into the food via the packaging, internal migration between different layers within the packaging material, or direct migration from the packaging into the food itself. Such processes may negatively impact the quality and safety of the food product [70,71]. In particular, the potential migration of nanomaterials is a concern when these materials are used in food-contact packaging [41]. However, reports on the migration testing necessary to ensure the safety assessment of nano-packaging materials are scarce [46,72]. Special attention should be given to migration in packaging films [21]. Table 1 shows that not all authors included tasks related to the migration process within the nanosafety aspect considered in their studies.

Evaluating cytotoxicity becomes relevant in studies of materials used in food science with applications such as food stabilizers, functional food ingredients, and food packaging [45,73]. Recent studies focus on developing active food packaging films with antimicrobial properties with NC as an additive [39,74,75].

Studies of NC as a food additive mention limited studies into the effects of NC on the gastrointestinal tract when used in food [76,77]. Long-term impacts, including effects on gut flora and nutrient absorption, require further investigation to ensure human safety [78].

An in-depth examination of research on NC toxicity has revealed a noticeable and growing body of studies [40,67,79]. However, all authors highlight the emergent nature of this field and underscore the need for further research to comprehensively understand the potential risks and implications associated with using NC in various applications. Some reviews concentrate on specific exposure routes, such as oral [67,80] or respiratory [81]. Oral or dermal exposure to CNC has demonstrated a lack of adverse health effects when compared to pulmonary toxicity [68]. Some *in vitro* and *in vivo* studies on the biological impacts of CNC and CNF concluded that they have limited toxic potential under realistic doses and exposure scenarios [79].

Manufacturers and users of NC need to stay informed about relevant regulations and guidelines, conduct appropriate risk assessments, and ensure compliance with applicable safety standards. CNF, CNC, and BNC have no specific directives for production and use as food ingredients [77] and have not yet been approved for food contact by the US Food and Drug Administration (FDA). In the European Union, the Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH) regulates the risks associated with chemicals or new substances to human health and the environment. REACH places responsibility on manufacturers and importers to ensure the safety of the substances they produce or bring into the Euro-pean market [82]. The European Commission has published extensively on specific requirements for nanoforms of materials, including technical reports and guidance on the implementation of these recommendations [83–85]. Several reviews have also been published recently on guidance on risk assessment of nanomaterials in the food and feed chain [86,87].

Although cellulose and derivates are considered safe, specific considerations may arise depending on the NC size, shape, surface chemistry, and intended use. It was suggested that bulk and nano forms of a material with the same chemical composition could be treated as the same substance in the context of REACH [88]. However, nanomaterials necessitate specific regulatory measures due to their unique physicochemical properties and potential associated risks. The safety of products containing NC should be analyzed case-by-case, considering the particular type of NC, potential exposure routes, and other relevant factors. NC has not yet been approved for food contact applications [52].

In the European Union and The United States, the specific exposure metrics for NC remain to be defined. China is engaged in the regulation of nanomaterials through the National Medical Products Administration (NMPA) and the Ministry of Ecology and Environment (MEE). There is agreement that currently available toxicity data are insufficient to determine the harmful consequences of NC oral exposure to humans [80].

On the other hand, there is a lack of information about the NC Occupational Exposure Limits Value (OEL) via inhalation or dermal exposure due to the small amount of data available regarding its risks [40,89,90]. Based on the precautionary principle, the 8-h OEL value has been suggested to be 0.01 fibers/cm^{-3}, the same as carbon nanofibers, due to the potential biopersistence of NC when inhaled [40,90].

The European Commission recently recommended a guide on the Safe and Sustainable-by-Design (SSbD) as a framework and criterion for design processes of chemicals and materials safe for human health and the environment with a sustainable life cycle [91,92]. The dimensions considered by the SSbD include health and environmental safety while also reflecting contemporary economic and social contexts.

Skin cosmetics products that include NC as a filler in their formulations currently researched by SSbD should agree with the REACH regulation [92,93].

Finally, a thorough understanding of the life cycle of a nanomaterial is imperative to comprehensively assess the potential risks to human health and the environment, adopting a holistic perspective [92]. The

life cycle encompasses various stages, starting from the initial production and manufacturing processes, extending through transportation, consumer utilization, and concluding with disposal methods or end-of-life [79]. Examining these stages is crucial for identifying and evaluating potential hazards, exposure pathways, and environmental impacts related to the entire life cycle of NC-associated materials. This holistic approach ensures a more robust and accurate assessment of the overall risk profile, facilitating the development of effective risk management strategies and informed decision-making. A recent review examining various aspects of NC production processes and life cycle assessments highlights key areas impacting the environmental performance of NC while also addressing considerations related to the end-of-life phase of the products [94]. The life cycle risk assessment (LCRA) is a methodology that identifies several stages during any manufactured nanomaterial life cycle. This method identifies and assesses potential risks from occupational, consumer, and environmental exposure throughout the product life cycle [95]. Fig. 5 summarizes the main aspects of each criterion considered in the LCRA and SSbD of NC.

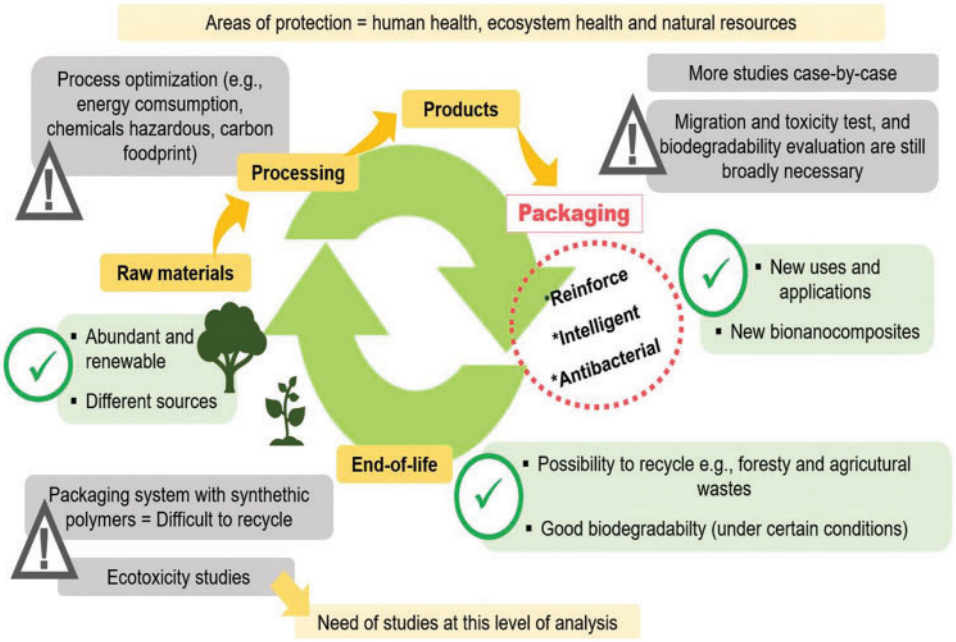

Figure 5: Analysis of steps included in the Safe and Sustainable-by-Design and the Life Cycle Risk Assessment of NC use for packaging applications, including positive and negative aspects and alert claims in each step

The SSbD and life cycle analysis of products derived from NC should be optimized for cradle-to-gate and cradle-to-grave assessments. Some strategies have been proposed for reducing energy demand and developing a low-carbon economy for the products. Currently, only laboratory data are available, so it is necessary to evaluate this process at the industrial level to assess the environmental impact of cellulose products on various aspects, such as air emissions, human health, and waste discharge. Among the final disposal options for these products (e.g., reuse, landfilling, recycling, combustion, storage), there is no data on the potential effects of each process. So, it is relevant to investigate and predict environmental consequences [96]. Furthermore, there is advice about the need for reagents used in cellulose and NC extraction process optimization to avoid environmental damage [27].

4.2 CNF Toxicity in Packaging Use

A considerable body of research supports the notion that wood-based nano-sized materials like NC, in various forms, are generally safe or non-toxic to humans [40,79]. However, due to their different characteristics, toxicity testing results are considered valid only within the specific context of each cellulose material, which has to be assessed case by case [55]. Factors such as particle size, shape, surface chemistry, and the biological model employed can influence the toxicity profile of nanomaterials [97,98]. Concerning size, most researchers indicate that smaller size (<50 nm) nanomaterials can produce severe nanotoxicity compared to bigger sizes (>50 nm) [98]. For a full toxicity assessment, it is essential to incorporate a comprehensive morphological characterization of nanomaterials using available instruments and methods (e.g., TEM, AFM), including a multi-scale characterization in which the authors can assess key physicochemical properties like size, shape, aspect ratio, surface area, agglomeration state.

Some factors, like the physicochemical features of the nanomaterial, e.g., thickness and length, as well as biopersistence, are determinants in the toxicity evaluations of CNF [19,56,99] (Fig. 6). CNFs have a long, entangled fibrous structure with a high aspect ratio and lower crystallinity [52]. The fiber paradigm has been adapted to evaluate the toxicity of high aspect ratio nanomaterials such as asbestos fibers. This model emphasizes factors like nanoscale dimension with a high length-to-diameter ratio, and biopersistence in biological environments, which can potentially affect the induction of chronic inflammation and fibrosis due to the incapacity of macrophages to phagocyte them [100].

Furthermore, as with other types of nanoparticles, it is essential to develop comprehensive strategies, including the optimization and standardization of safe and efficient synthesis protocols, identification and validation of sensitive and specific biomarkers of nanotoxicity, and development of protective measures for workers involved in the production and application [101]. For example, some pretreatments applied before NCF fibrillation (e.g., carboxymethylation and TEMPO-mediated oxidation) can influence their biological interaction and effects [67]. Aimonen et al. [56] highlight that few studies compare the toxicity of CNFs derived from the same resource but with different surface chemistry. Fig. 6 mentions the main determining factors and assessments for CNF toxicity analysis.

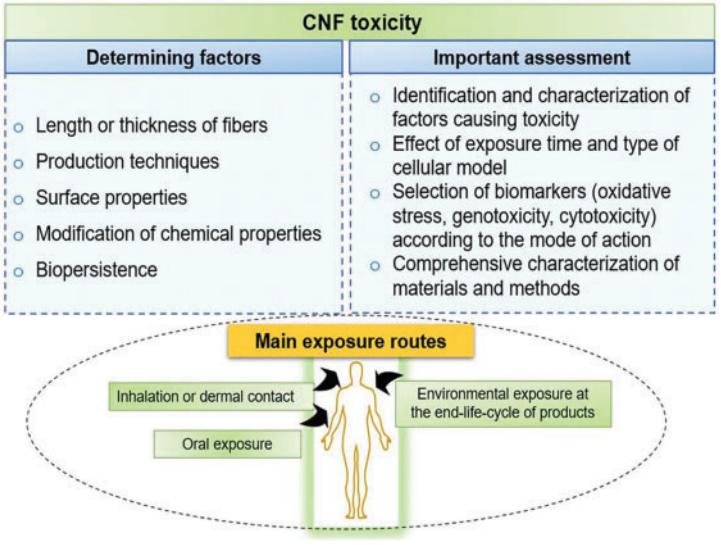

Figure 6: Featured considerations on CNF toxicity for its use in packaging

Upon contact with biological systems, CNFs can interact with cellular membranes and internalize through endocytic pathways, potentially leading to the generation of reactive oxygen species (ROS), lysosomal destabilization, and the activation of pro-inflammatory signaling pathways, all of which are mechanisms commonly associated with nanomaterial-induced toxicity. Overall, according to the main exposure way (Fig. 6), it is expected that the effect on the respiratory system is mainly manifested in oxidative stress, inflammation, epithelial damage, and genetic changes [102]. Considering oral exposure, CNFs can interact with the gastrointestinal tract, causing local inflammation, disruption of epithelial integrity, and gut microbiota disruption.

Assessing regulatory considerations is crucial before integrating NC into the food sector for NC-based materials commercialization. Food packaging materials must meet stringent requirements to protect and preserve food quality and safety. Even without a specific section about nanomaterials safety, all references follow the paradigm of sustainable packaging, indicating that the nanomaterials used for this application should not only consider safety during their use but also in their storage, transportation, market, and other options during their life cycle [5,27]. A recent study proposed that future packaging materials should incorporate essential attributes summarized by the acronym "PACKAGE": Property, Application, Cellulose, Keen, Antipollution, Green, and Easy [51]. The significance of PACKAGE lies in its potential to address the critical issues associated with traditional, non-degradable plastic packaging.

On the other hand, environmental issues are a chief part of a safety evaluation but remain poorly studied [90]. Table 1 shows that while many studies focus on safety implications such as biodegradability and toxicity, few address environmental factors such as ecotoxicity evaluations, a relevant assessment of the packaging utilization, and their end-of-life options [26].

In the packaging field, there is an increasing focus on nanocomposites. These innovative materials involve NC integrated with different substances to enhance and tailor specific properties. This combination allows for advanced packaging solutions that offer improved mechanical strength, barrier properties, and sustainability, aligning with the growing demand for eco-friendly and high-performance packaging materials. Some studies conclude that their toxicological aspects evaluation (e.g., cytotoxicity) is still incipient [48,52].

Incorporating reinforcing agents into biopolymers involves chemical and structural features that enable their commercial application [5,48,103]. Biodegradable polymers such as Polylactic Acid (PLA) and polyhydroxyalkanoate (PHA) have potential applications in food packaging and the potential to replace conventional plastics [31,104]. Other materials like chitosan, which has antimicrobial properties, or proteins obtained from animals and vegetables, can be modified and processed to complement packaging and provide greater barrier capacity [48]. There are abundant references regarding its uses, properties, and preparation methods [21,72]. Surface modification of NC can impart new beneficial properties, increasing their applicability. However, different functionalizations will determine differences in physical and chemical parameters (aggregation rate, hydrophobicity, surface charge, surface chemistry) and could also cause an impact on biological responses [19]. Some modifications of NC could make it less biodegradable or non-biodegradable or result in toxic or dangerous compounds [47].

4.3 3Rs Statement in Toxicological Evaluation

Nanotoxicology studies the toxicity caused by nanomaterials and become an emerging field of research. The assessment of these compounds relies on toxicological studies. They can use either *in vitro* models, where biological experiments occur in an artificial environment such as a test tube, or *in vivo* studies, where biological experiments use whole animal systems as the route of exposure and for evaluating effects [105]. There are fewer *in vivo* investigations than *in vitro* ones [40]. Generally, *in vitro* assessments are less

mandatory than *in vivo* studies to identify potential clinical trials in humans, but both are complementary. Also, given the recent emphasis on refining and reducing animal-based testing strategies, with the eventual goal of phasing them out over time, there is an urgent need to develop alternative testing models, e.g., *in silico* models.

Nowadays, the principles of the 3Rs implicate new alternative methods, which are emerging as trending topics in future investigations [106]. These approaches, which center on reduction, refinement, and replacement, seek to meet scientific goals while reducing the number of animals used in research. Fig. 7 presents an overview of the main aspects of the different testing models.

Computer simulations, which rely on *in silico* testing models, offer an alternative to complement experimental approaches [107,108]. However, it presents some difficulty in predicting the consequences of the interaction between nanomaterials and living matter [77].

The *in vitro* triculture small intestinal epithelial model is a methodology that provided substantial information on the potential cellular responses to ingested NC under relevant physiological conditions, as demonstrated by Cao et al. [109], and is likely to determine nano-bio interactions [110,111]. Multidimensional cellular models such as co-cultures and 3D cultures can help to enhance the reliability of hazard evaluation of CMs and their potential risk to human health. This type of study is relevant in future investigations about NC applications in the food industry and their implications for human health.

The cytotoxicity, which can be analyzed *in vivo* and (or) *in vitro* studies, involves enzymatic activity leaked from the damaged cells or using stains that only remark the damaged cells as an indicator through microscopic examination [105]. Results of cytotoxicity, viability, and impact on mammalian cell morphology seem to be prevalent in the current literature [109,111,112]. For a proper results interpretation, it is crucial to consider exposure systems (cell types), dosage, and NC type/treatment/origin and ensure a clear and thorough material characterization. Cytotoxicity is also a parameter with conflicting conclusions [68,90]. The subsequent part of this review shows a selection of relevant studies on this subject.

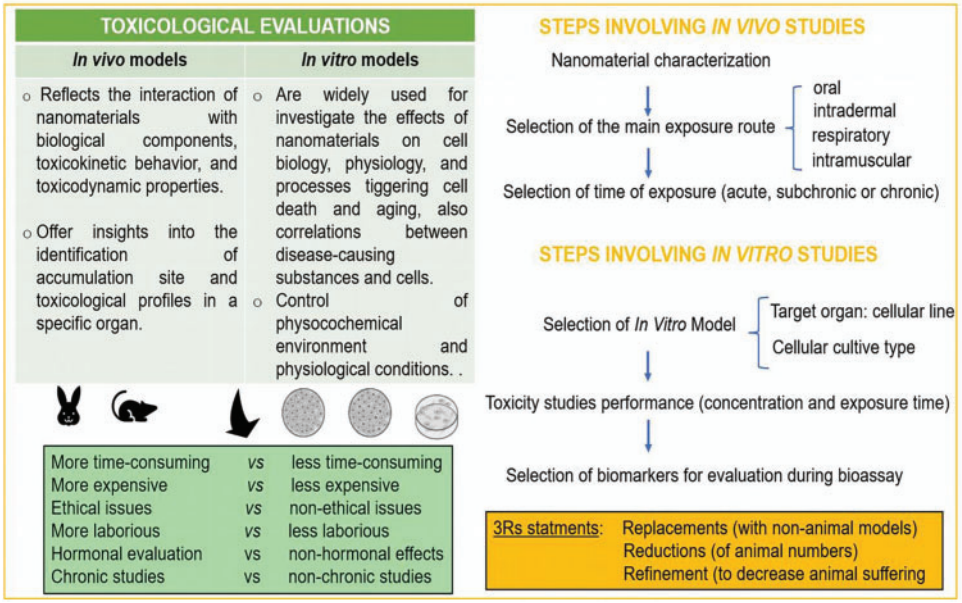

Figure 7: Key aspects of usual methods in toxicological evaluation in agreement with the 3Rs statement

Most outcomes concerning cytotoxicity, viability, and the impact on mammalian cell morphology that appear widespread in the current literature are benign [44,82]. The selection of the cell type for the assays depends on the toxicity of toxicants on the organ because each cell type has its function, responding in diverse ways to exposure to a specific nanomaterial [97,113].

The cytotoxic and genotoxic evaluations are necessary due to their association with carcinogenic effects. Nanomaterials can potentially induce genotoxic damage by direct interaction with DNA, by disturbing the process of mitosis, or by inducing reactive oxygen species production. When choosing an appropriate set of *in vitro* genotoxicity tests, the three chief endpoints are gene mutation, structural chromosome aberrations, and numerical chromosome aberrations [114]. Some evaluation methods used are Comet Assay, Micronucleus Assay, Chromosome Aberration Assay, and Ames Test, which are widely used in toxicological studies [115]. Finally, multiple endpoints and multiple cell types are preferred in laboratory studies to avoid false-negative results. Regarding genotoxicity tests, there are also evident discrepancies in the findings, as shown in Table 2 and described below.

Less addressed in the literature, immunotoxicity evaluates the effect of NC on the immune system. The biocompatibility of NC is related to monocyte/macrophage system response, so several *in vitro* model systems are used to delineate this interaction [16]. Most studies used mouse and human monocyte-derived macrophages [116,117]. Lopes et al. [62] assume that the inflammatory responses to CNF presence are due to material surface chemistry, thereby opening the possibility of designing safer materials. Despite some studies not evidencing inflammatory effects or cytotoxicity, contradictory effects highlight the need for additional evaluation, so inflammation as an immune defense mechanism requires further research [44].

The ecotoxicity evaluation of nanomaterials, relevant when using NC in packaging [118], is crucial for understanding their potential impact on individual organisms and entire populations [119]. These assessments employ various organisms suitable for simple and rapid ecotoxicity testing, with a recent trend toward reducing the use of vertebrate organisms. Ecotoxicity tests serve as tools to assess the risks associated with potential releases into the environment. Methods for evaluating the ecotoxicity of nanomaterials follow standardized tests outlined by organizations such as the OECD (Organization for Economic Cooperation and Development, ISO (International Organization for Standardization, EPA (United States Environmental Protection Agency) [120,121]. Boros and Ostafe [119] conducted a comprehensive review, highlighting commonly used organisms in ecotoxicological studies for assessing nanomaterial toxicity, which includes relevant criteria for selecting testing methods, descriptions of each organism, and the advantages and disadvantages of using them as a bioindicator.

The results obtained by the authors show a variety of responses for each biomarker (Table 2). Their interpretations do not allow for general assumptions necessary to achieve overall nanosafety. Even if the results vary, cytotoxicity seems not to be a result of the direct action of CNF on cells but rather due to secondary factors like endotoxin contamination, microbial contamination, adsorption of culture medium components to CNF, and aggregation or agglomeration and dispersion states of CNF [122]. The agglomeration is a common factor observed in related studies because it can alter the effective surface area and influence cellular uptake, potentially leading to misleading toxicity assessments.

The role of the size and shape of nanomaterials in the interaction with the gastrointestinal tract should be studied more to understand the potential for intestinal barrier dysfunction and its implications for ingestion [58]. The metabolic activity and integrity of Caco-2 cells could not be affected by CNF materials, even with different surface modifications. Although some concentration-dependent growth inhibition of *E. coli* could be observed when exposed to CNF materials, this was not the case for *L. reuteri* (Table 2). The bacterial growth in the gastrointestinal tract is an indicator of oral toxicity of CNF, and any changes in the gut microbiota have been related to gastrointestinal disease [59]. Furthermore, the size of nanomaterials plays a

critical role in their interaction with the gastrointestinal tract. Continued research is needed to understand how cellulose nanofiber (CNF) materials affect gut microbiota and intestinal cells. These interactions must be thoroughly examined when nanomaterials are intended for use in food-related products.

The phagocytosis or internalization of nanomaterial by a cell has a significant implication for both the cellular health and the biological fate of the material (e.g., biodistribution, degradation, and long-term accumulation within tissues). Menas et al. [53] found that CNC but not CNF particles were taken up by the cells. In other studies, the CNF uptake by cells occurs [56,66]. The last authors indicate that the chemical modifications of CNF and the fibrillation method employed can modify the direct contact with cells. Using TEM, Pinto et al. [57] detected phenotypic cellular changes (e.g., cytoplasmic or endocytic vacuoles presence, binucleation) in the exposed cells, providing evidence of particle internalization. Pitkänen et al. [55] selected the two finest CNF fractions (<10 μm) and reported no significant cytotoxic effects. Although many indicators suggest that the toxicity of nanofibers may be associated with their morphological characteristics, the specific molecular mechanisms underlying this toxicity still need to be elucidated.

Regarding cytotoxicity results, the cellular responses are diverse, highlighting the importance of validating the toxicity results only for the tested material, considering the structure, properties, and preparation methods. Some studies show signs of cytotoxicity demonstrated by some of the used biomarkers (Table 2) [53–55]. Conversely, other authors found non-cytotoxic effects nor oxidative stress [60,61,123].

Moreover, the human cell types chosen in each study are those most likely to be affected by potential exposure to CNF in occupational settings or by consumers [62]. They included immune surveillance cells and epithelial cells lining the skin and respiratory system, representing those most likely to be impacted by exposure routes to CNF [66].

Some authors indicate cytotoxic and genotoxic effects demonstrated by the exposure to CNF and CMF using different biomarkers [54,57,63]. The authors also suggested that differences in the DNA damage response of specific cell models highlight the importance of cellular models in genotoxicity studies. Some studies employ co-culture models, which are more realistic than monoculture [54,124] and also reveal different effects based on physicochemical properties. Likewise, a significant increase in micronucleated binucleated cells was induced by cellulose microfibrils produced by enzymatic hydrolysis [57]. Another critical task from this study is that CNC, unlike fibers, is not internalized into cells. The last authors also stated that the toxicity of the different cellulose nanomaterials (fibrils vs. crystals) is not comparable due to the distinct raw material origin, isolation techniques, processing/manufacturing techniques, drying processes, and others. This assessment is also supported by Menas et al. [53]. The potential for low CNF concentrations to induce cell overgrowth and genotoxicity raises significant concerns regarding occupational exposures and their implications for human health risk assessment [54]. The need for further studies targeting other genotoxic endpoints and cellular and additionally delving into cellular and molecular mechanisms are emphasized by Pinto et al. [57].

The exposure time is an additional parameter to consider. The consulted studies use short exposures for cell viability determination. So, there is a need for toxicological studies that use longer exposure times and encompass the low-dose range typically applied to *in vitro* studies [54]. Chronic studies have elucidated that cellulose fibers persist in rat lungs even after a year of exposure, suggesting the biodurability of these particles [125]. This finding highlights the need for long-term assessments in toxicological research to comprehensively understand the potential health implications associated with exposure to such materials over extended periods.

Several *in vivo* studies suggested the potential impact of NC aspiration on acute inflammatory responses and DNA damage at the pulmonary level, as detected in female rats [126]. However, other *in vitro* and *in vivo*

assays demonstrated neither cytotoxic nor genotoxic effects [64]. Numerous live studies can serve as anchor points to confirm its effects or cellular mechanisms of action. They offer valuable insights into how these materials interact with biological systems, influencing cellular absorption, interaction with cell membranes, and with subcellular components [81].

4.4 Gaps in the Knowledge and Need for Future Studies

When considering CNF for food-related applications, oral exposure becomes crucial to evaluate [59]. Studies have highlighted the need for further investigation into potential adverse health effects, such as immunotoxicity and genotoxicity, after oral exposure to NC [58,80]. Before concluding that any material is completely safe for applications such as packaging, the biodegradability aspects, potential migration, toxicity, and environmental impact (ecotoxicity) must be thoroughly clarified.

Several relevant points emerge following the completion of the review that we consider relevant to indicate: 1) It is imperative to include an exhaustive characterization of the study material, providing a solid foundation for subsequent analyses and interpretations; 2) Apply a broad array of parameters (biomarkers) to evaluate during *in vitro* studies, prioritizing those most relevant to our specific research objectives. The same applies to the cell type and cultivation methods, which should be carefully chosen for the exposure mechanism under investigation and those expected to generate a response; 3) Recognizing that the toxicity of individual materials differs from that of bionanocomposites is a relevant area for future research; 4) Use the information provided by available *in vivo* studies to enhance the interpretation of *in vitro* results that offer valuable insights into cellular mechanisms and responses.

5 Conclusions

Although there is a tendency to prefer biobased materials over traditional petroleum-based ones, there is disagreement about whether NC's broad use is safe. Addressing concerns about its environmental and toxicological impact is critical to ensure a safe integration in various applications without compromising consumer health or ecological integrity. Evaluating the nanosafety of cellulose nanofibers in packaging must account for key factors such as biodegradability, migration into food products, and overall toxicity. Due to the lack of consistent toxicological data supported by international regulations, the safety of the exposure or ingestion of CNF because of its presence in packaging is still an open question. Despite the increasing research in this field in recent years, the number of available studies is limited, particularly given the variety of nanomaterials emerging in the market. The risks associated with the exposure or ingestion of cellulose nanofibers require focused investigation, as current knowledge remains limited. Comprehensive research is needed to clarify the potential toxicological mechanisms of NC and its derivatives, including NC-based bio-nanocomposites, which may exhibit distinct properties compared to individual components.

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