In book series: Advances in Experimental Medicine and Biology, Springer Nature Switzerland AG.
Publication date: Jun 2021.

Nanocelluloses – Nanotoxicology, safety aspects and 3D bioprinting

Gary Chinga-Carrasco[1]*, Jennifer Rosendahl[2], and Julia Catalán[3,4]

[1] RISE PFI, 7491 Trondheim, Norway

[2] RISE, Division Materials and production, Department chemistry, biomaterials and textiles, Section Biological Function, 504 62 Borås, Sweden

[3] Occupational Safety, Finnish Institute of Occupational Health, 00250 Helsinki, Finland

[4] Department of Anatomy, Embryology and Genetics, University of Zaragoza, 50013 Zaragoza, Spain

*Corresponding author: Gary Chinga Carrasco, gary.chinga.carrasco@rise-pfi.no

In book series: Advances in Experimental Medicine and Biology, Springer Nature Switzerland AG.
Publication date: Jun 2021.

Abstract

Nanocelluloses have good rheological properties that facilitate the extrusion of nanocellulose gels in micro-extrusion systems. It is considered a highly relevant characteristic that makes it possible to use nanocellulose as an ink component for 3D bioprinting purposes. The nanocelluloses assessed in this book chapter include wood nanocellulose (WNC), bacterial nanocellulose (BNC), and tunicate nanocellulose (TNC), which are often assumed to be non-toxic. Depending on various chemical and mechanical processes, both cellulose nanofibrils (CNF) and cellulose nanocrystals (CNC) can be obtained from the three mentioned nanocelluloses (WNC, BNC, and TNC). Pre/post-treatment processes (chemical and mechanical) cause modifications regarding surface chemistry and nano-morphology. Hence, it is essential to understand whether physicochemical properties may affect the toxicological profile of nanocelluloses. In this book chapter, we provide an overview of nanotoxicology and safety aspects associated with nanocelluloses. Relevant regulatory requirements are considered. We also discuss hazard assessment strategies based on tiered approaches for safety testing, which can be applied in the early stages of the innovation process. Ensuring the safe development of nanocellulose-based 3D bioprinting products will enable full market use of these sustainable resources throughout their life cycle.

Keywords: Nanocellulose, 3D printing, bioprinting, toxicology, medical devices, regulatory frameworks.

1. Introduction

Several types of nanocelluloses can be obtained from different raw materials, including wood, annual plants, agro-industrial side streams, bacteria, and marine resources. The most common nanocelluloses are obtained from hard- and softwood chemical pulp fibers, e.g. kraft and sulfite pulp fibers and will be referred to as wood nanocelluloses (WNC) in this chapter. Wood pulp fibers are roughly 1-5 mm in length and 15-50 μm in width.

Wood pulp fibers are processed with chemical and enzymatic pre-treatments to facilitate the structural deconstruction of the fibers into two main types of WNCs, i.e., cellulose nanofibrils (CNF) and cellulose

nanocrystals (CNC). Depending on the pre-treatments, wood CNFs have dimensions of roughly >1 μm in length and <100 nm in width, while wood CNC are shorter nano-objects with lengths of <200 nm and widths<50 nm (Table 1). For simplicity, in this book chapter, we will apply the term CNF in general, including cellulose nanofibrils, cellulose nanofibers, microfibrillated cellulose, and nanofibrillated cellulose.

Compared to wood-derived nanocelluloses, bacterial nanocellulose (BNC) is obtained from bacterial biosynthesis and is commonly composed of longer and nano-sized fibrils (diameters < 100 nm). Also, tunicate nanocellulose (TNC) is another type of nanomaterial obtained from marine animals (Tunicates). BNC and TNC are composed mainly of cellulose, while WNC may contain hemicellulose and residual lignin due to the plant origin.

Various types of nanocelluloses have been assessed from a biomedical perspective. Applications include wound dressings, scaffolds for tissue engineering, neural guidelines, to name a few [1-5]. These applications would benefit from a controlled deposition of nanocellulose and additional components to form specific and personalized constructs. It would make it possible to fabricate biomedical devices that are tailor-made for particular patients and situations. Such technology is also most valuable for constructing tissue models that mimic tissues such as skin, tumors, and human organs. Here is where three-dimensional (3D) printing will play a significant role, i.e., the development of tailor-made model constructs for testing drugs, medicines and, in the long run, for replacing malfunctioning body organs with fully functioning vascularized 3D printed constructs.

3D printing is an additive manufacturing process to create a 3D object layer-by-layer, aided by a pre-defined computer model. There exist various types of 3D printing processes applied to the fabrication of biomedical devices and tissue models, e.g., fused deposition modeling (FDM), stereolithography, inkjet printing, and micro-extrusion (also called direct-ink-writing) [6-8]. The fabrication of tissue models or organoids can comprise the 3D printing with biomaterial inks (e.g., nanocelluloses, collagen, and alginates) to construct scaffolds and then load the scaffolds with cells to form a tissue model that is maturated in a bioreactor. A more direct approach is to utilize a biomaterial ink directly loaded with

cells (defined as bioink) and deposit the bioink layer-by-layer following a pre-defined design, also termed as 3D bioprinting [9].

This book chapter will describe various nanocelluloses intended for biomedical applications, focusing on physicochemical properties that may determine the toxicological profile. More attention to 3D bioprinting of nanocellulose-based bioinks and the requirements necessary to fulfill from a regulatory point of view will be given.

2. Overview of nanocelluloses

Good overviews have been recently published about several types of nanocelluloses for biomedical applications and 3D bioprinting [8, 10, 11]. The various studies are mostly based on wood nanocelluloses with different physicochemical characteristics that may affect the toxicological profile and the 3D printability. Note that the nanocelluloses WNC, BNC, and TNC differ on the source of cellulose (wood, bacterial biosynthesis, and tunicate, respectively). Depending on the raw materials pre-treatment, the nanocelluloses may have different structural and surface chemical characteristics (Table 1 and Fig. 1).

WNCs are probably the most common type of cellulose nanomaterial proposed for biomedical applications. Wood CNF is one type of WNC and is characterized by being high-aspect-ratio nano-objects with diameters in the nanoscale (<100 nm) and lengths in the micrometer-scale (roughly >1 micrometer) [12-14]. CNF is composed of amorphous and crystalline domains [15]. CNF for biomedical applications and testing have been obtained by mechanical nanofibrillation by using, e.g., homogenizers, fluidizers, and grinders [16-18]. Before the mechanical nanofibrillation, various chemical and enzymatic pre-treatments can be applied to ease the fibrillation of fibers into homogeneous nanofibril dispersions. Such pre-treatments affect not only the physical characteristics of the nanofibrils but also the surface chemistry. Contrary to enzymatic pre-treatments [19], chemical pre-treatments introduce e.g. carboxyl, carboxymethyl and aldehyde groups [12, 13, 20, 21]. Phosphorylation has also been applied to introduce phosphoryl side groups on the surface of CNF [22].

Wood CNCs are another type of WNC and are low aspect ratio nano-objects prepared by chemical hydrolysis (through HCl and H_2SO_4) of the amorphous parts of the nanofibrils (Fig. 1D). Although the mechanical strength (Young modulus) of CNC has been reported to be high, this may vary considerably depending on the source of cellulose, methodology, and direction of measurement, e.g., transversal or longitudinal [23].

Table 1 Some characteristics of nanocellulose produced from woody biomass. Reprinted and adapted with permission from [8]. Copyright (2018) American Chemical Society.

Sample	Carboxyl (μmol/g)	Carboxy-methyl (μmol/g)	Aldehyde (μmol/g)	Sulphate half ester (μmol/g)	DP	Nanofibril diameter (nm)	Nanofibril length (μm)	References
M-CNF	100				890	<100	>1	[16, 24, 25]
E-CNF	24				913	~20	>1	[19, 26]
T-CNF	765-1800		211		250-620	<20	0.2-1.1	[5, 14, 24, 27, 28]
C-CNF	58	346-515				<20	<1	[5, 13, 16, 24]
C-P-CNF		393	1202		<80	<20	<0.2	[21]
CNC				300	90	<20	<0.2	[20, 27]

*The CNF materials were produced without pre-treatment (M-CNF) and with enzymatic (E-CNF), TEMPO mediated oxidation (T-CNF), carboxymethylation (C-CNF), and carboxymethylation/periodate oxidation (C- P-CNF) pre-treatments, respectively. DP is the degree of polymerization.

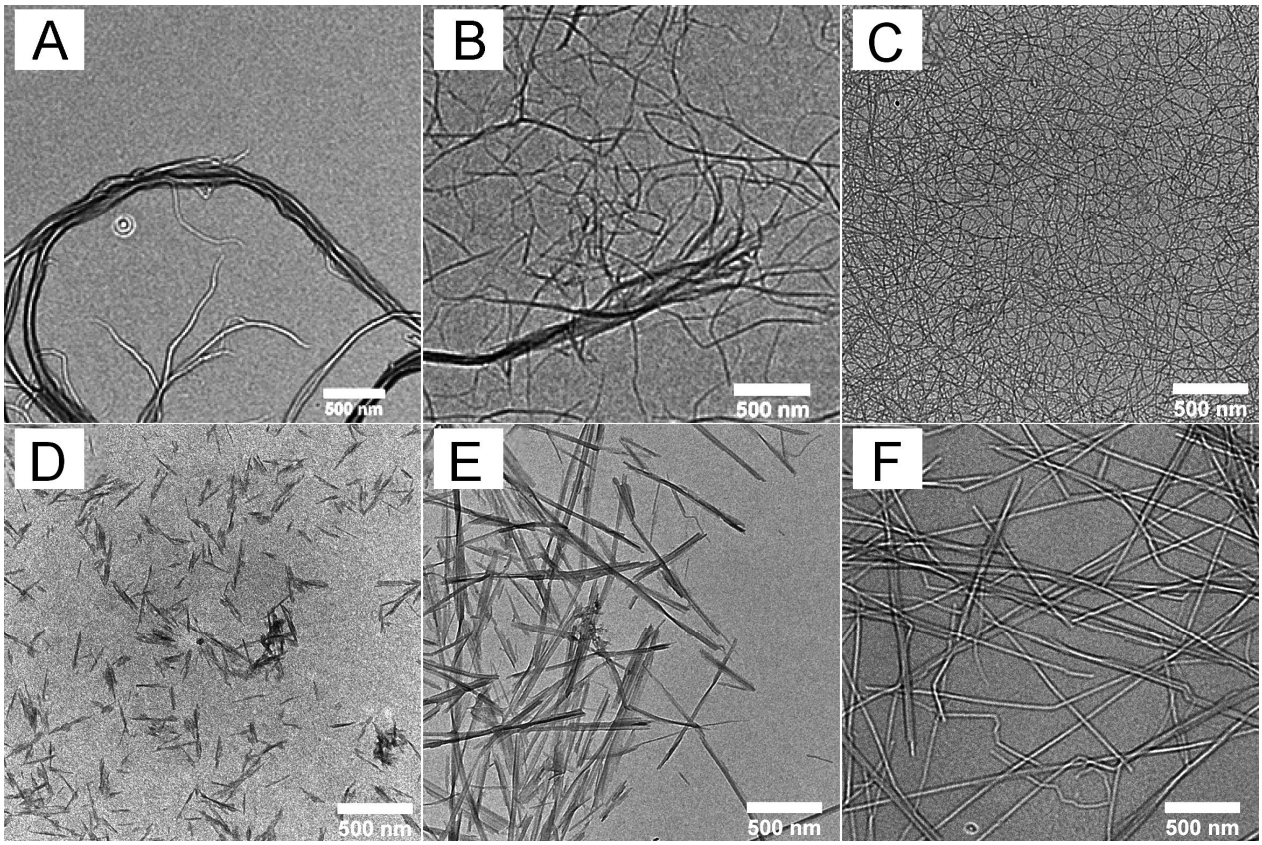

Fig. 1 Transmission electron microscopy images of wood CNFs: (A) mechanical grade, (B) enzymatic pre-treated, and (C) TEMPO mediated oxidized. (D) wood CNC, (E) BNC, and (F) TNC have been treated with H_2SO_4. Reproduced and modified with permission from [29]. Copyright (2014) American Chemical Society.

BNC consists of only glucose monomers, which are biologically extruded into cellulose nanofibrils by Gram-Negative acetic acid bacteria, resulting in extracellular cellulose pellicles [30]. Various carbon sources are utilized and fermented in the acidic-neutral pH range by the bacteria [31]. The BNC materials have a series of advantageous characteristics: high water holding capacity, a large degree of polymerization, high crystallinity, and excellent mechanical properties. Compared to WNC, BNC has a low production yield, being thus an expensive biomaterial. However, based on its purity (only cellulose chains), BNC has been proposed for a series of high-value applications within the biomedical sector [10]. BNC per se is not 3D printable as the material is produced as cellulose pellicles. Hence, additional post-processing of BNC has been applied to produce nanocrystals through acid hydrolysis [32] and

(mechanical disintegration and homogenization) to make a 3D printable BNC dispersion [33].

TNC is derived from tunicate animals, which contain cellulose in the tunic tissues, i.e., on the surface of the epidermis of the tunicate marine animals. The cellulose nanofibrils are synthesized by enzyme complexes involved in the synthesis of glucan chains [34]. TNC is obtained by processing the tunicate material with alkali process, similar to kraft pulping of woody material [35], is composed almost entirely of cellulose, and the nanofibrils are highly crystalline and with a high aspect ratio (Fig. 1F).

3. Nanotoxicology and safety aspects

Due to their natural origin, cellulosic materials (e.g., WNC, BNC, and TNC) are often assumed not to be toxic. However, the induction of lung diseases by cellulose fiber-containing dust in textile workers and the pulp and paper-producing industry has been well recognized since the last century [36], leading to several studies investigating the possible health risks associated with cellulosic materials. Although the toxicological findings were contradictory among studies, all of them agreed on the high biopersistence of cellulose fibers. According to the fiber pathogenicity and frustrated phagocytosis paradigms, biopersistent long (>10 μm) fibers may have the potential to be carcinogenic [37, 38], leading to lung cancer [39]. Although it is expected that long and stiff fibers (e.g., carbon nanotubes, the base of the frustrated phagocytosis paradigm) may negatively affect phagocytosis, CNFs are considered softer and flexible, which may facilitate the phagocytosis by macrophages. However, features, such as nanoscale sizes (at least one dimension less than 100 nm), larger surface area, and modified surface chemistry may impart novel material properties and biological behavior compared with conventional materials [40]. Therefore, it is necessary to address the human health and environmental safety aspects of nanocelluloses before scaling up their production [41].

Various studies and reviews have addressed the potential toxicity of nanocelluloses. According to Stoudmann et al. [42], the studies revealed variations and some contradictory findings attributed to several factors, e.g., cellulose source, pre-treatments, and incomplete material characterization. It

seems that compared to CNC, various grades of CNFs have caused the most variable results when it comes to endpoints such as cytotoxicity, genotoxicity, and inflammation. It is important to emphasize that CNFs vary considerably depending not only on the source but also on the pre-treatment (e.g., enzymatic and chemical) and mechanical nanofibrillation (Table 1). It is thus essential to be specific when the toxicological profile of CNF materials is assessed and describe the physicochemical aspects in detail, including, e.g., the size (width and length), the nanofibrillation yield (fraction of nanofibrils concerning the total mass), and the surface chemistry (which depends on the pre-treatment) (Table 1).

Keep in mind that nanocelluloses include a wide range of CNC and CNF materials, and most studies are based on lab-scale production of nanocelluloses, which may raise concerns about the reproducibility of the assessed materials. Hence, an appropriate comparison of the toxicological findings of different studies may be difficult. Also, samples of wood CNFs may contain micrometer-sized residual fibers [43], depending on the pre-treatments and mechanical equipment (grinders, homogenizers, and fluidizers) been applied during production. Hence, it is also essential to quantify the nanofibrillation yield [44].

Unfortunately, in some previous studies concerning toxicology, CNFs have mostly been grouped and generalized as one nano-object, and no adequate characterization or description of the CNF materials has been provided. This may lead to confusion and misleading conclusions that have to be taken with care. Hence, this observation raises a significant concern previously emphasized, i.e., "proper characterization of structural, chemical and biological aspects should be a requirement in scientific publications in order to document the characteristics of nanocelluloses and their biological impacts" [8].

Hence, in this section, to the best of our knowledge and depending on the information provided by the specific reviewed studies, we have tried to identify the various specific CNF grades according to the terminology used in Table 1 to provide more insight into the physicochemical effects on the corresponding toxicological profile.

3.1. Routes of exposure

The route of exposure may determine the toxicological responses to nanocelluloses. The main portals of entry to the human body include the gastrointestinal tract, skin, systemic circulation, and the lung, through inhalation [36]. The latter is considered the primary route of exposure for humans for any nanoparticle released into the environment, especially in occupational settings [45]. A life cycle risk assessment of nanocelluloses identified inhalation of dry nanocellulose powders or, in the case of wet slurry, airborne wet nanocellulose-containing particles during the production and manufacturing of nanocelluloses as the most relevant exposure scenarios [46]. Also, nanocelluloses seem to have long pulmonary biopersistence, as supported by in vitro experiments with artificial lung airway lining and macrophage phagolysosomal fluids [47], and by in vivo evidence [48-53]. As previously mentioned, the biopersistence of fibers has been identified as a critical feature governing the toxicological response following chronic inhalation exposures. Therefore, the release and inhalation of cellulose/polymer particles during processing steps, such as drilling, cutting, and sanding of polymer nanocomposites, in addition to possible liquid aerosols in wet operations, might be a concern [41].

Although inhalation has been pointed out as the main route for human exposure to nanomaterials, there is little information about exposure concentrations. When M-CNF were properly handled, no significant increases of particles in the air, compared to background levels, were observed during friction, grinding and spray drying [54]. Dustiness measurements were used in another study to simulate occupational exposure to spray-dried CNC [55]. The authors estimated that the mass fractions of inhalable, thoracic, and respirable particles were moderate. The National Institute for Occupational Safety and Health (NIOSH, USA) conducted an exposure characterization study of the production of CNC that had been tagged with cesium [56]. The analyses of filter-based air samples for elemental cesium indicated that CNCs were aerosolized during centrifugation and manipulation of the dry product without exceeding the occupational exposure limit (OEL) values for cellulose dust. There is not enough data on occupational exposure or inhalation toxicity for nanocelluloses to determine material-specific occupational exposure limit (OEL) values for airborne dust [56, 57]. As for other nanomaterials,

lower exposure levels may be expected to be harmful to nano-sized fibers, compared to bulk forms.

Exposure to nanocelluloses may also happen by the oral route, as nanocelluloses are intended to be incorporated into food (e.g., as a rheological agent), as well as in food packaging [18, 58, 59]. No clear demonstration of release from packages has been shown to date [46]. Recently, fluorescently labeled E-CNFs were used for monitoring leaching in laboratory papers (100% E-CNFs and E-CNF-fiber blended papers). The results showed loss values below 3 wt% E-CNFs, as fibrillation of E-CNF increased, improving paper stability, and reducing overall cellulose nanofibril loss [59].

Most of the potential 3D printed applications involving nanocelluloses are meant to be biomedical applications [8, 11]. Dermal exposure is the potential route for wound dressings- assuming that nanocelluloses are released from the dressings- especially as the absorption may be higher through the damaged skin barrier (e.g., burn skin). In the case of constructs for tissue engineering and drug delivery systems, nanocellulose-based products are expected to be directly delivered into the human body. Hence, the toxicity of the nanocelluloses will be determined by their potential release from the products, translocation through different body compartments, and clearance rate from the body [60].

3.2. Human toxicological studies

Knowledge of the potential adverse biological impact of nanocelluloses is still scarce, despite the increasing number of studies addressing the toxic effects of these materials in the last few years. The excellent reviews of Roman [60], Endes et al. [36] and Seabra et al. [61], followed by the more recent ones of Ventura et al. [62], Čolić et al. [63] and Stoudmann et al. [42], summarize the existing studies, showing that conflicting conclusions are reached.

Most toxicological studies on nanocelluloses have been performed in vitro, using mammalian cell cultures. These studies (summarized in the previously reported reviews) indicated absence or low cytotoxicity for CNCs, whereas more contradictory responses seemed to be reported for CNFs. As mentioned above, this is most probably due to the complex physicochemical characteristics of different CNFs (Table 1). Regarding genotoxic effects, the existing studies are too scarce to allow clear

conclusions. Although in vitro models are appropriate for identifying acute effects and elucidating mechanisms of action, they cannot provide information on the behavior of the materials in complex systems, such as whole organisms [62]. In vitro methods are neither well-suited for studying long-term effects. Hence, in vivo studies using animal models are still needed to get full understanding the toxic effects of nanocelluloses.

Most of the few existing in vivo studies have been performed by administrating the nanocelluloses through the respiratory tract, mainly by intratracheal instillation [52] or (oro)pharyngeal aspiration [48-51, 64-66]. Regarding CNC, they elicited an acute inflammatory response in mice 24 h after a single administration [64]. Pulmonary exposure to repeated doses of CNC resulted in reprotoxic effects in male mice three months after the last administration [65]. Some CNFs appear to be highly inflammogenic 24 h following pulmonary exposure, but the inflammatory response subsides within a month [51, 52]. Interestingly, different inflammatory pathways seem to be involved in response to CNC or CNF exposures [66]. On the other hand, some CNFs seem to show a genotoxic potential [49, 50, 52], which raises concerns about their possible carcinogenicity. Only one study has assessed the toxicity of CNC by inhalation [55]. Rats were exposed to aerosolized CNCs at a maximum concentration of 0.26 mg/L for 4 hours. After monitoring the animals for mortality, gross toxicity, and behavioral changes for a period of 14 d, they were euthanized and subjected to autopsy. No adverse effects were observed.

In all previous studies, the maximum post-treatment period analyzed was up to one month. Shvedova et al. [48] investigated the effects of repeated doses of CNC (resulting in an accumulated dose of 240 µg/mouse) after three months post-exposure, showing an inflammatory response more pronounced in females than male mice. More recently, one study investigated the pulmonary toxicity exerted by BNC nanofibrils after a total period of 6 months [53]. C57BL/6 mice were intratracheally instilled with repeated doses (for three consecutive weeks) of 100 µg/mouse of BNC nanofibrils. Histological analyses revealed a chronic bronchoalveolar inflammation together with alterations in the lung tissue after six months.

As concerns, animal experiments performed by other routes, no skin sensitization, corrosion, or

irritation was demonstrated for CNC using standardized Organization for Economic Co-operation and Development (OECD) Test Guidelines [55]. The same authors did not find acute or sub-chronic toxic effects after oral administration of CNC. Although still very limited in number, the available studies suggest that CNCs are non-toxic upon ingestion or contact with the skin [60]. On the other hand, rats administrated with M-CNF (produced by grinding) by gavage showed no significant differences in hematological and serum markers and histopathological analyses than control animals [18]. In another study, no adverse effects were observed in rats fed with CNF for 90 consecutive days [67]. The CNF was produced by mechanical homogenization, i.e., apparently a type of M-CNF (Table 1).

To date, no human biomonitoring studies specifically dealing with nanocellulose exposure have been performed. The few toxicological studies on nanocellulose-based products in humans are clinical trials for some specific biomedical applications. For instance, CNF (homogenized and without pre-treatment reported by the authors, M-CNF) was applied to develop a wound dressing assessed in a clinical trial on burn patients. No allergic reaction or inflammatory response was observed [17]. On the other hand, a BNC-containing wound dressing incorporating sericin and polyhexamethylene biguanide was assessed by applying it on the skin of healthy volunteers. No signs of irritation were shown on the skin of any of the individuals [68].

3.3. The effect of physicochemical properties

It is well-recognized that physicochemical features of nanomaterials may affect their toxicity [40, 69, 70]. CNC and CNF are produced using different techniques, which dramatically affect their physicochemical characteristics (see Table 1). Hence, they also show different hazard features. CNC, which is internalized by macrophags and lung epithelial cells, triggers an inflammatory response. On the other hand, some types of CNFs, which may not be so efficiently phagocytized or taken up, elicit none or milder inflammatory reaction [62, 71]. Furthermore, both CNC and CNF show less hazardous effects than those produced by other nanofibres that also display a high aspect ratio and show long pulmonary biopersistence, such as multiwalled carbon nanotubes (MWCNTs)[51, 72]. E-CNF appeared

to be more potent than MWCNTs in inducing systemic acute phase response in one in vivo study [52]. Therefore, differences in other physicochemical properties, such as rigidity or metal impurities, may explain the more severe effects of MWCNTs [66].

Differences in some physicochemical properties are also affecting the biological behavior of nanocelluloses belonging to a similar category (e.g., CNC vs. CNF). For instance, the interaction of CNF with dendritic cells depended on the thickness and length of the material [73]. On the other hand, the magnitude of the immune response triggered by three different CNCs in the human lung alveolar epithelial cell line A549 was directly related to their effective particle sizes [71]. Moreover, surface functionalization is indeed one of the key features [60]. Surface functionalized nanocelluloses are increasingly proposed for several applications, such as healthcare products and food packaging, due to the new beneficial properties imparted by the surface modifications [58]. The abundance of hydroxyl and carboxyl groups on the surface of nanocellulose, allowing subsequent modification with polymers, has made this material attractive for drug delivery applications [74, 75]. However, different functionalization will determine differences in the agglomeration rate, hydrophobicity, surface charge, and surface chemistry of nanocelluloses, affecting their cellular uptake, interaction with subcellular organelles downstream biological responses [62]. Surface functionalization, which affects the material surface chemistry and the size (width) and morphology, has been reported to drive the inflammatory response to CNF [16]. A pro-inflammatory response, measured by cytokine secretion, was detected in THP-1 macrophages treated with an E-CNF. However, such an effect was not observed when the surface charge groups carboxymethyl (C-CNF) and hydroxypropyltrimethylammonium were introduced into CNF [16]. On the other hand, the same group has recently assessed the effects of CNFs with different surface modifications (carboxymethylation, hydroxypropyltrimethylammonium substitution, phosphorylation, and sulfoethylation) on the intestinal cell line Caco-2 [58]. In this case, CNF surface functionalization did not have an impact on the cell metabolic activity and cell membrane integrity. The effect of surface charge on the immunological response evoked by two differentially functionalized CNCs has been investigated in a mouse macrophage-like cell line (J774A.1) and human

THP-1 macrophages [76]. The cationic CNCs-poly (aminopropylmethacrylamide, APMA) showed a more robust secretion of inflammatory cytokines in the murine cell line, while the anionic CNCs-poly (N-iso-propylacrylamide, NIPAAm) showed a significant NLRP3 inflammasome-dependent and independent immunological response in human macrophages. Furthermore, mitochondrial function was differentially affected by both types of CNCs. Differential induction of cell morphology changes was previously reported for the same CNCs, with CNCs-poly (NIPAAm) causing cell enlargement and elongation [77]. In another study, the capacity of one type of unmodified CNC and four cationic derivatives of it to stimulate NLRP3-inflammasome-dependent immunological response and enhance the production of mitochondrial reactive oxygen species (ROS) was analyzed in the J774A.1 cell line [78]. Only one of the cationic derivatives activated the inflammatory response, being the presence of amide linkage and fewer cationic polymer brushes the potential modulating factors. On the opposite, nanocellulose (denominated as CNC by the authors) bearing negatively charged carboxylic groups (introduced through TEMPO mediated oxidation), which were used as precursors to obtain hydrogel patches by cation-induced gelation, did not show cytotoxic effects on a human melanoma cell line[79].

A short-term repeated oral toxicity study revealed hepatotoxicity of a CNC modified with oxalate ester in the exposed rats [75]. As pointed out by the authors, the introduction of this functional group at the surface of CNC might increase its hydrophobicity, a surface characteristic that has been associated with increased cytotoxicity and inflammatory response. Unfortunately, the unmodified CNC was not included in the study of Otuechere et al. [75], which precludes raising conclusions on whether the observed deleterious effects were exclusively due to the modification.

Hadrup et al. [52] studied the adverse effects induced in mice exposed to E-CNF and carboxylated CNF, provided by different manufacturers, by intratracheal instillation. They concluded that carboxylation of CNF was associated with reduced pulmonary and systemic toxicity, and suggested the involvement of hydroxy groups in the inflammatory and acute phase responses. This conclusion has to take into account that carboxylation (probably through TEMPO mediated oxidation, T-CNF, Table 1) causes a higher nanofibrillation of pulp fibers, compared to enzymatically pre-treated CNF (E-CNF), i.e., E-CNF

may have a larger fraction of residual fibers, lower nanofibrillation yield and thicker and longer nanofibrils. Hence, conclusions about toxicity and considering only the surface modifications and chemistry of CNFs have to be taken with caution as in most cases, the effect of the surface modification cannot be decoupled from the CNF morphology and physical properties (e.g., width, length, nanofibrillation yield, and residual fibers). This is a crucial point, usually not considered in toxicological studies.

Similar results were found in other studies where the same materials and C-CNF were administrated to mice by (oro)pharyngeal aspiration [50, 51]. CNFs (apparently M-CNF and E-CNF based on the data provided by the authors and producers) were more prone to trigger inflammation [51] and to induce DNA strand breaks in the lungs [50] than those modified by carboxymethylation (C-CNF). Interestingly, when tested in vitro, E-CNF was the only material showing high cytotoxicity and significant increased production of the pro-inflammatory cytokines IL-1β and TNF-α in exposed THP-1 macrophages [51]. But none of the materials induced cytotoxicity or genetic damage in human bronchial epithelial BEAS-2B cells, suggesting that the mechanisms involved in the genotoxic effects detected in vivo are not present in the in vitro model [50]. Alternatively, the increase of pro-inflammatory cytokines caused by E-CNF in this particular study may have been triggered by the relatively high endotoxin levels (1.27 EU/ml) reported by the authors [51].

Far from being an obstacle, the possibility of moderating biological responses by modifying the properties of the materials opens up the option of designing them safer [40, 80]. As concerns biomedical applications, the consideration at early stages of the design of those attributes linked to the safety and efficacy of the product are the pillar of current quality-by-design approaches [81]. In that sense, the characterization needs, which are addressed in currently available regulatory documents for nanotechnology-enabled health products, have been extracted and categorized by the REFINE project [82]. Hence, requirements for characterization were suggested as endpoints for quality and safety assessments. Most of the extracted parameters refer to the nanoscale properties specific

for or associated with the materials, such as size, shape, morphology, or surface properties [82]. However, the success of those strategies requires the existence of validated characterization methods for nanomaterials [70], which are still lacking for a reliable characterization of nanocelluloses, both in complex liquid media used in in vitro cellular models [69] and real occupational settings [46, 83].

3.4. Regulatory frameworks

Nanomaterials are explicitly or implicitly covered by the European Union's (EU) regulatory framework, which consists of several pieces of horizontal and sector-specific legislation, each of them with a defined purpose and scope. For each regulation, specific provisions for the safety assessment and authorization of nanomaterials are applied [84].

As concerns the production of nanocelluloses, they are exempted from the Registration, Evaluation, Authorization, and Restriction of Chemical (REACH) regulation (1907/2006/EC). Cellulose is a natural polymer, and natural polymers (including nanoforms) are exempted from the REACH registration.

Worker protection is regulated in the EU through the directive on safety and health at work (89/391/EEC), the directive on risks related to chemical agents at work (98/24/EC), and their related national legislations. Occupational exposure limit (OEL) values are set to any chemical agent relevant to the work environment, except for carcinogen (regulated by the carcinogens and mutagens directive, 2004/37/EC). As mentioned above, no specific OEL values exist for nanocelluloses. The Permissible Exposure Limit allowable by the Occupational Safety and Health Administration (OSHA, USA) for cellulose dust is 5 mg/m3 for the respirable fraction, expressed as 8-h time-weighted average, TWA [57]. No OEL values for cellulose dust are available at the EU. Instead, several countries (e.g., Finland) use the OEL value for unspecific organic dust as inhalable fraction (5 mg/m3, TWA). On the other hand, an OEL value of 0.01 fibers/cm3 has been recommended for nanocelluloses [85], which is the same value suggested for other biopersistent fibrous nanomaterials, e.g., carbon nanofibers. However, the same authors recommended minimizing the exposure as far as reliable methods for quantitative measurement of air sample concentrations, which would allow comparison with the suggested OEL

value, are available.

Although polymers are exempted from the REACH registration, they should comply with food-related regulations. Both cellulose fibers (millimetric scale) and micro-cellulose (micrometric scale) have been evaluated by the European Food and Safety Agency (EFSA) [86]. As neither intestinal absorption nor toxicity was observed, both materials were considered as safe. A similar statement applies to the chemically modified celluloses that were included in the same assessment. However, other types of modified celluloses should be considered as a different material than those authorized, and a new process to obtain the pre-marketing authorization must start. In the case of nanocelluloses, their nano features may affect the interaction with biological systems. Hence, a specific assessment is required during their safety evaluation, as described by the EFSA Guidance on Nanomaterials [86], which is currently under revision.

To date, no specific regulatory framework exists for nanomaterial-based medical products and devices. Instead, nanotechnology-enabled health products follow current regulatory frameworks for medicinal products or medical devices. However, they may require additional quality and safety assessments triggered by the nanomaterial's unique characteristics [82]. Furthermore, the classification of a product into a medicinal product or a medical device depends on the primary mode of action. Such classification may not always be clearly defined for nanotechnology-enabled health products due to their increasing complexity and high diversity. A product's components may have different modes of action, which are governed by different regulations [82]. Recently, a draft guideline on the quality requirements for drug-device combinations has been released [87].

Most of the 3D printing applications involving nanocelluloses are related to medical devices, which are regulated by the Medical Devices Regulation (EU) 2017/745 (MDR), and the In Vitro Diagnostic Medical Devices Regulation (EU) 2017/746 (IVDR). Both Regulations entered into force in May 2017 and have a staggered transitional period of 4 and 5 years, respectively. The latter does not contain specific requirements regarding nanomaterials, whereas several provisions on nanomaterials are included in the former [82]. The MDR requires special attention when devices have or consist of nanomaterials

that can be released into the patient's or user's body unless they only come into contact with intact skin [82, 84]. An indication informing on the presence of nanomaterials on the device should be included on the device's label. Nanomaterials also require an independent safety assessment, even if the corresponding non-nano sized substance is already authorized. The biological evaluation -included in the safety assessment- can be performed according to the ISO/TC 194 (2012) [88]. The MDR also specifies that medical devices incorporating or consisting of a nanomaterial belong to class III, i.e., the highest risk class, unless the nanomaterial is encapsulated or bound in such a manner that it cannot be released into the patient's or user's body when the device is used within its intended purpose [84]. The guidance adopted by the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) on the Determination of Potential Health Effects of Nanomaterials Used in Medical Devices [89] can help to clarify how the MDR should be applied in practice. A summary of it can be found in Annex B of the REFINE white paper [82].

3.5. Safety assessment and testing strategies

Safety assessment is required in all the above-described regulations, most of which agree on the human health and environmental effects that should be addressed [90]. Hazard assessment generally relies on several toxicological endpoints that are assessed using validated test guidelines or guidance documents, most of them still requiring animal experiments. However, new alternative methods, in agreement with the 3R principles, may replace them in the future [91]. 3R means that all animal tests should be replaced by alternative methods when this is possible (Replace), reduce the number of animals used much as possible without compromising the quality of the data (Reduce), and that all experimental procedures are performed in a way that minimizes suffering, stress and pain of the animals (Refine).Besides, nanotechnology-enabled health products always require clinical trials before their use in clinical practice can be approved [82]. Therefore, the production and commercialization of nanocelluloses and nanocellulose-based products will have to comply with the corresponding

regulatory requirements, depending on the products' intended final use. Nevertheless, it is highly advised to screen the toxic potential of the nanocelluloses, based on in vitro assays, at the pre-commercialization or pre-clinical stages, which allows supporting safe-by-design and quality-by-design strategies [41]. Figure 2 summarizes the different steps of the testing strategies that have been proposed, which would allow a safety assessment of nanocelluloses before their regulatory approval for medical applications.

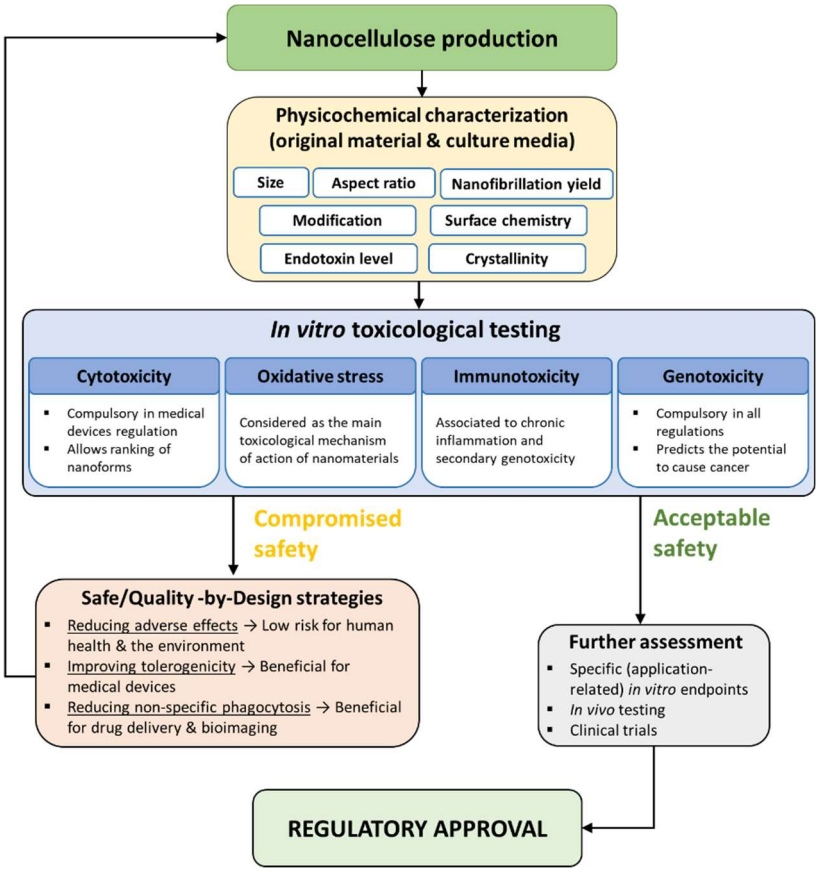

Fig. 2 A testing strategy for the safety assessment of nanocelluloses used in medical applications (based on Ventura et al. [62] and Čolić et al. [63]).

One of the challenges in testing nanomaterials is that the observed toxic effects may change from one nanoform to another similar one that shows a slight variation in any of its physicochemical features [92]. Hence, a thorough characterization of the tested nanomaterials is required [70, 92]. Although protocols for characterizing nanocelluloses have recently been proposed [93], not well-validated methods are still available, especially concerning characterization in culture media [69].

Bacterial endotoxins are lipopolysaccharides originating from the outer wall of Gram-negative bacteria. Endotoxins are known to trigger inflammation, and they may induce oxidative stress and, subsequently, other toxic effects (e.g., DNA damage) [94]. Therefore, demonstration of a level of endotoxins sufficiently low in the material is recommended when investigating the toxicity of nanomaterials [95], especially for immunotoxicity testing, and is required for biomedical applications [3, 94]. As bacterial endotoxins are common contaminators of naturally derived materials, endotoxin testing is especially relevant in the case of nanocelluloses [3]. However, endotoxin testing is challenging as nanomaterials may interfere with the endotoxin assays [94]. Polymyxin B has sometimes been used in parallel immunotoxicity experiments to inhibit the potential effects of any endotoxin present in the CNF samples [16]. However, it is more desired to produce and handle the nanocelluloses in an environment as much endotoxin-free as possible. An illustrative example is the method that Nordli et al. [3] developed to obtain ultrapure T-CNF suitable for wound dressings.

Several testing strategies suitable for nanomaterials have been proposed in the last years [96-98]. All of them suggest a battery of assays to assess key endpoints involved in nanomaterials-induced adverse cellular effects [91]. Nanomaterials can induce cells to produce reactive oxygen species (ROS), which may lead to pro-inflammatory effects. Increased ROS levels produced directly by the nanomaterial or as a consequence of the inflammatory response can result in DNA or chromosome damage [62, 91]. Each of these endpoints can be assessed using methods already existing for conventional chemicals. However, many of the methods need to be modified when applied to nanomaterials, mainly due to the materials' interference with the assays [84].

Cytotoxicity is usually the first step in assessing the toxicity of nanomaterials. It is one of the endpoints requested for testing of medical devices to obtain regulatory approval [Medical Devices Regulation (EU) 2017/745 (MDR)], and it is also required as a pre-test for establishing the range of doses to be evaluated in the genotoxicity assays [96]. However, although cytotoxicity assays are useful for the early screening and ranking of nanomaterials, they do not provide information on the type of hazardous

event and the possible mechanism of action. Furthermore, the lack of cytotoxicity does not mean lack of hazardous effects [41]. For instance, Lopes et al. [16] reported inflammatory effects induced by E-CNFs (Table 1) in macrophages at doses that did not impair the cells' viability.

Generation of ROS is a normal mechanism in maintaining cellular metabolism, but when produced in excess (oxidative stress) results in adverse effects [75]. As oxidative stress is the prevailing paradigm in explaining how nanomaterials induce adverse cellular effects [91], this endpoint is included in most of the testing strategies proposed for nanomaterials, including the one suggested by Endes et al. [99] to mimic the inhalation of high aspect ratio nanoparticles in a 3D lung model. Endes et al's and other strategies also recommend assessing immunotoxicity [97, 99], as inflammation is one of the initial steps that may give rise to lung fibrosis, secondary genotoxic effects, and carcinogenesis after inhaling biopersistent nanofibers [41]. Furthermore, immunotoxicity testing is part of the regulatory assessment of nanotechnology-enabled health products [82]. Finally, genotoxicity is a critical endpoint in the toxicity testing of nanomaterials [96, 97], as it is a hazard endpoint required in all the regulations previously described. It is because of the critical consequences of mutations on human health, as they play a crucial role in the initiation and progression of carcinogenesis, and reproductive and developmental abnormalities [41].

Current in vitro toxicological testing of nanomaterials is limited by the the ability of the present assays to deal with secondary toxic mechanisms and organ specificity that are fully present only in a whole organism in vivo [41]. Co-culture of, e.g., inflammatory and target cells and 3D tissue models may help in detecting secondary effects of nanomaterials, although the number of studies utilizing these techniques is scarce. In the case of nanocelluloses, an in vitro multicellular model of lung epithelium using an air-liquid interface cell exposure system was used to assess the specific fibre-cell interactions of two types of CNCs [100]. On the other hand, Ventura et al. [44] used a co-culture of A549 and THP-1 cells to assess the toxicity of T-CNFs. More recently, an advanced intestinal co-culture model consisting of Raji B, Caco-2, and HT29- MTX cells has been used for toxicological testing of CNFs [101]. Furthermore, omics approaches can present a supporting tool in elucidating the prevailing

mechanisms of nanomaterials' toxicity. To date, only one study has assessed gene expression changes in the lung tissue of mice three months post-exposure to CNC using a high-throughput mRNA microarray [48]. However, more data is needed to validate these methods before making a clear conclusion and recommendations about their applicability [91].

4. 3D bioprinting of biomaterials and model tissue constructs

Significant advances have been made regarding 3D printing of biomaterials, and 3D bioprinting, including cell-laden bioinks, as well as supporting components into complex 3D functional scaffolds [102]. Hence, 3D bioprinting, which involves additional complexities, should consider the technical printing and the interaction of the biomaterial with the cells. However, there is still a lack of knowledge concerning the materials for bioprinting [103].

3D bioprinting implies requirements regarding the biomaterial preferences: the material must be dispensable in the technologies used for deposition and patterning, it needs to quickly solidify after material dispersing, maintain volume during and after 3D printing (not swell or shrink). A biomaterial to be tested for 3D bioprinting of, e.g., tissue models, medical devices, and vehicles for drug delivery, should be biocompatible and safe, and this should be ensured as described in the previous sections. During 3D bioprinting, each layer should be joined together to keep the structure, mechanical characteristics should be similar to the in vivo situation, and it should be stable in a growth environment for cells, often 37 °C. Further, the material must allow cells to attach, migrate and proliferate; it should not be cytotoxic (as explained in the previous sections), and depending on the application, it should be degradable [102, 104].

Materials for 3D printing falls under two main categories, distinguished by their components and means of production, naturally derived and principally synthetic. Examples of naturally derived biomaterials for 3D printing are nanocellulose, collagen hydrogels, gelatin, agarose, hyaluronic acid-based hydrogels, alginate, and chitosan-alginate composite scaffolds [105]. Synthetic inks often consist of poly(ethylene glycol) (PEG), Polyvinylpyrrolidone (PVP), poly(lactic-co-glycolic acid) (PLGA) or

Polycaprolactone (PLC).

Biomaterials are often combined to optimize a bioink; for example, nanocellulose has been mixed with alginate to obtain better shear thinning, cell survival, cellular differentiation, and to keep a stable construct during culturing [26, 106]. This exemplifies an essential aspect of biomaterials, i.e., that a single biomaterial cannot meet all the criteria necessary to fabricate a functional bioink for bioprinting [107].

Bioinks are utilized to fabricate scaffolds with specific shapes, sizes, and geometrical complexities to create 3D tissue constructs that may mimic the human body [108]. 3D structures enable different cell responses, compared to the corresponding 2D structures, e.g., integrin expression, cell migration, cell mechanics, proliferation, differentiation, stemness [109-113]. The possibility to bioprint scaffolds to mimic the human microenvironment as in vitro models makes it possible for various applications, such as tissue engineering, regenerative medicine, drug screening with high-throughput assays, wound dressing, transplantation, and clinical application [108]. Different tissue constructs that mimic native tissues and organs have been successfully bioprinted utilizing several 3D printing approaches, for example; skin [114], cardiac [115], bone [116], cartilage [117], liver [118], and lung [119]. However, the fabrication of fully functional tissue models and organs is still demanding due to limitations regarding, e.g., vascularization [120].

3D bioprinting has been an alternative for tissue engineering and regenerative medicine and a robust drug screening tool and discovery tool. 3D bioprinting will facilitate these in vitro models that potentially represent the specific pathological environment of patients. Remember that the lack of relevant human mimicking pre-clinical models is a primary reason for drug candidates failing in clinical trials [121, 122]. Additionally, multiple cancer cell lines have exhibited more drug resistance when cultured in 3D than 2D [123-125], and drug resistance in the 3D cultures has also been shown more similar to in vivo models [126-130]. It is also important to emphasize that there are increasing legal requirements and public opinions for the use of alternative, non-animal models in the regulatory safety assessment of chemicals, drugs, and medical devices [131]. It is considered one of the significant

driving forces regarding the research community's efforts to develop fully functional tissue models by 3D bioprinting technologies. It is important to emphasize that the research community, pharmaceutical companies, and regulatory instances are striving to work according to 3R (Replace, Reduce, Refine).

4.1. Nanocellulose-based inks for 3D printing

Specifically, nanocelluloses have demonstrated a considerable potential to be utilized in 3D printing of several medical devices and tissue models, including wound dressings, tissue engineering models, and drug delivery. The performance of nanocellulose inks on 3D printing operations depends on various factors, including i) the concentration, ii) the rheology, iii) surface chemistry, and iv) the cellulose nanofibril and cellulose nanocrystal physical characteristics.

The research and development of nanocelluloses have advanced, and this is exemplified by various companies that are presently offering nanocelluloses that can be used for 3D printing applications. Companies were requested to provide information about their corresponding (semi)commercial products, and the information provided by the companies that kindly responded to this request is listed in Table 2.

The commercial nanocelluloses are obtained from different sources from woody biomass (soft- and hardwood) and marine animals (Tunicates), applying various pre-treatments and consequently have different characteristics (Table 2). It is crucial to provide such specifications relevant to the 3D printing process and understand the application of nanocellulose-based inks for specific 3D bioprinting purposes and the corresponding biological effects.

Various types of nanocelluloses can be applied for 3D bioprinting processes [26, 132-135]. The concentration of nanocellulose inks can also be tuned, which may affect the structure of the scaffolds. The porosity and pore connectivity in scaffolds are essential for the diffusion of nutrients during the

maturing of tissue models. The structures of the 3D printed scaffolds are observed in Fig. 3. Note the differences regarding the porosity and pore wall roughness, which are most probably caused by the nanofibril morphology.

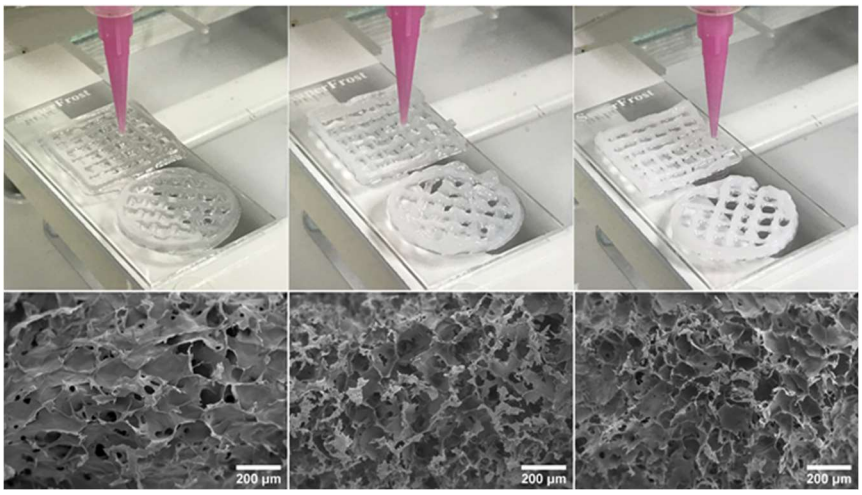

Fig. 3 3D printing of different CNFs and the corresponding pore structures of the printed and freeze-dried structures. Left) T-CNF (2.5 wt%, produced by RISE PFI). Middle) TUNICELL ETC CNF (2.5 wt produced by Ocean TuniCell AS). Right) Exilva CNF (5 wt%, produced by Borregaard). All the scaffolds were 3D printed with a Regemat3D printing unit, utilizing a nozzle of 0.41 mm and a speed of 3 mm/s. The same settings were used in all the printing operations, and no attempt was made to improve the 3D print quality.

Compared to BNC, which has been extensively developed for wound dressings [136], T-CNFs have been proposed as a good alternative as it can form translucent structures, with good liquid absorption, adequate mechanical strength in wet conditions [137], some antimicrobial properties [138, 139] and particular immunogenic properties [140, 141]. Also, nanocelluloses are in general 3D printable (Fig. 3), which opens the possibility to 3D bioprint skin constructs, e.g., testing wound dressings or for medical use in wound healing situations.

As mentioned above, one of the potential and promising applications of nanocellulose-based inks is the fabrication of tissue models for, e.g., cancer research (Fig. 4, Fig. 5). Scaffolds can be 3D printed,

freeze-dried, and seeded with cancer cells. The scaffolds are incubated for a limited time, and laboratory testing can be performed, e.g., gene expression analysis, western blot, flow cytometry, and functional testing (Fig. 4).

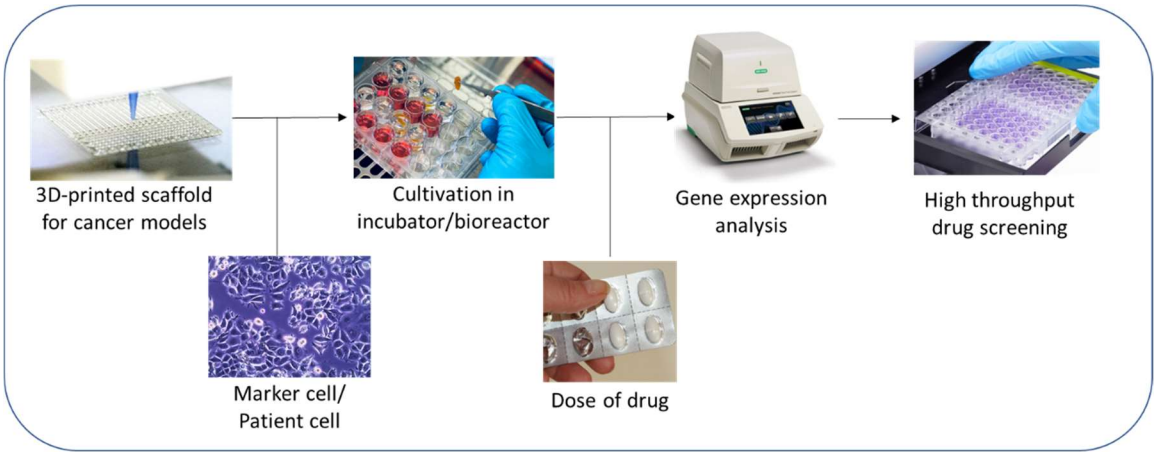

Fig. 4 Workflow of drug screening using 3D printed tissue scaffold and gene expression analysis.

In Fig. 5, breast cancer cells have been grown on T-CNF and imaged using scanning electron microscopy (SEM). It clearly shows that cells are attaching to the surface, and there are many different phenotypes of cells. Note the different shapes such as elongated and rounded up. Further, it also shows that cell interconnection exists between adjacent cancer cells and cells that are far from each other. It is considered a promising observation as cancer cells can be grown on T-CNF scaffolds. The next steps currently being explored are the maturation of such constructs and the cancer cells' corresponding characterization through gene expression analysis. This approach will potentially facilitate cancer tissue models that can be applied for drug screening and, thus, developing personalized medicine for cancer treatment.

It is important to note that nanocelluloses used for 3D printing of tissue models, e.g., drug screening, could be easier to commercialize than nanocelluloses that will be applied for, e.g., tissue engineering

and medical devices that will be in direct contact with human tissue. This is due to the extensive regulations applied to nanomaterials (Regulation (EU) 2017/745) that can be released into the patient's or user's body. However, the development of nanocellulose for 3D printing of tissue models should follow the Regulation (EU) 2017/746 on In-Vitro Diagnostic Devices.

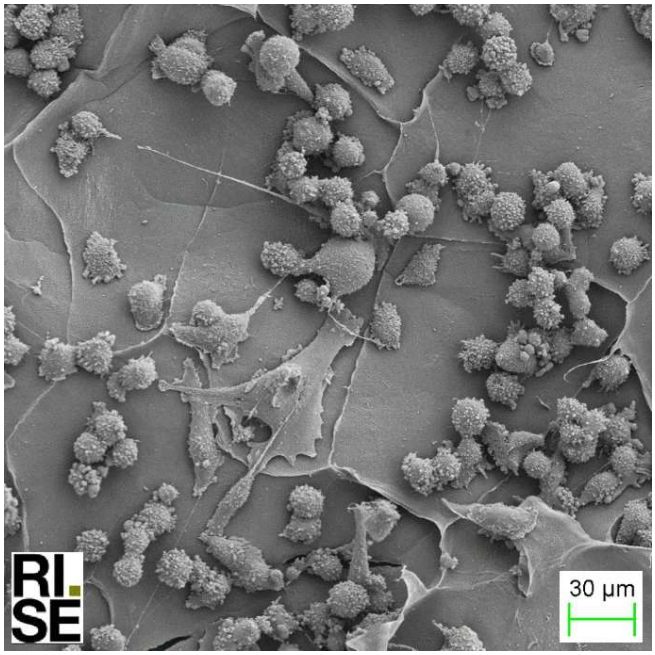

Fig. 5 Cancer cells grown on T-CNF 3D printed scaffolds. The concentration of the T-CNF was 1%, and the scaffolds were freeze-dried.

5. Concluding remarks

This book chapter has provided an overview of nanotoxicology and safety aspects associated with different types of nanocelluloses, including CNC and CNF. Although the assessed nanocelluloses are mainly obtained from woody biomass, different kinds of nanocelluloses can be produced from tunicates or bacterial biosynthesis. Relevant regulatory requirements were considered. Finally, a short overview was provided of (semi)commercial nanocelluloses that are or can be used as ink components for 3D bioprinting, and a specific example towards bioprinting of cancer tissue model was exemplified.

This demonstrates the potential of nanocellulose as a natural biopolymer for biomedical applications, also considering aspects related to regulatory approval before commercialization.

Acknowledgments

The authors would like to thank Dr Piia Taxell (Finnish Institute of Occupational Health) and the European Chemicals Agency (ECHA) for providing information on the workers' regulations and the current status of nanocelluloses under the REACH regulation, respectively. Sarunas Petronis (RISE) is acknowledged for acquiring the SEM image with cancer cells. This paper was funded by the Finnish Work Environment Fund (grant no. 117146), Swedish foundation for strategic research and by the OxyPol project, Nano2021 program, Research Council of Norway (grant no. 309178).

References

1. Mertaniemi, H., et al., *Human stem cell decorated nanocellulose threads for biomedical applications.* Biomaterials, 2016. **82**: p. 208-20.
2. Lou, Y.R., et al., *The Use of Nanofibrillar Cellulose Hydrogel As a Flexible Three-Dimensional Model to Culture Human Pluripotent Stem Cells.* Stem Cells and Development, 2014. **23**(4): p. 380-392.
3. Nordli, H.R.C.-C., G.; Rokstad, A. M.; Pukstad, B., *Producing ultrapure wood cellulose nanofibrils and evaluating the cytotoxicity using human skin cells.* Carbohydr Polym, 2016. **150**: p. 65-73.
4. Kuzmenko, V., et al., *Tailor-made conductive inks from cellulose nanofibrils for 3D printing of neural guidelines.* Carbohydr Polym, 2018. **189**: p. 22-30.
5. Rashad, A., et al., *Cytocompatibility of Wood-Derived Cellulose Nanofibril Hydrogels with Different Surface Chemistry.* Biomacromolecules, 2017. **18**(4): p. 1238-1248.

6. Chimene, D., R. Kaunas, and A.K. Gaharwar, *Hydrogel Bioink Reinforcement for Additive Manufacturing: A Focused Review of Emerging Strategies.* Adv Mater, 2020. **32**(1): p. e1902026.

7. Chiulan, I., et al., *Recent Advances in 3D Printing of Aliphatic Polyesters.* Bioengineering (Basel), 2017. **5**(1).

8. Chinga-Carrasco, G., *Potential and Limitations of Nanocelluloses as Components in Biocomposite Inks for Three-Dimensional Bioprinting and for Biomedical Devices.* Biomacromolecules, 2018. **19**(3): p. 701-711.

9. Marga, F., et al., *Toward engineering functional organ modules by additive manufacturing.* Biofabrication, 2012. **4**(2): p. 022001.

10. Wang, X., Q. Wang, and C. Xu, *Nanocellulose-Based Inks for 3D Bioprinting: Key Aspects in Research Development and Challenging Perspectives in Applications-A Mini Review.* Bioengineering (Basel), 2020. **7**(2).

11. Xu, W., et al., *Three-Dimensional Printing of Wood-Derived Biopolymers: A Review Focused on Biomedical Applications.* ACS Sustain Chem Eng, 2018. **6**(5): p. 5663-5680.

12. Saito, T., et al., *Homogeneous suspensions of individualized microfibrils from TEMPO-catalyzed oxidation of native cellulose.* Biomacromolecules, 2006. **7**(6): p. 1687-91.

13. Wagberg, L., et al., *The build-up of polyelectrolyte multilayers of microfibrillated cellulose and cationic polyelectrolytes.* Langmuir, 2008. **24**(3): p. 784-95.

14. Fukuzumi, H., T. Saito, and A. Isogai, *Influence of TEMPO-oxidized cellulose nanofibril length on film properties.* Carbohydr Polym, 2013. **93**(1): p. 172-7.

15. Habibi, Y., L.A. Lucia, and O.J. Rojas, *Cellulose nanocrystals: chemistry, self-assembly, and applications.* Chem Rev, 2010. **110**(6): p. 3479-500.

16. Lopes, V.R.S.-M., C.; Strømme, M.; Ferraz, N., *In vitro biological responses to nanofibrillated cellulose by human dermal, lung and immune cells: surface chemistry aspect.* Part Fibre Toxicol, 2017. **14**(1): p. 1.

17. Hakkarainen, T., et al., *Nanofibrillar cellulose wound dressing in skin graft donor site treatment.* J Control Release, 2016. **244**(Pt B): p. 292-301.

18. DeLoid, G.M.C., X.; Molina, R. M.; Silva, D. I.; Bhattacharya, K.; Ng, K. W.; Loo, S. C. J.; Brain, J. D.; Demokritou, P., *Toxicological effects of ingested nanocellulose in in vitro intestinal epithelium and in vivo rat models.* Environ Sci Nano, 2019. **6**(7): p. 2105-2115.

19. Fall, A.B., A. Burman, and L. Wagberg, *Cellulosic nanofibrils from eucalyptus, acacia and pine fibers.* Nordic Pulp & Paper Research Journal, 2014. **29**(1): p. 176-184.

20. Liimatainen, H., et al., *Enhancement of the Nanofibrillation of Wood Cellulose through Sequential Periodate-Chlorite Oxidation.* Biomacromolecules, 2012. **13**(5): p. 1592-1597.

21. Chinga-Carrasco, G. and K. Syverud, *Pretreatment-dependent surface chemistry of wood nanocellulose for pH-sensitive hydrogels.* J Biomater Appl, 2014. **29**(3): p. 423-32.

22. Kokol, V., et al., *Characterisation and properties of homo- and heterogenously phosphorylated nanocellulose.* Carbohydr Polym, 2015. **125**: p. 301-13.

23. Dri, F.L.H.J., L. G.; Moon, R. J.; Zavattieri, P. D., *Anisotropy of the elastic properties of crystalline cellulose Iβ from first principles density functional theory with Van der Waals interactions.* Cellulose, 2013. **20**: p. 2703–2718.

24. Heggset, E.B., G. Chinga-Carrasco, and K. Syverud, *Temperature stability of nanocellulose dispersions.* Carbohydr Polym, 2017. **157**: p. 114-121.

25. Le Van, H., *Properties of nano-fibrillated cellulose and its length-width ratio determined by a new method.* Cellulose Chemistry and Technology, 2017. **51** ((7-8)): p. 649-653.

26. Markstedt, K., et al., *3D Bioprinting Human Chondrocytes with Nanocellulose-Alginate Bioink for Cartilage Tissue Engineering Applications.* Biomacromolecules, 2015. **16**(5): p. 1489-96.

27. Moberg, T., et al., *Rheological properties of nanocellulose suspensions: effects of fibril/particle dimensions and surface characteristics.* Cellulose, 2017. **24**(6): p. 2499-2510.

28. Tanaka, R., et al., *Determination of nanocellulose fibril length by shear viscosity measurement.* Cellulose, 2014. **21**(3): p. 1581-1589.

29. Sacui, I.A., et al., *Comparison of the Properties of Cellulose Nanocrystals and Cellulose Nanofibrils Isolated from Bacteria, Tunicate, and Wood Processed Using Acid, Enzymatic, Mechanical, and Oxidative Methods.* Acs Applied Materials & Interfaces, 2014. **6**(9): p. 6127-6138.

30. Gorgieva, S. and J. Trcek, *Bacterial Cellulose: Production, Modification and Perspectives in Biomedical Applications.* Nanomaterials (Basel), 2019. **9**(10).

31. Molina-Ramirez, C., et al., *Effect of Different Carbon Sources on Bacterial Nanocellulose Production and Structure Using the Low pH Resistant Strain Komagataeibacter Medellinensis.* Materials, 2017. **10**(6).

32. Choi, S.M. and E.J. Shin, *The Nanofication and Functionalization of Bacterial Cellulose and Its Applications.* Nanomaterials (Basel), 2020. **10**(3).

33. Gutierrez, E., et al., *3D Printing of Antimicrobial Alginate/Bacterial-Cellulose Composite Hydrogels by Incorporating Copper Nanostructures.* Acs Biomaterials Science & Engineering, 2019. **5**(11): p. 6290-6299.

34. Kimura, S.I., T. , *New cellulose synthesizing complexes (terminal complexes) involved in animal*

cellulose bio, synthesis in the tunicate Metandrocarpa uedai. Protoplasma, 1996. **194**: p. 151-163

35. Zhao, Y., et al., *Cellulose Nanofibers from Softwood, Hardwood, and Tunicate: Preparation-Structure-Film Performance Interrelation.* ACS Appl Mater Interfaces, 2017. **9**(15): p. 13508-13519.

36. Endes, C.C.-E., S.; Mueller, S.; Foster, E. J.; Petri-Fink, A.; Rothen-Rutishauser, B.; Weder, C.; Clift, M. J., *A critical review of the current knowledge regarding the biological impact of nanocellulose.* J Nanobiotechnology, 2016. **14**(1): p. 78.

37. Donaldson, K.M., F. A.; Duffin, R.; Poland, C. A., *Asbestos, carbon nanotubes and the pleural mesothelium: a review of the hypothesis regarding the role of long fibre retention in the*

parietal pleura, inflammation and mesothelioma. Particle and Fibre Toxicology, 2010. **7**(5): p. 1-17.

38. Stanton, M.F., *Some etioligical considerations of fibre carcinogenesis., 289-294.* In: 'Biological effects of asbestos' WHO IARC, 1973. **LyonBogovski P, Gilson JC, Timbrell V, Wagner JC**

39. Donaldson, K. and C.A. Poland, *Nanotoxicology: new insights into nanotubes.* Nat Nanotechnol, 2009. **4**(11): p. 708-10.

40. Lynch, I., C. Weiss, and E. Valsami-Jones, *A strategy for grouping of nanomaterials based on key physico-chemical descriptors as a basis for safer-by-design NMs.* Nano Today, 2014. **9**(3): p. 266-270.

41. Catalán, J. and H. Norppa, *Safety Aspects of Bio-Based Nanomaterials.* Bioengineering (Basel), 2017. **4**(4).

42. Stoudmann, N., et al., *Human hazard potential of nanocellulose: quantitative insights from the literature.* Nanotoxicology, 2020: p. 1-17.

43. Chinga-Carrasco, G., *Cellulose fibres, nanofibrils and microfibrils: The morphological sequence of MFC components from a plant physiology and fibre technology point of view.* Nanoscale Res Lett, 2011. **6**(1): p. 417.

44. Ventura, C.L., A. F.; Sousa-Uva, A.; Ferreira, P. J. T.; Silva, M. J., *Evaluating the genotoxicity of cellulose nanofibrils in a co-culture of human lung epithelial cells and monocyte-derived macrophages.* Toxicol Lett, 2018. **291**: p. 173-183.

45. Ede, J.D., et al., *Risk Analysis of Cellulose Nanomaterials by Inhalation: Current State of Science.* Nanomaterials (Basel), 2019. **9**(3).

46. Shatkin, J.A. and B. Kim, *Cellulose nanomaterials: life cycle risk assessment, and environmental health and safety roadmap.* Environmental Science: Nano, 2015. **2**(5): p. 477-499.

47. Stefaniak, A.B., et al., *Lung biodurability and free radical production of cellulose nanomaterials.* Inhal Toxicol, 2014. **26**(12): p. 733-49.

48. Shvedova, A.A.K., E. R.; Yanamala, N.; Farcas, M. T.; Menas, A. L.; Williams, A.; Fournier, P. M.; Reynolds, J. S.; Gutkin, D. W.; Star, A.; Reiner, R. S.; Halappanavar, S.; Kagan, V. E., *Gender differences in murine pulmonary responses elicited by cellulose nanocrystals.* Part Fibre Toxicol, 2016. **13**(1): p. 28.

49. Catalán, J.R., E.; Aimonen, K.; Hannukainen, K. S.; Suhonen, S.; Vanhala, E.; Moreno, C.; Meyer, V.; Perez, D. D.; Sneck, A.; Forsström, U.; Højgaard, C.; Willemoes, M.; Winther, J. R.; Vogel, U.; Wolff, H.; Alenius, H.; Savolainen, K. M.; Norppa, H., *Genotoxic and inflammatory effects of nanofibrillated cellulose in murine lungs.* Mutagenesis, 2017. **32**(1): p. 23-31.

50. Lindberg, H.K.C., J.; Aimonen, K.J.; Wolff, H.; Wedin, I.; Nuopponen, M.; Savolainen, K.M.; Norppa, H., *Evaluation of the genotoxic potential of different types of nanofibrillated celluloses.* TechConnect Briefs, 2017: p. 229–232.

51. Ilves, M.V., S.; Aimonen, K.; Lindberg, H. K.; Pesonen, S.; Wedin, I.; Nuopponen, M.; Vanhala, E.; Højgaard, C.; Winther, J. R.; Willemoës, M.; Vogel, U.; Wolff, H.; Norppa, H.; Savolainen, K.; Alenius, H., *Nanofibrillated cellulose causes acute pulmonary inflammation that subsides within a month.* Nanotoxicology, 2018. **12**(7): p. 729-746.

52. Hadrup, N.K., K. B.; Berthing, T.; Wolff, H.; Bengtson, S.; Kofoed, C.; Espersen, R.; Højgaard, C.; Winther, J. R.; Willemoës, M.; Wedin, I.; Nuopponen, M.; Alenius, H.; Norppa, H.; Wallin, H.; Vogel, U., *Pulmonary effects of nanofibrillated celluloses in mice suggest that carboxylation lowers the inflammatory and acute phase responses.* Environ Toxicol Pharmacol, 2019. **66**: p. 116-125.

53. Silva-Carvalho, R.S., J. P.; Ferreirinha, P.; Leitão, A. F.; Andrade, F. K.; Gil da Costa, R. M.; Cristelo, C.; Rosa, M. F.; Vilanova, M.; Gama, F. M., *Inhalation of Bacterial Cellulose Nanofibrils Triggers an Inflammatory Response and Changes Lung Tissue Morphology of Mice.* Toxicol Res, 2019. **35**(1): p. 45-63.

54. Vartiainen, J.P., T.; Sirola, K.; Pylkkänen, L.; Alenius, H.; Hokkinen, J.; Tapper, U.; Lahtinen, P.; Kapanen, A.; Putkisto, K.; Hiekkataipale, P.; Eronen, P.; Ruokolainen, J.; Laukkanen, A., *Health and environmental safety aspects of friction grinding and spray drying of microfibrillated cellulose.* Cellulose, 2011. **18**(3): p. 775-786.

55. O'Connor, B.B., R.; Goguen, R. , *Commercialization of Cellulose Nanocrystal (NCC™) Production: A Business Case Focusing on the Importance of Proactive EHS Management*, in *Nanotechnology Environmental Health and Safety*, M. Hull, Bowman, D., Editor. 2014, Elsevier Inc.: Oxford, UK. p. 225–246.

56. Martinez KF, E.A., Rudie A, Geraci C *Occupational exposure characterization during the manufacture of cellulose nanomaterials. In: Production and applications of cellulose nanomaterials*. 2014, TAPPI Press. p. 61–64.

57. Shatkin, J.A.O., G., *Comment on Shvedova et al. (2016), "gender differences in murine pulmonary responses elicited by cellulose nanocrystals".* Part Fibre Toxicol, 2016. **13**(1): p. 59.

58. Lopes, V.R.S., M.; Ferraz, N., *In vitro biological impact of nanocellulose fibers on human gut bacteria and gastrointestinal cells.* Nanomaterials, 2020. **10**(6).

59. Reid, M.S., M. Karlsson, and T. Abitbol, *Fluorescently labeled cellulose nanofibrils for detection and loss analysis.* Carbohydrate Polymers, 2020. **250**: p. 116943.

60. Roman, M., *Toxicity of Cellulose Nanocrystals: A Review.* Industrial Biotechnology, 2015. **11**(1): p. 25-33.

61. Seabra, A.B.B., J. S.; Fávaro, W. J.; Paula, A. J.; Durán, N., *Cellulose nanocrystals as carriers in medicine and their toxicities: A review.* Carbohydr Polym, 2018. **181**: p. 514-527.

62. Ventura, C.P., F.; Lourenço, A. F.; Ferreira, P. J. T.; Louro, H.; Silva, M. J., *On the toxicity of cellulose nanocrystals and nanofibrils in animal and cellular models.* Cellulose, 2020. **27**(10): p. 5509-5544.

63. Čolić, M.T., S.; Bekić, M., *Immunological aspects of nanocellulose.* Immunology Letters, 2020. **222**: p. 80-89.

64. Yanamala, N.F., M. T.; Hatfield, M. K.; Kisin, E. R.; Kagan, V. E.; Geraci, C. L.; Shvedova, A. A., *In vivo evaluation of the pulmonary toxicity of cellulose nanocrystals: A renewable and sustainable nanomaterial of the future.* ACS Sustainable Chemistry and Engineering, 2014. **2**(7): p. 1691-1698.

65. Farcas, M.T.K., E. R.; Menas, A. L.; Gutkin, D. W.; Star, A.; Reiner, R. S.; Yanamala, N.; Savolainen, K.; Shvedova, A. A., *Pulmonary exposure to cellulose nanocrystals caused deleterious effects to reproductive system in male mice.* J Toxicol Environ Health A, 2016. **79**(21): p. 984-997.

66. Park, E.J., et al., *Fibrous nanocellulose, crystalline nanocellulose, carbon nanotubes, and crocidolite asbestos elicit disparate immune responses upon pharyngeal aspiration in mice.* J Immunotoxicol, 2018. **15**(1): p. 12-23.

67. Ong, K.J., et al., *A 90-day dietary study with fibrillated cellulose in Sprague-Dawley rats.* Toxicol Rep, 2020. **7**: p. 174-182.

68. Napavichayanun, S., R. Yamdech, and P. Aramwit, *The safety and efficacy of bacterial nanocellulose wound dressing incorporating sericin and polyhexamethylene biguanide: in vitro, in vivo and clinical studies.* Arch Dermatol Res, 2016. **308**(2): p. 123-32.

69. Bitounis, D., et al., *Dispersion preparation, characterization, and dosimetric analysis of cellulose nano-fibrils and nano-crystals: Implications for cellular toxicological studies.* NanoImpact, 2019. **15**.

70. OECD, *Physical-chemical decision framework to inform decisions for risk assessment of manufactured nanomaterials,.* ENV/JM/MONO(2019)12, Series on the Safety of Manufactured Nanomaterials, 2019. **90**.

71. Menas, A.L.Y., N.; Farcas, M. T.; Russo, M.; Friend, S.; Fournier, P. M.; Star, A.; Iavicoli, I.; Shurin, G. V.; Vogel, U. B.; Fadeel, B.; Beezhold, D.; Kisin, E. R.; Shvedova, A. A., *Fibrillar vs crystalline nanocellulose pulmonary epithelial cell responses: Cytotoxicity or inflammation?* Chemosphere, 2017. **171**: p. 671-680.

72. Clift, M.J.D.F., E. J.; Vanhecke, D.; Studer, D.; Wick, P.; Gehr, P.; Rothen-Rutishauser, B.; Weder, C., *Investigating the interaction of cellulose nanofibers derived from cotton with a sophisticated 3D human lung cell coculture.* Biomacromolecules, 2011. **12**(10): p. 3666-3673.

73. Tomić, S., et al., *Native cellulose nanofibrills induce immune tolerance in vitro by acting on dendritic cells.* Scientific Reports, 2016. **6**(1): p. 31618.

74. Khine, Y.Y.B., R.; Raveendran, R.; Stenzel, M. H., *Photo-Induced Modification of Nanocellulose: The Design of Self-Fluorescent Drug Carriers.* Macromol Rapid Commun, 2020. **41**(1): p. e1900499.

75. Otuechere, C.A.A., A.; Adebayo, O. L.; Ebigwei, I. A., *In vivo hepatotoxicity of chemically modified nanocellulose in rats.* Hum Exp Toxicol, 2020. **39**(2): p. 212-223.

76. Despres, H.W.S., A.; Anderson, P.; Hemraz, U. D.; Boluk, Y.; Sunasee, R.; Ckless, K., *Mechanisms of the immune response cause by cationic and anionic surface functionalized cellulose nanocrystals using cell-based assays.* Toxicol In Vitro, 2019. **55**: p. 124-133.

77. Jimenez, A.S., et al., *Effect of surface organic coatings of cellulose nanocrystals on the viability of mammalian cell lines.* Nanotechnol Sci Appl, 2017. **10**: p. 123-136.

78. Sunasee, R.A., E.; Pyram, D.; Hemraz, U. D.; Boluk, Y.; Ckless, K., *Cellulose nanocrystal cationic derivative induces NLRP3 inflammasome-dependent IL-1β secretion associated with mitochondrial ROS production.* Biochem Biophys Rep, 2015. **4**: p. 1-9.

79. Meschini, S.P., E.; Maestri, C. A.; Condello, M.; Bettotti, P.; Condello, G.; Scarpa, M., *In vitro toxicity assessment of hydrogel patches obtained by cation-induced cross-linking of rod-like cellulose nanocrystals.* J Biomed Mater Res B Appl Biomater, 2020. **108**(3): p. 687-697.

80. Kraegeloh, A., et al., *Implementation of Safe-by-Design for Nanomaterial Development and Safe Innovation: Why We Need a Comprehensive Approach.* Nanomaterials (Basel), 2018. **8**(4).

81. Bastogne, T., *Quality-by-design of nanopharmaceuticals – a state of the art.* Nanomedicine: Nanotechnology, Biology and Medicine, 2017. **13**(7): p. 2151-2157.

82. Halamoda Kenzaoui, B., Box, H., Van Elk, M., Gaitan, S., Geertsma, R., Gainza Lafuente, E., Owen, A., Del Pozo, A., Roesslein, M. and Bremer, S., *Anticipation of regulatory needs for nanotechnology-enabled health products*. 2019, EUR 29919 EN: Publications Office of the European Union, Luxembourg,.

83. Roberts, R.G., K.; Stebounova, L. V.; Anne Shatkin, J.; Peters, T.; Johan Foster, E., *Collection of airborne ultrafine cellulose nanocrystals by impinger with an efficiency mimicking deposition in the human respiratory system.* J Occup Environ Hyg, 2019. **16**(2): p. 141-150.

84. Rauscher, H., K. Rasmussen, and B. Sokull-Klüttgen, *Regulatory Aspects of Nanomaterials in the EU.* Chemie Ingenieur Technik, 2017. **89**(3): p. 224-231.

85. Stockmann-Juvala, H.T., P.; Santonen, T., *Formulating Occupational Exposure Limits Values (OELs) (Inhalation & Dermal)*. 2014, Helsinki, Finland: Finnish Institute of Occupational Health.

86. Additives, E.P.o.F., et al., *Re-evaluation of celluloses E 460(i), E 460(ii), E 461, E 462, E 463, E 464, E 465, E 466, E 468 and E 469 as food additives.* EFSA J, 2018. **16**(1): p. e05047.

87. EMA., *Guideline on the quality requirements for drug-device combinations* https://www.ema.europa.eu/en/documents/scientific-guideline/draft-guideline-quality-requirements-drug-device-combinations_en.pdf, 2019.

88. 194., I.T., *Biological evaluation of medical devices- Part 22: Guidance on nanomaterials. .* ISO /TR 2012. **10993-22**.

89. SCENIHR, *Opinion on the Guidance on the Determination of Potential Health Effects of Nanomaterials Used in Medical Devices. .* Final Opinion, 2015. **January**: p. 1–77.

90. Ong, K.J.S., J. A.; Nelson, K.; Ede, J. D.; Retsina, T., *Establishing the safety of novel bio-based cellulose nanomaterials for commercialization.* NanoImpact, 2017. **6**: p. 19-29.

91. Drasler, B., et al., *In vitro approaches to assess the hazard of nanomaterials.* NanoImpact, 2017. **8**: p. 99-116.

92. Gao, X. and G.V. Lowry, *Progress towards standardized and validated characterizations for measuring physicochemical properties of manufactured nanomaterials relevant to nano health and safety risks.* NanoImpact, 2018. **9**: p. 14-30.

93. Foster, E.J., et al., *Current characterization methods for cellulose nanomaterials.* Chemical Society Reviews, 2018. **47**(8): p. 2609-2679.

94. Giannakou, C., et al., *Sensitive method for endotoxin determination in nanomedicinal product samples.* Nanomedicine (Lond), 2019. **14**(10): p. 1231-1246.

95. Agency, E.C., *Guidance on information requirements and chemical safety assessment.* Appendix R7-1 for nanomaterials applicable to Chapter R7a, 2017. **Version 2.0**.

96. Dusinska, M., et al., *Towards an alternative testing strategy for nanomaterials used in nanomedicine: lessons from NanoTEST.* Nanotoxicology, 2015. **9 Suppl 1**: p. 118-32.

97. Dusinska, M., et al., *Immunotoxicity, genotoxicity and epigenetic toxicity of nanomaterials: New strategies for toxicity testing?* Food Chem Toxicol, 2017. **109**(Pt 1): p. 797-811.

98. EFSA Scientific Committee, H.A., Benford D, Halldorsson T, Jeger MJ, Knutsen HK, More S, Naegeli H, Noteborn H, Ockleford C, Ricci A, Rychen G, Schlatter JR, Silano V, Solecki R, Turck D, Younes M, Chaudhry Q, Cubadda F, Gott D, Oomen A, Weigel S, Karamitrou M, Schoonjans R and Mortensen A, *Guidance on risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain: Part 1, human and animal health.* EFSA Journal, 2018. **16**(7): p. 95.

99. Endes, C., et al., *An in vitro testing strategy towards mimicking the inhalation of high aspect ratio nanoparticles.* Part Fibre Toxicol, 2014. **11**: p. 40.

100. Endes, C., et al., *Fate of Cellulose Nanocrystal Aerosols Deposited on the Lung Cell Surface In Vitro.* Biomacromolecules, 2015. **16**(4): p. 1267-1275.

101. Pradhan, S.H., et al., *Physical, chemical, and toxicological characterization of fibrillated forms of cellulose using an in vitro gastrointestinal digestion and co-culture model.* Toxicol Res (Camb), 2020. **9**(3): p. 290-301.

102. Murphy, S.V. and A. Atala, *3D bioprinting of tissues and organs.* Nat Biotechnol, 2014. **32**(8): p. 773-85.

103. Gungor-Ozkerim, P.S., et al., *Bioinks for 3D bioprinting: an overview.* Biomater Sci, 2018. **6**(5): p. 915-946.

104. Hölzl, K.L., S.; Tytgat, L.; Van Vlierberghe, S.; Gu, L.; Ovsianikov, A., *Bioink properties before, during and after 3D bioprinting.* Biofabrication 2016. **8**(3): p. 032002.

105. Rijal, G. and W. Li, *3D scaffolds in breast cancer research.* Biomaterials, 2016. **81**: p. 135-156.

106. Jessop, Z.M., et al., *Printability of pulp derived crystal, fibril and blend nanocellulose-alginate bioinks for extrusion 3D bioprinting.* Biofabrication, 2019. **11**(4): p. 045006.

107. Ojansivu, M., et al., *Wood-based nanocellulose and bioactive glass modified gelatin-alginate bioinks for 3D bioprinting of bone cells.* Biofabrication, 2019. **11**(3): p. 035010.

108. Matai, I., et al., *Progress in 3D bioprinting technology for tissue/organ regenerative engineering.* Biomaterials, 2020. **226**: p. 119536.

109. Birgersdotter, A., R. Sandberg, and I. Ernberg, *Gene expression perturbation in vitro--a growing case for three-dimensional (3D) culture systems.* Semin Cancer Biol, 2005. **15**(5): p. 405-12.

110. Kleinman, H.K. and G.R. Martin, *Matrigel: basement membrane matrix with biological activity.* Semin Cancer Biol, 2005. **15**(5): p. 378-86.

111. Kim, J.B., *Three-dimensional tissue culture models in cancer biology.* Semin Cancer Biol, 2005. **15**(5): p. 365-77.

112. Bissel, M.J.K., P. A.; Radisky, D. C. , *Microenvironmental Regulators of Tissue Structure and Function Also Regulate Tumor Induction and Progression: The Role of Extracellular Matrix and Its Degrading Enzymes.* Cold Spring Harbor Symposia on Quantitative Biology, 2005. **70**: p. 343-356.

113. Landberg, G., et al., *Patient-derived scaffolds uncover breast cancer promoting properties of the microenvironment.* Biomaterials, 2020. **235**: p. 119705.

114. Cubo, N., et al., *3D bioprinting of functional human skin: production and in vivo analysis.* Biofabrication, 2016. **9**(1): p. 015006.

115. Duan, B., *State-of-the-Art Review of 3D Bioprinting for Cardiovascular Tissue Engineering.* Ann Biomed Eng, 2017. **45**(1): p. 195-209.

116. Singh, Y.P., et al., *Hierarchically structured seamless silk scaffolds for osteochondral interface tissue engineering.* J Mater Chem B, 2018. **6**(36): p. 5671-5688.

117. Lee, C.H., et al., *Tissue formation and vascularization in anatomically shaped human joint condyle ectopically in vivo.* Tissue Eng Part A, 2009. **15**(12): p. 3923-30.

118. Faulkner-Jones, A.F., C.; Cornelissen, D.-J.; Gardner, J.; King, J.; Courtney, A.; Shu, W., *Bioprinting of human pluripotent stem cells and their directed differentiation into hepatocyte-like cells for the generation of mini-livers in 3D.* Biofabrication, 2015. **7**(4): p. 044102.

119. Horvath, L., et al., *Engineering an in vitro air-blood barrier by 3D bioprinting.* Sci Rep, 2015. **5**: p. 7974.

120. Tomasina, C., et al., *Bioprinting Vasculature: Materials, Cells and Emergent Techniques.* Materials (Basel), 2019. **12**(17).

121. Perrin, S., *Preclinical research: Make mouse studies work.* Nature, 2014. **507**(7493): p. 423-425.

122. Hutchinson, L.K., R., *High drug attrition rates—where are we going wrong?* . Nature Reviews Clinical Oncology, 2011. **8**: p. 189.

123. Ma, H.L.J., Q.; Han, S.; Wu, Y.; Tomshine, J. C.; Wang, D.; Gan, Y.; Zou, G.; Liang, X.-J. , *Multicellular Tumor Spheroids as an in Vivo–Like Tumor Model for Three-Dimensional Imaging of Chemotherapeutic and Nano Material Cellular Penetration.* Molecular Imaging, 2012. **11**(6): p. 7290.

124. Abuelba, H., et al., *In vitro evaluation of curcumin effects on breast adenocarcinoma 2D and 3D cell cultures.* Rom J Morphol Embryol, 2015. **56**(1): p. 71-6.

125. Lovitt, C.J., T.B. Shelper, and V.M. Avery, *Evaluation of chemotherapeutics in a three-dimensional breast cancer model.* J Cancer Res Clin Oncol, 2015. **141**(5): p. 951-9.

126. David, L., et al., *Hyaluronan hydrogel: an appropriate three-dimensional model for evaluation of anticancer drug sensitivity.* Acta Biomater, 2008. **4**(2): p. 256-63.

127. Chitcholtan, K., P.H. Sykes, and J.J. Evans, *The resistance of intracellular mediators to doxorubicin and cisplatin are distinct in 3D and 2D endometrial cancer.* J Transl Med, 2012. **10**: p. 38.

128. Karlsson, H., et al., *Loss of cancer drug activity in colon cancer HCT-116 cells during spheroid formation in a new 3-D spheroid cell culture system.* Exp Cell Res, 2012. **318**(13): p. 1577-85.

129. Masuda, S. and J.C. Izpisua Belmonte, *The microenvironment and resistance to personalized cancer therapy.* Nat Rev Clin Oncol, 2013. **10**(2).

130. Iseri, O.D., et al., *Drug resistant MCF-7 cells exhibit epithelial-mesenchymal transition gene expression pattern.* Biomed Pharmacother, 2011. **65**(1): p. 40-5.

131. Mostrag-Szlichtyng, A.Z.C., J.-M.; Worth, A.P., *Computational toxicology at the European commission's joint research centre.* Expert opinion on drug metabolism & toxicology, 2010. **6**(7): p. 785-792.

132. Heggset, E.B.S., B. L.; Sundby, K. W.; Simon, S.; Chinga-Carrasco, G.; Syverud, K., *Viscoelastic properties of nanocellulose based inks for 3D printing and mechanical properties of CNF/alginate biocomposite gels.* Cellulose, 2018. **26**(1): p. 581-595.

133. Xu, W., et al., *On Low-Concentration Inks Formulated by Nanocellulose Assisted with Gelatin Methacrylate (GelMA) for 3D Printing toward Wound Healing Application.* ACS Appl Mater Interfaces, 2019. **11**(9): p. 8838-8848.

134. Chinga-Carrasco, G.E., N. V.; Pettersson, J.; Vallejos, M. E.; Brodin, M. W.; Felissia, F. E.; Håkansson, J.; Area, M. C., *Pulping and Pretreatment Affect the Characteristics of Bagasse Inks for Three-dimensional Printing.* ACS Sustainable Chem. Eng., 2018. **6**(3): p. 4068-4075.

In book series: Advances in Experimental Medicine and Biology, Springer Nature Switzerland AG. Publication date: Jun 2021.

135. Martinez Avila, H.S., S.; Rotter, N.; Gatenholm, P. , *3D bioprinting of human chondrocyte-laden nanocellulose hydrogels for patient-specific auricular cartilage regeneration.* Bioprinting, 2016. **1-2**: p. 22-35.

136. Czaja, W., et al., *Microbial cellulose--the natural power to heal wounds.* Biomaterials, 2006. **27**(2): p. 145-51.

137. Sun, F., et al., *Mechanical characteristics of nanocellulose-PEG bionanocomposite wound dressings in wet conditions.* J Mech Behav Biomed Mater, 2017. **69**: p. 377-384.

138. Powell, L.C., et al., *An investigation of Pseudomonas aeruginosa biofilm growth on novel nanocellulose fibre dressings.* Carbohydr Polym, 2016. **137**: p. 191-197.

139. Jack, A.A., et al., *The interaction of wood nanocellulose dressings and the wound pathogen P. aeruginosa.* Carbohydr Polym, 2017. **157**: p. 1955-1962.

140. Nordli, H.R.P., B.; Chinga-Carrasco, G.; Rokstad, A. M., *Ultrapure Wood Nanocellulose—Assessments of Coagulation and Initial Inflammation Potential.* ACS Appl. Bio Mater., 2019. **2**(3): p. 1107-1118.

141. Basu, A.H., J.; Ferraz, N., *Hemocompatibility of Ca2+-Crosslinked Nanocellulose Hydrogels: Toward Efficient Management of Hemostasis.* Macromol Biosci., 2017. **17**(11): p. 1700236.

In book series: Advances in Experimental Medicine and Biology, Springer Nature Switzerland AG. Publication date: Jun 2021.

Table 2 Characteristics of some (semi)commercial nanocelluloses. All the values in this table has been kindly provided by the corresponding companies.

Commercial name	**Raw material**	**Pre-treatment/Type of nanocellulose**	**Concentration (wt%)**	**Purity (%)**	**Crystallinity (%)**	**Degree of polymerization**	**Nanofibril morphology (nm)**	**Endotoxin content (EU/ml)**	**Commercial/ Semi-commercial**	**Company**
TUNICELL ETC	Tunicates	Enzymatic	2.5	Glucose 99.2±0.3	89.07±1.6	3900-4200	Width: 8.5 Length: 2519	≤0.5	Commercial	Ocean TuniCell, Norway
Exilva	Softwood sulfite pulp	Not stated	2-10	Hemicellulose <10 Lignin <1	Not stated	800-2000	Diameter range: 5-1000 nm	Not stated	Commercial	Borregaard, Norway
Corbiocel	Hardwood Kraft Bleached Pulp	TEMPO mediated oxidation	1-2	Extractives: 0.4 Lignin: 0.3 Glucan: 73.7 Xylan: 19.5	Not stated	250-270	Width:2–3 Length: 400–600	Not stated	Semi-Commercial	Regemat3D, Spain

				Ashes: 0.7						
GrowInk™	Hardwood kraft pulp	"native" nanofibrillar cellulose and anionic nanofibrillar cellulose	< 3 wt%	Contains hemicellulose	~70	500 – 1800	Width: 4-20 Length: 1000-20000 (depending obviously somewhat on grade)	<5	Commercial product, potential for use in clinical environment.* Manufactured according to ISO13485 standard.	UPM, Finland