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Elucidating the Potential Biological Impact of Cellulose Nanocrystals

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Abstract: Cellulose nanocrystals exhibit an interesting combination of mechanical properties and physical characteristics, which make them potentially useful for a wide range of consumer applications. However, as the usage of these bio-based nanofibers increases, a greater understanding of human exposure addressing their potential health issues should be gained. The aim of this perspective is to highlight how knowledge obtained from studying the biological impact of other nanomaterials can provide a basis for future research strategies to deduce the possible human health risks posed by cellulose nanocrystals.

Keywords: nanocellulose; nanomaterial; human health effects; risk; exposure; hazard; characterisation; testing strategies; cellulose nanocrystals

1. Introduction

Cellulose is the most abundant organic polymer on earth, and can be found in plants, algae, bacteria, amoeba, and even some marine animals. The polymer is composed of β -(1 \rightarrow 4) D-glucose monomers [1], and in its natural state, cellulose is a hierarchically structured material with different layers of organization. At the lowest level, the polymer chains are organized in highly ordered and uniaxially oriented crystalline domains, which are disrupted by disordered amorphous regions. This structure is the basis for the isolation of different types of nanocellulose from natural cellulosic materials.

Several distinct forms of nanocellulose types, where at least one of the dimensions is on the nano-scale, exist. The most commonly studied and used forms are bacterial cellulose (BC), microcrystalline cellulose (MC), microfibrillated cellulose (MFC) and cellulose nanocrystals (CNCs) [2,3]. CNCs, which are also referred to as cellulose nanowhiskers (CNWs) or nanocrystalline cellulose (NCC), are produced by hydrolysis of cellulose pulp with a mineral acid, such as hydrochloric acid [4], sulphuric acid [5] or phosphoric acid [6]. During the acid treatment, the amorphous portions of the hierarchically structured material, which are more prone to hydrolysis than the crystalline domains,

are disintegrated so that only the crystalline parts remain in the form of 'needle-shaped' nanofibers. Cellulose nanocrystals thus made exhibit a length between hundred nm and several μm and a width between 10 and 50 nm [7,8], dependent on the cellulose source used [9].

CNCs are receiving considerable interest within the research community due to their interesting and desirable set of properties, which include the renewable nature of their sources, and a combination of high stiffness and strength and low density [2]. Thus, CNCs have been widely used as reinforcing filler for a variety of polymers to yield nanocomposites with improved mechanical properties [2,3,10]. In addition, the surface chemistry, made up almost exclusively of hydroxyl groups, renders nanocellulose as an interesting substrate whose surface can be readily and freely functionalized. This propensity, together with their biologically benign nature, is driving the use of nanocellulose within different (bio)materials [10–12]. CNCs have further been used in a broad range of other new materials applications, including optically [13] and electrically [14,15] active materials, aerogels [16–18], and mechanically adaptive materials [7,19–25], just to name a few examples.

Fueled by promising outcomes of research projects, and great potential of pilot studies, an industrial-scale production of CNCs is being undertaken [26], and commercial exploitation of this nanomaterial has begun. Whilst such an outlook can be seen as advantageous from an application point of view, i.e., new materials that are cost-effective and that provide advanced, as well as enhanced qualities over their alternative counterparts, there remain open questions [27] concerning the human exposure to CNC-based nanomaterials, and furthermore, what the (potentially adverse) human health effects are following such an exposure.

Over the past three decades, during which the field of nanotechnology witnessed constant expansion, there has been heightened emphasis placed upon the need to develop a thorough understanding of the biological impact of nano-sized materials. Although the above highlighted examples illustrate the potential effectiveness of nanocellulose as an application, there remains a necessity to holistically deduce their possible adverse biological impact due to their nanoscale properties [28], taking into consideration the pitfalls associated with studying possible nanomaterial hazard [29]. Thus, with nanocellulose, it is essential to build upon the already formed knowledgebase of nanomaterial hazard, even via read-across techniques, wherein structurally similar analogues are used to hypothesize toxicity without experimental testing [30], in order to progress both understanding and perception of the biological impact of such 'new' nanomaterials effectively.

The objective of this perspective is, therefore, to consider how the advancements of nanocellulose applications have been studied through both *in vitro* and *in vivo* investigation, and how this knowledge within may be attributed towards clarity of current understanding, and future activities regarding the use of, and biological impact of CNCs.

2. Life-Cycle and Human Exposure of CNCs

As with any other (biodegradable) material, CNCs have a life-cycle [31,32] which, as shown in Figure 1, is initiated with the growth and harvesting of the natural raw material (the most viable source for commercial use at this point appears to be wood, although for research purposes many other sources are being used, including cotton [6,33,34], banana stems [8], and tunicates) [7,35] and continues with its isolation, the modification and integration into a material system (e.g., compounding with a polymer), and further processing in order to create a final 'product', which, eventually, is placed on the market. The life-cycle continues thereafter with further processing prior to disposal, which may occur through biodegradation or incineration. Throughout this life-cycle, there is the possibility of exposure to humans, eventually after nanocellulose is released from the product and through a number of environments and scenarios. In each of these there are different modes of human exposure, which include the respiratory tract (inhalation), skin contact, eye contact, ingestion and possible interaction with the bloodstream (i.e., via direct injection through medical application, or via translocation from the lung following inhalation [32,36]) resulting in possible secondary organ exposure, i.e., liver, heart, brain, and/or kidney.

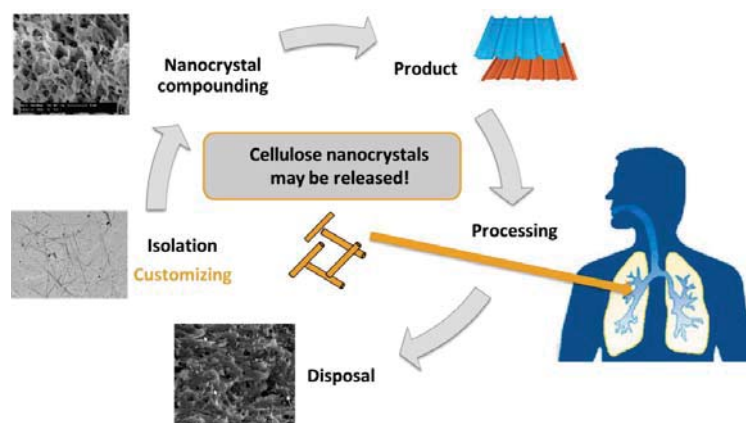


Figure 1. Schematic of the life-cycle of cellulose nanocrystals and products made from these nanoparticles. There are five main points in the life-cycle of CNCs; i. isolation, ii. compounding, iii. product formation, vi. post manufacturing processing and use, and v. disposal. All stages of the life-cycle pose a potential human exposure scenario for which both the exposure level and the hazard associated, and thus the risk of CNCs to human health, are currently not fully understood. It must be emphasized that inhalation exposure remains the assumed primary route of entry to the human body for CNCs.

However, only two major exposure routes have been observed as pertinent to humans during life-cycles involving anisotropically shaped nanomaterials of this type; inhalation and skin exposure. This knowledge originates from studies by Maynard and colleagues [37], as well as more recently by others [38], involving carbon nanotubes (CNTs) and not CNCs. Due to the significant differences between the production, properties and anticipated fields of use of CNTs [39] and nanocellulose [3], it must be considered that the exposure routes towards humans could be different, although one can speculate that inhalation probably would remain the primary form of uptake due to the potential aerosolisation of the CNCs at this point in their life-cycle. A pertinent association could also be made with the isolation of bulk cotton fibres [40], although this would arguably only be relevant to cotton-based CNCs, the exposure risk and routes remain the same (i.e., inhalation and skin exposure). Naturally, if workers are adequately protected then such exposures can be reduced [41]. However, despite such attention to worker safety, since workers would be exposed to repeated doses of nanocellulose, over a chronic period of time such an understanding is necessary, as is the specific concentrations that they are exposed to. Therefore, to progress knowledge in this area, (i) the human exposure routes must be confirmed for CNCs at the isolation stage of their life-cycle; and furthermore (ii) understanding of the occupational exposure levels should be confirmed.

In order to determine the human exposure routes within a nanocellulose production environment, a number of lessons can be learned from air pollution, as well as those studies focusing on other nanomaterials [42,43]. It must be noted, however, that the specific identification of aerosolised or otherwise released nanomaterial fractions, especially fibrous nanomaterials, are highly problematic and such particles are difficult to measure in any environment due to limitations in the currently available technology, e.g., with a scanning mobility particle sizer (SMPS) [44]. It is currently unknown to what extent CNCs can be detected with available methods. Thus, as a starting point, it would be important to confirm the usefulness of existing analytical tools or develop new methodologies that permit the accurate measurement of the actual CNC concentration in air, so that these particles can be detected efficiently right from their origin.

The issue of human exposure levels to nanomaterials is, in general, an important issue within the field of nanotoxicology. Recently, intense efforts have been made by the National Institute for Occupational Safety and Health (NIOSH) in the United States of America. Although it has established occupational exposure levels for silica dust and titanium dioxide, NIOSH has predominantly focused

on CNTs, and in a recent central intelligence bulletin suggested an exposure limit for CNTs as $1 \mu\text{g}/\text{m}^3$ for an eight hour working day [45]. Although this recommended exposure limit (REL) could be considered as an overload situation over a workers' life-time [46], this metric has been suggested based on a plethora of in vivo and some in vitro testing strategies using solely CNTs in order to comprehend specificity for these nanomaterials. This concept therefore reduces somewhat the applicability towards an REL for CNCs. However, if the physical characteristics of the nanocellulose sample in question are remotely comparable to those of the CNTs, then it could be, or might be considered apt. Nonetheless, the US Occupational Safety and Health Administration had previously set a specific permissible exposure limit (PEL) of $200\text{--}750 \mu\text{g}/\text{m}^3$ over an eight hour timed weighted average (TWA) for cotton dust. Irrespective of the issues surrounding both exposure limits, they do provide a significant basis for research to dictate that investigations undertake exposures at 'realistic' concentrations/doses so that extrapolation towards human exposure can be made [47]. Furthermore, such exposure limit values provide a valuable 'stop-gap' until regulatory bodies are able to provide direction towards the use and exposure of nanomaterials [48]. It is also prudent to note that the REL TWA provided by NIOSH for silica dust ($0.05 \text{mg}/\text{m}^3$) [49] could also be used as a 'highest exposure scenario' for CNCs, due to the heightened crystalline fraction (which is the fraction known to drive the heightened inflammatory responses caused following (most) silica exposures) [50]. This concept further highlights an important note, in general, for the nanotox community regarding the need for the appropriate use of positive particle controls to use as a comparison for determining the biological impact of nanomaterials, such as CNCs.

For the subsequent compounding and usage (i.e., product) of CNC-based materials there is also a risk of exposure, albeit it can be assumed to be much smaller than during the initial isolation of CNCs. During these latter stages of the life-cycle, the risk of exposure can mostly be attributed towards the possible abrasion of the product, which could result in the release of individual CNCs, small CNC aggregates, or nanocellulose-polymer composite (nano)particles, which could be subsequently inhaled or penetrate through the skin upon contact. Recent research on this matter has again focused upon CNTs [51–53]. From these initial studies it has been postulated that the release of CNTs, at least in their bare form and also combined with polymer matrix is relatively low. Specific exposure levels are not yet known and therefore additional research must be conducted. Furthermore, in terms of usage, it should also be noted that there could be direct exposure to the human body via ingestion (e.g., nanocellulose in contact with food products, such as in food packaging) and also there is the potential injection into the human bloodstream (e.g., the use of nanocellulose as a tool within nanomedicine). These latter aspects, however, are currently of minor importance, as the use of nanocellulose as main components in such food-related and/or medical devices do not appear to be imminent. However, due to their potential application in these contexts, hazard assessment of these scenarios should be undertaken in order to obtain clear risk analysis data, as previously shown by Bergin and Witzmann (ingestion of nanomaterials) [54], as well as for medical application (i.e., injection) [55].

Finally, understanding of the human exposure effects during the disposal of nanocellulose, in whatever format, is severely limited. A recent study into the incineration of nanomaterials in a waste plant showed that at a variety of different locations within the building, no or only small amounts of nanomaterials were found following their incineration [56]. Whilst this could also be true for nanocellulose, it is safe to assume that, very much like wood, cotton and other raw cellulosic materials from which CNCs are extracted will end in similar ash once burnt.

Thus, from the currently available information and relevant application of nanocellulose, it can be summarized that during the entire life-cycle the human exposure routes can be stated in order of importance as i.e., inhalation > skin > others (e.g., eye contact, ingestion, injection). Such a perspective is vital towards determining which exposure route hazard analyses should focus upon. This however, is by no means new information. It can be considered that the entire discipline of nanotoxicology is predominantly based upon the consideration that most nanomaterials are inhaled and therefore the lung is the primary human target organ, as is the case within the particle toxicology field [57]. However,

when focusing upon these exposure routes, emphasis should be upon which forms of nanocellulose to study. Since the potential for inhalation of nanocellulose is most paramount at the isolation stage, it is fundamental that the biological impact of bare and functionalised CNCs are studied initially. Such information would then act as a building block in assessing the hazard posed by nanocellulose released from polymer composites (or a combination thereof), and subsequently the human health implications during their disposal. For the success of such an outlook however, all nanocellulose samples would need to undergo essential and thorough characterization.

3. Characterising CNC Exposure

Since the mid-2000s, it has been necessary that a thorough characterisation of the specific, pristine nanomaterial being tested for their biological impact is performed [58]. In fact, it is mandatory for most journals nowadays that such information is contained within all original research manuscripts. This significant change within the field of nanotoxicology is evident from the continual association and significant influence that the physico-chemical characteristics of nanomaterials were noted to (significantly) contribute to the biological effects observed [59]. Although widely accepted, this concept did however raise multiple discussions as to which physical and/or chemical characteristics must be studied for each nanomaterial. Due to the diverse nature of nanomaterials, it has so far been too difficult to define a precise set of characterisation standards (i.e., which characteristics must researchers assess?). Mostly the characteristics of shape, size, (chemical) composition, surface material, surface charge density and surface area [58] have been considered paramount. However, due to analytical challenges associated with some nanomaterials [60] it has predominantly been accepted that as much information on the physico-chemical characteristics are provided as possible. Furthermore, assessment of the physico-chemical characteristics within the biological environment (e.g., for in vitro based investigations, it is important to determine the impact that the cell culture medium and associated proteins has upon nanocellulose) studied is desirable [61], yet challenging [62].

Currently there is limited understanding as to the biological impact of nanocellulose in relation to their physical attributes (throughout their life-cycle), thus developing such knowledge will lend itself to determining their biocompatibility. Furthermore, such information is important for the future of nanocellulose hazard assessment, since in a number of previous studies an intimate characterisation is unfortunately absent, making it difficult to correlate across different studies and to address, if any, the key parameters that influence different cell responses following nanocellulose exposure [10].

In order to address this, Table 1 highlights many of the key physico-chemical parameters that should, ideally, be investigated when studying nanocellulose and CNCs in particular. Furthermore, the problems associated with each different technique and analytical endpoint is highlighted, with subsequent suggestions as to how to mitigate such issues. Although all the parameters highlighted in Table 1 are essential, it is again important to note that the potential hazard of CNCs would likely be related to (i) their dimensions (i.e., in the nanoscale); and (ii) their 'fibre-like' appearance (i.e., long, straight, and often 'needle-like'). Whilst the first hurdle, their nanoscale dimension, is suitably covered by the suggested analyses given in Table 1, the latter (i.e., fibre-like appearance) can be related to the 'fibre paradigm' [63].

The fibre paradigm itself is associated with the findings of both glass [64] and asbestos fibers [63]. It was originally shown by Davis and colleagues [65] that long, stiff amosite asbestos fibers, unlike short amosite asbestos fibres, can lead to serious damage to the lungs of rats when inhaled or following intraperitoneal injection. Effects noted were chronic inflammation leading to eventual granuloma formation and in some cases mesothelioma (the hallmark cancer of long fibre asbestos exposure). In regards to glass fibers, often used in construction as an insulating material and fire retardant, similar heightened negative health effects towards both workers and consumers have been shown over an increased period [66]. Further research has shown that the specific health related issues following exposure to both glass and asbestos fibres include inflammation, alveolitis and reduced pulmonary functions [67]. Importantly, all of this work could only be reported in the manner it was

due to the specific physical and chemical characterisation of the fibres investigated. More recently, CNTs, which are potentially advantageous components for a number of different consumer, industrial, and technological applications, were shown to induce asbestos-like effects when introduced into the peritoneal cavity of mice [68]. These results however were attributed to specific physicochemical characteristics i.e., increased length and stiffness as well as biopersistence.

For CNCs, concerns associated with the fibre paradigm are debatable as their average lengths do not fit the required characteristics to fit the paradigm [63]. Indeed, the minimum length for nanomaterials, or high aspect ratio nanomaterials (HARN), to fit the fibre paradigm is $>5 \mu\text{m}$ [69]. Average dimensions for typical CNCs isolated from cotton ($100\text{--}200 \times 5\text{--}15 \text{ nm}$) soft-wood pulp ($100\text{--}150 \times 5\text{--}15 \text{ nm}$), and tunicates ($1000\text{--}2000 \times 10\text{--}20 \text{ nm}$) are significantly below this threshold [70]. This is, however, not to say that the population of fibres that are longer than $5 \mu\text{m}$ is zero (especially in long CNC types such as tunicate CNCs [71]) and that therefore such materials should not elucidate effects associated with the fibre-paradigm. Indeed, this aspect suggests that CNCs demand special attention considering their proposed application and possible human exposure. Further need to study nanocellulose in this notion is that their width ($<5 \mu\text{m}$) certainly fits the fibre paradigm [63]. The final aspect of this paradigm however, which remains the most difficult to decipher for any (nano)fibre type, especially nanocellulose, is their biopersistence (or biodurability [72]).

Table 1. Overview of the most commonly used analytical methods for the characterization of the physico-chemical properties of nanocellulose, in particular CNCs. Details as to the limitations of each method, with concepts towards mitigation of such limitations also given.

Characterization Method	Feature of Nanocellulose Characterised	Limitation Regarding Nanocellulose	Limitation Mitigation	References
Electron Microscopy (TEM)	Shape & dimension (Best for overall structural analysis, for most samples)	Drying effects when spotting onto EM grids	Alter drying conditions, concentration, BSA-based techniques [73]	[6,13,74]
Atomic Force Microscopy (AFM)	Shape & dimension	AFM tip has the potential to overestimate sizes if sharpness is lost	Use height (more accurate), not measured width	[75–77]
Dynamic Light-Scattering (DLS)	Overall dimensions	Tough to elucidate exact dimensions	Modify with an accurate form factor	[78,79]
Optical Photographs	Dispersion/colloidal stability. Observation of aggregates (larger than 300 nm)	Limited by Abbe diffraction limit	Must use electron microscopy for smaller (less than 300 nm)	[80,81]
Conductometric Charge Titration	Charge density (Best for surface half ester content determination)	Small ($<20 \text{ mmol/Kg}$) is within noise limit	Larger sample size,	[6,82]
Elemental Analysis	Elemental content of sample	Common for C, H, N, S, P analysis only	Must be correlated to predicted chemical structure	[6,83,84]
Infrared Spectroscopy (IR)	Functional groups (bonds)	Only looks at chemical bonds	Limited to IR active chemical bonds, sensitivity	[78,85]
X-ray Photoelectron Spectroscopy	Elements on the surface	Voxel does not allow individual CNC analysis	Does not elucidate groups, only elements	[86]
Brunauer, Emmet and Teller method (BET)	Surface area	Cellulose naturally aggregates when dried	Aggregation will lead to lower than individualized CNCs	[87,88]
Dye Adhesion	Surface area	Limited by size of dye	Use in conjunction with other techniques (e.g., rough estimation by length \times dimension analysis)	[70,89]
Inverse Gas Chromatography (IGC)	Surface properties	Cellulose naturally aggregates when dried	Aggregation will lead to lower than individualized CNCs	[71]

Previously, biopersistence (associated with exposure to the human lung) of any fibrous material has been deduced via direct assessment (mostly in acellular and in vivo environments) over a chronic period. However, in order to reduce animal experimentation in vitro based analyses have also previously focused on a single-cell system (e.g., macrophages), as well as using different biological-based buffers at different pH and under flow conditions. Yet, despite these efforts there is currently no clear method to efficiently and effectively elucidate the potential biopersistence or biodurability of a (nano) fibre. Currently, knowledge of the biopersistence, or biodurability of nanocellulose is severely lacking, although several studies are ongoing [31], in order to truly understand its biological impact, efforts must be made to comprehend this important biological-based characteristic of the material.

4. How to Determine the Potential Biological Impact of Nanocellulose

The biological impact of the bulk form of non-nanoscale cellulose fibres [90] (e.g., microfibrillated nanocellulose) as well as cellulose dust (usually micron sized ($>10\ \mu\text{m}$)) has been widely studied in the past [91]. Due to the inherent differences between these materials and nanocellulose, it is difficult to make any clear correlations between them. However, it must be emphasized that non-nanosized cellulose materials, when compared to other fibrous types, such as asbestos, commonly showed limited adverse biological effects [92].

Focusing upon CNCs however, a first study on the biological impact of CNCs isolated from cotton and tunicates was reported by Clift and colleagues in 2011 [93]. Further insightful research studies have followed (Table 2), and have contributed to the current understanding of the biological impact of CNCs and other nanocellulose types. Despite the increasing number of studies published on this new nanomaterial in the past few years, in most of the cases a first biocompatibility analysis was performed to assess the possible lethality of the nanocellulose. Yet, a detailed mechanistic toxicological assessment that is necessary to determine their potential human health effects (over time) remains lacking. Such analyses are vital, especially considering the landmarks of nanotoxicological research strategies; i.e., considering the potential for nanocellulose to cause oxidative stress [31,94,95], and possibly genotoxicity [96]. Such understanding is imperative towards conceiving any understanding as to the potential (chronic) adverse effects of nanocellulose towards human health. Thus, in order to achieve such investigations representative models must be utilized, and often in collaboration with other, complimentary testing strategies.

Commonly, in the past, in vivo studies (e.g., rodent models) have been used to study the toxicology of nanomaterials, since these allow for whole body exposure scenarios and permit assessing the biodurability, dissolution and secondary organ toxicity of any test substance. However, in view of the recent calls for the refinement and reduction of such animal based testing strategies (with a view to eventually replacing them over time) there is an immediate need to develop alternative testing models, such as in vitro, in silico and computational models [97]. In a recent review, Hartung and Sabbioni highlighted the 'alternative' models currently available within the field of nanotoxicology [98]. Recently, several advanced and multi-cellular in vitro systems have been used with the objective to determine the mechanisms behind the possible hazard associated with nanomaterials [99]. Whilst these models show a different biochemical/biomolecular response to monoculture systems (whether it be a similar trend, but different concentration-based effects, or a completely different biological effect) [100], there is still much debate and unknown as to how they correlate to the in vivo scenario, albeit efforts are underway to address this knowledge gap [101]. Such systems have recently been shown to be advantageous in determining the hazard posed by nanocellulose [47], as well as its interaction with cellular systems [102].

Table 2. An overview over the published studies focussing on the hazard assessment of nanocellulose. Details regarding the specific form of nanocellulose used, the biological system employed and the specific biochemical endpoint analysed are given.

Nanocellulose Form Studied	Biological Model Used	Endpoint Assessed	Reference
Bacterial cellulose nanofibres (BC-NF)	3T3 fibroblasts, CHO cells	mutagenicity, proliferation, genotoxicity	[103]
Bacterial cellulose nanofibres	HUVEC, C57/Bl6 mice	viability, cytotoxicity, apoptosis/necrosis, cell cycle	[104]
Cellulose nanocrystals (CNCs)	<i>Oncorhynchus mykiss</i> hepatocytes, <i>Daphnia magna</i> , <i>Ceriodaphnia dubia</i> , <i>Pimephales promelas</i> , <i>Vibrio fischeri</i> , <i>Pseudokirchneriella subcapitata</i> , <i>Hydra attenuata</i> , <i>Danio rerio</i>	genotoxicity, reproduction, survival, growth	[105]
CNCs isolated from flay	HEK 293, Sf9 cells	uptake, cytotoxicity	[106]
CNCs isolated from cotton and tunicates	3D model of the pulmonary epithelial airway barrier	cytotoxicity, (pro)inflammatory response	[93]
Cellulose nanofibers isolated from caraua/cotton	<i>Allium cepa</i> , primary lymphocytes, 3T3 fibroblasts	Genotoxicity	[107]
Plant derived CNCs	HBMEC, bEnd.3, RAW 264.7, MCF-10A, MDA-MB-231, MDA-MB-468, KB, PC-3, C6 cells	uptake, cytotoxicity	[108]
Nanofibrillated cellulose (NFC)	BEAS 2B cells	Genotoxicity	[109]
CNCs isolated from cotton, flax, hemp	V79 fibroblast, Sf9 cells	Cytotoxicity	[110]
Cotton cellulose nanofibres (CNF)	Bovine fibroblasts	cytotoxicity, stress response, apoptosis	[111]
CNCs isolated from cotton	BEAS 2B cells, monocyte-derived macrophages	cytotoxicity, genotoxicity, inflammatory response	[112]
CNCs isolated from MCC	NIH3T3 fibroblasts, HCT116 cells	cell viability	[113]
CNFs isolated from cotton	<i>Chlorella vulgaris</i>	cell viability, growth	[114]
CNCs isolated from wood	C57BL/6 mice	pulmonary outcome	[115]

Whilst the specific mechanisms associated with and driving any of the observed biochemical and biomolecular reactions measured are a necessity, it is vital that a specific understanding of how nanocellulose interacts with different biological systems can be developed, and how this relates to the biochemical response measured. The use of state-of-the-art microscopy approaches will be necessary due to the innate difficulties in identifying nanocellulose within cellular structures. A recent study by Endes et al. showed the possibility to achieve imaging of CNCs within cells by fluorescently labelling the nanocellulose used, with the assumption that small amounts of dye, in this case fluorescein, does not have any significant effect on the associated tests, such as uptake or cytotoxicity [102]. Analytical techniques should be sought out, which can directly identify cellulose, without the need of modification to easily identify nanocellulose inside cells so that a toxicodynamic approach can be undertaken. Finally, once all this information is gained, then correlatory analysis against the specific physical and chemical characteristics of the nanocellulose sample so that efforts can be made to negate the production of such materials exhibiting these characteristics (i.e., safe-by-design nanomaterials).

It should be noted, that this article focuses on cellulose nanocrystals. One of the greatest challenges in addressing the effect of nanocellulose, is that it comes in many different forms, not only with respect to source, aspect ratio and surface chemistry, and processing methods. These different forms usually focus on different systems, in different ways. Although the above table suggests the majority of research is focused on CNCs, there are others, which is beyond the scope of this paper.

Nonetheless, the discussion contained within the present perspective would certainly fit towards any and all nanocellulose types.

5. Summary and Outlook

Nanocellulose is an interesting tool for material scientists as the platform to engineer desired functions into polymeric and biological systems. Many research groups have found a plethora of ways to use cellulose in both its bare, and functionalized form. These applications have shown that both in vitro and in vivo applications are viable, and do not create any measurable negative effects. Along with its production/isolation, the commercialization of products containing nanocellulose has begun and is constantly increasing. Several avenues of use seem to be emerging for nanocellulose, from high end smart and biocompatible materials, to large-scale use in commodity products. All these exciting properties of cellulose nanocrystals and cellulose based nanomaterials seem to be to the beginning of a new concept of enhanced commodity materials and specialized biomedicine in which materials science and biology are closely related; giving the opportunity to engineer the desired material using nanomaterials.

With all of these obvious advantages, there remains a lack of knowledge concerning the potential hazard nanocellulose may pose to human health, and furthermore at how, if, and at what dose humans would be exposed, given the wide range of potential life-cycle scenarios. Although there has been much research-based emphasis on deducing this unknown, and so far no adverse acute effects have been reported when using realistic concentrations, a variety of compounding factors disallow any meaningful wide-ranging understanding to be gained from the research currently available. Varying characteristics of the investigated sample deriving from differing production protocols, sources, dimensions, purity, concentration, application mode and exposure time can strongly influence the biological response observed in vitro or in vivo and may not reflect a realistic assessment of the potential hazard of cellulosic fibres in general and in particular for cellulose nanocrystals.

Therefore, in order to realize these aspects, and overcome these issues, it is suggested to consider the following points in order to fully, and holistically deduce the potential human health risk of nanocellulose;

- Assess and quantify what and if the released dose at each stage of the material's life-cycle is a potential mode for environmental as well as human exposure (e.g., inhalation and skin contact).
- At each stage of the life-cycle of nanocellulose undertaken, thorough characterisation of the released nanomaterial (if any) and decipher between single nanocellulose nanofibers, polymer composite released nanocellulose nanofibers and micron-sized particles. Several parameters need to be analyzed, the most relevant factors being: the dimensions (width, length, aspect ratio), colloidal stability on the studied medium, surface chemistry, specific surface area and degree of crystallinity (directly related to the stiffness of the material).
- In order to achieve the characterisation of the materials at every life-cycle stage, reliable and representative methods must be used (as suggested in Table 1). The need to develop alternative or adapted methods for every nanomaterial, especially nanocellulose remains and is the responsibility of the field to progress. New protocols need to be established for the facile characterization and determination of nanoparticle size and determination of surface chemistry on the nanoscale, which allow for a simple and realistic comparison between studies.
- Understanding of the acute and chronic effects of nanocellulose exposure, particularly during occupational exposure (i.e., isolation stage) in order to comprehend the ability for nanocellulose to either contribute to, or exacerbate pre-existing disease states.
- Determine the biomolecular and biochemical mechanisms that drive, if any, the (adverse) biological effects following nanocellulose exposure.
- The application of realistic doses in contrast to overload situations on target organ (in vitro) or related systems has to be the aim in any hazard assessment study.

- Relate the exposure dose effect and associated biochemical effects to the specific characteristics of the nanocellulose investigated in order to determine the specific physical and/or chemical characteristics that might be driving the possible hazardous response measured.

It is the hope that such suggestions towards the assessment of biological interactions and impact of nanocellulose to human health provides coherent and effective knowledge and understanding that can be put towards the development of regulatory guidelines for the production, use and disposal of nanocellulose. Further to this, elucidation of the biological impact of nanocellulose will only serve towards realizing the plethora of advantages posed by this naturally occurring material.

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