Risk assessment of released cellulose nanocrystals – mimicking inhalatory exposure

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Abstract. Cellulose nanocrystals (CNCs) exhibit advantageous chemical and mechanical properties that render them attractive for a wide range of applications. During the life-cycle of CNC containing materials the nanocrystals could be released and become airborne, posing a potential inhalatory exposure risk towards humans. Absent reliable and dose-controlled models that mimic this exposure *in situ* is a central issue in gaining an insight into the CNC-lung interaction. Here, an Air Liquid Interface Cell Exposure system (ALICE), previously designed for studies of spherical nanoparticles, was used for the first time to establish a realistic physiological exposure test method for inhaled fiber shaped nano-objects; in this case, CNCs isolated from cotton. Applying a microscopy based approach the spatially homogenous deposition of CNCs was demonstrated as a prerequisite of the functioning of the ALICE. Furthermore, reliability and controllability of the system to nebulise high aspect ratio nanomaterials (HARN, *e.g.* CNCs) was shown. This opens the potential to thoroughly investigate the inhalatory risk of CNCs *in vitro* using a realistic exposure system.

1. Cellulose nanocrystals: composition and applications

Cellulose is the most abundant organic polymer on earth [1]. Individual cellulose molecules consist of linear chains, each of about 10,000 linked D-glucose units, and are the main component produced by condensation reactions of glucose during photosynthesis within the primary cell wall of green plants [1, 2]. Cellulose microfibrils contain both a crystalline and an amorphous fraction. The repeated crystalline domains that are formed via hydrogen bonds are disrupted by disordered regions of cellulose chains, which is the amorphous part. The ratio between the two fractions varies by species specificity, although in each case forms a natural composite material that provides a highly rigid structure (*e.g.* plant cell walls) [1]. The amorphous part can be degraded via controlled hydrolysis with mineral acids creating high aspect ratio cellulose nanocrystals (CNCs). Depending upon the source (*e.g.* cotton, soft wood pulp, rice and banana husks, bacteria, tunicates) rod shaped crystals with typical diameters of 5-50 nm and a length of 100-2,000 nm can be obtained [3-7]. Thus, CNCs

represent high aspect ratio nanoparticles (HARNs) with an aspect ratio typically between 10 and 100 [8].

These characteristics, together with outstanding material properties, *i.e.* high strength and stiffness (elastic modulus ~80-150 GPa) give rise to the potential use of CNCs in many applications, including for example high-performance polymer nanocomposites [9], mechanically adaptive materials [10], membranes for water purification [11] and photonic films [12]. Overall CNCs may be viewed as an oil-independent, lightweight and inexpensive alternative to carbon nanotubes (CNTs) [8].

2. Opportunities and risks of CNCs

Due to their low cost and ease of availability, combined with their properties and the possibility to simply modify the surface, CNCs have gathered increased attention within the field of material science in the past decades [2]. Taking into consideration the high potential for their industrial exploitation, it is essential to look into the potential risk posed by this nanomaterial [13].

The life-cycle of CNCs that are produced from different sources and embedded into polymers to form composites for specific applications includes the isolation of CNCs, compounding with polymers, incorporation into a product, processing and eventually their disposal. During these steps the materials undergo mechanical mixing processes as well as possible finishing steps such as sanding, drilling, abrasion and degradation. Thus, contained CNCs may be released as single nanocrystals, small agglomerates or even composite dust into the air and therefore potentially reach the human body via inhalation [14, 15]. There are three main exposure routes for nano-objects into the human body that highlight occupational, consumer related and environmental exposure: via the skin, the gastrointestinal tract and the respiratory tract. The lung, due to its large surface area is widely accepted as the primary entry route for ambient particulate materials into the human body [16]. Therefore, CNCs pose not only an occupational but also a consumer related and an environmental exposure risk [17]. One reason for a thorough investigation into the potential inhalatory risk of CNCs is due to their similarity to CNTs, especially in terms of their physico-chemical characteristics. Previously it has been reported that long, straight and stiff CNTs that were injected into the abdomen of mice caused severe adverse effects (*i.e.* inflammatory granulomas), which highlighted the discussion as to whether or not the 'asbestos-like effects' reported should be considered for any fiber shaped particle in the nano-dimension [18]. In accordance with previous fiber toxicology research that formed the 'fiber paradigm' (*i.e.* fibers must be long, thin and biopersistent) [19, 20], it has emerged in recent years that to fully understand the potential advantages of nanofibers, the interaction of HARN with biological systems must be taken into consideration.

In addition, it should also be highlighted that there is considerable investigation into the adverse health effects with humans following exposure to cotton dust. Workers of cotton mills, weaving and soft tissue paper-producing industries have been shown to suffer from reduced lung function, airway obstruction, 'flu-like' symptoms, also called byssinosis (brown lung disease), eye and skin problems due to exposure to cotton dust when compared with silk mill workers [21, 22]. A distinction of the observed health effects within occupational settings induced from the different fractions of cotton dust, including cotton fibers, is currently limited. Furthermore the role of bacteria, fungi [23] and related contents such as endotoxin [24, 25] and spores existing in the air due to growing on the cotton material is under debate for the determined occupational related health problems. These aspects indicate that not only the shape and size of CNCs must be taken into consideration but also the material itself (cotton), its composition and purity, when assessing the potential associated adverse effects of CNCs.

Studies for the release kinetics of CNCs from nanocomposites do currently not exist, since these materials are not yet produced at a large scale. In the case of CNTs, however, which are at a state of mass production, the potential aerosol exposure to carbon nanofibers and multiwalled carbon nanotubes (MWCNTs) during production was shown in several cases due to poorly controlled transfer and bagging of the material or within a blending laboratory [26, 27]. Wohlleben et al. investigated the potential release of nanofillers such as CNTs and silica from different polymer matrices [28]. The authors were unable to show, however, that a countable fraction of single CNTs are released into the air following different abrasion scenarios. In contrast, Schlagenhauf et al. showed the release of CNTs

in an abrasion scenario with CNT nanocomposites, freeing single CNTs as well as agglomerates in the size range of 13 nm up to 20 µm [29].

Therefore, according to the potential heightened use and broad interest in the application of CNCs in nanocomposites, due to their outstanding characteristics and renewable nature, their potential health risk must be investigated. The comparability of CNTs and CNCs in terms of dimensions, stiffness and application justifies the need for a thorough investigation of the potential impact of CNCs on biological systems in general and on the human respiratory system in particular.

2.1. In vitro and in vivo interactions of CNCs: an evaluation of recent findings

Up to now scientists have mainly focussed upon the improvement of protocols for CNCs in terms of their mechanical, thermal or chemical characteristics [5, 30-34]. Risk assessment of any CNC-based nanomaterial or CNCs themselves has so far been limited. Despite this, due to the plethora of applications associated with CNCs, research is beginning to focus upon the biological interaction of CNCs.

One of the first studies to address the potential risk upon inhalation of CNCs was performed using CNCs derived from cotton (220±67 x 15±5 nm) and assessed their effects with a novel 3D triple cell co-culture model of the human epithelial airway barrier [35]. This model consists of a layer of a human bronchiolar epithelial cell line (16HBE14o$^-$) forming a polarised cell layer and specific characteristics such as tight junctions after differentiation [36]. Human peripheral blood monocyte derived macrophages (MDMs) and dendritic cells (MDDCs) are added to form a stratified, *in vivo* like multicellular layer, enabling the interaction between the different cell types [37-39]. Applying the different test materials via suspensions (0.005-0.03 mg/mL) to the apical side (MDM and epithelial cell side) of the cell model it was shown that upon interaction with the MDM contained in the multicellular system, MWCNTs and crocidolite asbestos fibers (CAFs) induced a significantly higher (pro-)inflammatory response when compared to the cotton derived CNCs used in the study. In contrast to CNCs, CAFs showed classical frustrated phagocytosis, a phenomenon commonly associated with asbestos fibers due to the inability of the leukocyte (*e.g.* macrophage) to adequately engulf a long, stiff fiber [40]. In addition to this, other studies have also shown induction of cell alterations and genotoxicity by nanofibers derived from cotton and curaua (0.01-1 %; 130-180 x 6-14 nm) [41]. Jeong and co-workers could not detect any adverse effects upon the exposure to bacterial cellulose under the investigated conditions *in vitro* (0.1-1.0 mg/mL) nor *in vivo* (0.5-5 mg/mL) [42]. A set of classical toxicological tests conducted with nanocrystalline cellulose (NCC, typically 200 x 10 x 5 nm; at a range of concentrations from 0.03 to 10 g/L) by Kovacs et al. revealed no concerning effects with organisms representing different trophic levels of the aquatic ecosystem, representing the receiving environment for NCC production site effluents [43].

In addition, several research groups performed animal experiments with cellulosic fibers, more precisely microfibrillated cellulose, of different dimensions (mass median aerodynamic diameter 4.8 µm up to 15 µm), origin (cotton dust, commercial cellulose powder, mechanical wood pulp dusts), doses (0.75 mg/100 g animal - twice per week - 6 weeks, 15 mg single dose, 300-757 fibers/cm^3 within 2 weeks, 10^6-10^9 fibers) and application mode (inhalation, intratracheal instillation, intraperitoneal injection) [44-51]. The various exposures showed that instillation into the lung, pleura or peritoneal cavity or inhalation of the cellulosic materials can lead to inflammation, fibrosis, granulomata, sarcomas as well as fibrosing bronchio-/alveolitis under the employed conditions with rats and hamsters [45-52]. For all detected effects the authors associated the biopersistence of the cotton fibers within the lungs. *In vivo* for example, Muhle et al. reported that cellulose fibers (4.2 µm x 0.87 µm, instillation dose 2 mg) were present in the lung after one year, with a calculated half-time of fiber clearance of about 1,000 days [52]. Compared to the existing number of publications concerning the risk of CNTs and their biological interactions, knowledge as to their potential interactions and effects of CNCs is severely limited. As previously highlighted, although CNCs could pose (potential) risk as CNTs, cellulose and its derivatives are perceived as benign materials, which is subverted by the findings of the research discussed above.

Despite such results, however, these studies [35, 41-52] have to be carefully evaluated. Due to different production protocols (milling vs. acid hydrolysis) or sources (cotton, wood pulp, paper,

plants, bacteria) the dimensions and composition (surface charge, surface groups) of the CNCs vary dramatically and were often not reported in detail. This lack of characterisation and the application of the potentially inappropriate techniques can have a significant influence on the evaluation of resultant biological effects following exposure. When characterising high aspect ratio nanomaterials there is controversial debate as to the gold standard methods that should be used, such as employing measurements of zeta potential for the materials' surface charge, or dynamic light scattering for its dimensions (underlying algorithms for these methods are generated on spherical particle behaviour in an electric field and the diffusion motion in liquid, respectively). Generally, the importance of close material characterisation has been highlighted and a minimum set of physico-chemical characteristics, such as dimensions, surface area, chemical composition (production protocols, purity) or aggregation behaviour have been widely accepted in order to properly conduct a risk assessment of any nanomaterial [53]. Furthermore, instillation of extremely high doses, so-called 'overload situations', might not necessarily reflect realistic exposure scenarios, but on the other hand might reveal the risk potential in a worst case scenario or highlight possible translocation (if exposed to a material over an elongated period of time), as shown with CNTs [18]. Furthermore, detailed knowledge is lacking as to whether observed effects are provoked solely from the fiber fraction of the investigated substances or are due to a combination with impurities (*e.g.* bacteria, fungi, pesticides, wood matter) contained within the sample [50, 51]. It is also important to highlight that results from *in vivo* and *in vitro* studies are also not directly comparable, taking into consideration the close interplay of different cell types within the exposed organ. In the process of understanding a potential risk in occupational, consumer related or environmental exposure scenarios towards nanoparticles the route of inhalation should be especially focussed upon as previously highlighted. Therefore, with regards to assessment of the exposure risk of CNCs, a relevant *in situ* exposure system, realistic doses and systematic characterisation of the material, as well as a suitable *in vitro* model of the investigated organ (*e.g.* the human lung) are of prime importance when investigating their biological impact.

3. *In situ* scenarios and the 3R`s concept

With emerging nanotechnology products there are more and more materials to be tested for their potential risks towards human health [54]. Efficient and reliable tests are necessary to evaluate in which context these materials can be used or should be modified in order to avoid any harmful effects to humans and the environment [55]. Alternatives to animal testing are, therefore, a required prerequisite not only from an ethical perspective but also when considering significant differences when comparing reactions of the rodent and human immune system (*i.e.* inflammatory reactions) [56, 57]. Within the aim of the three R's concept (refinement, reduction and replacement) [58] it is therefore important to develop, validate and use *in vitro* models. In addition, high throughput test strategies are of highest importance regarding the growing field of nanotechnology and the emerging need for risk assessment [55].

In conjunction, several *in vitro* systems have been under investigation to copy the *in vivo* situation (*e.g.* in the lung). Researchers simulated the alveolar-capillary barrier *in vitro* as principle biological barrier in the lower respiratory tract [59]. Rothen-Rutishauser et al. described a triple cell co-culture model forming the defined architecture of the human epithelial airway barrier (section 2.1) [37]. Other approaches focus on the interplay of different cell types with little regard to the *in vivo* structure of the lung [60]. Investigation of such systems highlight the differences in the biological response when comparing mono- and co-culture techniques (two, three or four cell types) and show different reactions toward nanoparticle exposure which have to be closely discussed and compared as is widely highlighted in literature [35, 36, 57, 61]. An additional aim when using optimal *in vitro* systems is to realistically investigate the potential biological impact of nanoparticle exposure. This can be achieved by the simulation of the *in situ* application of nanoparticles. For studying the potential risks of inhalation of nanoparticles many systems have already been established for spherical or atmospheric (nano)particles including a glove-box system for occupational exposure [62] and air–liquid interface cell exposure systems for exposure of engineered nanoparticles [63], as well as to study the environmental exposure to both exhaust emissions and brake wear particles [64-66]. Other possibilities to generate nanoparticle aerosols and facilitate the investigation of inhaled nanoparticles

either in the field of basic research or for risk oriented purposes were highlighted by Creutzenberg et al., including spark generators, dry dispersion techniques, using pressurised air or aerosolising particle suspensions to form droplet aerosols [67]. These systems have the advantage to overcome artefacts derived from suspension related exposure set-ups that include agglomeration and sedimentation of nanoparticles or cell medium interactions (protein interactions), which can influence observed effects as shown in different studies [68, 69]. Additionally, the direct comparison of aerosol and suspension exposure *in vitro* revealed considerably different outcome concerning the dose-response relationship and effective moment of maximum development of the investigated parameters following exposure to zinc oxide nanoparticles [70]. On the other hand, the comparison offers the possibility to distinguish exposure to nanoparticle containing aerosols composed of particulate and gaseous phase versus exposure in suspension to solely the particle-associated effects [70]. These studies contribute to the trend aiming at the most realistic exposure scenario depending on the research focus (occupational exposure, basic research) overcoming experimental artefacts (aggregation, sedimentation, interaction with cell medium), together with sophisticated *in vitro* systems, although, as of yet none have been used with HARN.

4. The Air Liquid Interface Cell Exposure system (ALICE) for HARN

As previously mentioned, the use of realistic *in vitro* models in combination with a relevant exposure system is of the utmost importance when evaluating the effects of the nanoparticle-cell interaction. In order to mimic the exposure to airborne nanoparticles via inhalation, an Air Liquid Interface Cell Exposure system (ALICE) was used [63]. The ALICE consists of an exposure chamber in which a perforated vibrating membrane nebuliser (customised eFlow, PARI Pharma GmbH, Germany) equipped with a class 45 nominal size aerosol head which produces a dense cloud of 6.00 µm (mass median diameter) droplets that fills the chamber and settles gently onto the cells. The chamber is connected to a continuous air-flow system that is generated by a pump. Humidity and temperature are controlled to create a stable physiological environment for the cells. Filters present at the inlet and outlet permit a 'semi-sterile' environment. Additionally, the deposition via ALICE nebulisation of any material can be monitored by a quartz crystal microbalance (detection limit 90 ng/cm^2) which is inserted into the chamber prior to the nebulisation process. This system has previously been described in accordance with studies of *in vitro* exposure to spherical particles (*e.g.* gold nanoparticles [71]) where it has been shown to provide an efficient, repeatable, dose controlled and spatially uniform deposition [63].

For the first time, the ALICE was applied for the nebulisation of suspended HARN, specifically CNCs derived from cotton (168±72 nm x 19±7 nm; Figure 1). CNCs were obtained from Whatman No.1 filter paper via hydrolysis with concentrated sulphuric acid following the protocol of Dong et al. [72]. Subsequent sonication for 4 h led to a well dispersed CNC suspension. Since anisotropic particles differ much in their physical properties from spherical particles, a close investigation of the nebulisation process is indispensable to ensure the previously described functioning of the ALICE [64]. A schematic drawing of the system is given in Figure 2.

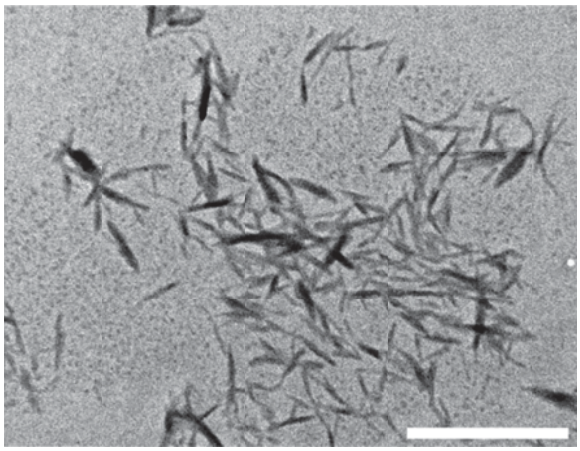

Figure 1: Transmission electron microscopy (TEM) image of 1 mg/mL cotton cellulose nanocrystals after 4 h sonication in ultrapure water. Scale bar represents 1 µm.

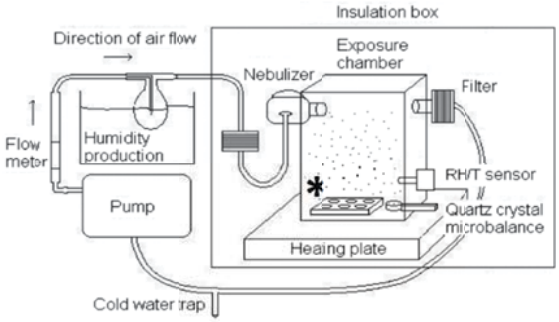

Figure 2: Schematic depiction of the Air Liquid Interface Cell Exposure system (ALICE), consisting of a closed air flow system including humidity production, an exposure chamber with aerosol generator, heating plate and a quartz crystal microbalance. * Equates to the position of 6-well plates with inserts containing glass slides, TEM grids or cells. Image is adapted under open access availability and with the permission of the authors of reference [64].

To document the spatially uniform deposition of CNCs after nebulisation as an important prerequisite for reliability and cornerstone of the ALICE, a microscopic analytical approach was used. Initially, 1 mL of an aqueous suspension of cotton CNCs (1 mg/mL) was nebulised via the nebuliser of the ALICE. After complete nebulisation onto glass slides (Ø 20 mm, Fisher Scientific AG, Switzerland) contained within 6-well plate inserts (BD Falcon™ Cell Culture Inserts, 3 µm pores, BD USA), the dried droplets comprising CNCs were analysed using confocal laser scanning microscopy (Zeiss LSM 710, Carl Zeiss AG, Germany). The dried CNC residuals were stained with a 0.2 % aqueous Calcofluor solution (Fluorescent Brightener 28, Sigma-Aldrich, USA). This fluorescent label is widely used and can be easily observed at an emission of 435 nm (excitation wavelength of 345 nm) [75-77]. Homogeneously distributed dry residuals of deposited CNCs with a bimodal size distribution were found arranged in a 'halo' shape (Figure 3 (a)).

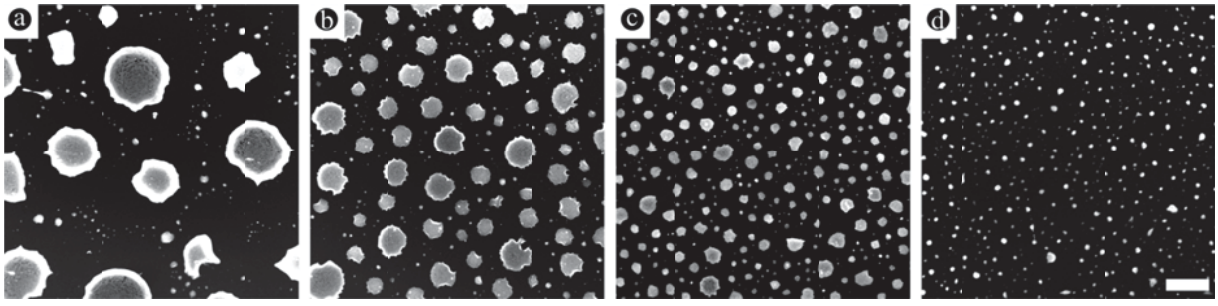

Figure 3: Confocal laser scanning microscopy images showing dried residuals of the nebulised 1 mg/mL cotton cellulose nanocrystal (CNC) suspension on glass slides after nebulisation times of (a) 80 s, (b) 40 s, (c) 20 s, (d) 10 s using the Air Liquid Interface Cell Exposure system. All images were taken at 20x magnification and the scale bar represents 100 µm.

To further investigate this observation time resolved nebulisation of the 1 mg/mL suspension of cotton CNCs was conducted. The nebulisation process was stopped after 40, 20 and 10 seconds respectively, and compared to a complete nebulisation (80 s). Figure 3 (a-d) shows that the deposition pattern and the size of dried residuals on the exposed glass slides changes as a function of exposure time. All residual particles appear as irregular shaped compact particles due to surface tension effects during the drying process. It is evident that the deposition pattern is spatially uniform for all nebulisation times. However, the size of the residual particles increases substantially from ~3 µm up to ~150 µm with increasing nebulisation time. Due to the continuing deposition process the dried residual particles are much larger than the CNC residuals expected from a single droplet. With decreasing nebulisation time, *i.e.* decreasing deposition of droplets, the fraction of single droplets increases which explains the reduction in the size of the residual particles reaching a minimum of ~3 µm for 10 s. The drying process also explains why surrounding each large droplet a 'halo' of very small droplets is visible (Figure 3 (a-b)). During drying, evaporating water pulls the included CNCs to a central point leaving an empty space between the 'halo' and the central, larger, dried residual of deposited CNCs. With less time of nebulisation this effect dissipates (Figure 3 (c-d)). It should be noted that the highly uniform distribution of droplets disregarding the nebulisation time highlights the efficient function of the ALICE, even with HARN. The hypothesis of an observed drying effect was supported by exposing copper grids (Plano AG, Germany) for transmission electron microscopy (TEM; Hitachi H-7100, Hitachi, Japan, at 75 kV equipped with a Morada 11 MPix digital CCD camera, Olympus, Japan) in the same manner as the glass slides for confocal microscopy. After complete nebulisation of 1 mg/mL cotton CNCs this reveals droplets in the nanometer size regime (Figure 4), pointing at the production of small droplets.

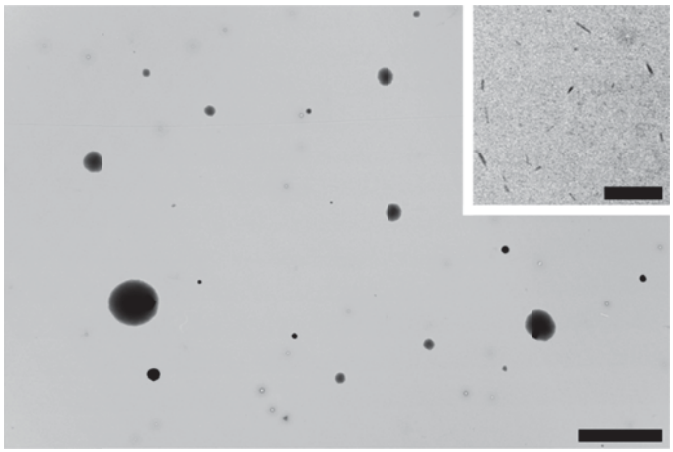

Figure 4: Transmission electron microscopy (TEM) image of 1 mg/mL cotton cellulose nanocrystals after being nebulised using the Air Liquid Interface Cell Exposure system onto a copper grid for TEM (Plano GmbH, Germany). Scale bar represents 10 µm. Inset shows single deposited crystals after exposure, scale bar represents 500 nm.

In summary, it has been shown with a microscopic approach that the ALICE provides spatially uniform deposition of aerosolised HARN (CNCs) in transwell inserts for cell culturing. An adverse effect of fiber shape on the spatial uniformity of particle deposition can be ruled out. Also it was shown that independent of concentration or exposure time (leading to varying aerosolised volumes of CNC suspensions) uniform distribution can be achieved as proven with repeated nebulisations of the HARN (n•3, data not shown). Due to merging of droplets on the glass slides measurements of the exact size of the produced aerosol droplets from imaging the dried residual particles on the slide cannot be achieved with this method (see Figure 3 and 4). Imaged dry residuals after deposition suffer from a variety of artefacts (*e.g.* drying, surface tension and contact angle). It is important to note that Figure 3 and Figure 4 are not representative for the CNC particles deposited onto the surface of a cellular *in vitro* model especially since cellular membranes, even exposed to the air liquid interface, never pose a dry surface, *i.e.* the compaction due to the drying process will not occur on the cells. The fluidic model of the double phospholipid layer of cell membranes with liquid coating will, therefore, encounter the entity produced by the nebuliser. Detailed analysis will elucidate if these are still fiber-

shaped or already compacted in spherical shaped droplets both observed by TEM after exposure (Figure 4, inset).

Nevertheless, the proof of spatially uniform deposition of aerosolised CNCs with the ALICE is of greatest importance when subsequently analysing deposited mass with the integrated quartz crystal microbalance and also the exposure of *in vitro* systems to study the biological impact of CNC-interactions in a controlled and dose-dependent manner.

5. Conclusion

The emerging field of nanotechnology gathers much attention due to the outstanding properties of nanoparticles that can be used in many different applications. CNCs in particular propose high chances for industrial exploitation due to their advantageous physico-chemical characteristics. In the field it is widely accepted that new materials are required to be closely investigated in regard to their potential adverse health effects prior to application. Only with the combination of novel, sophisticated cell culture models and realistic exposure systems together with close characterisation of the material and appropriate material doses close to the reality of occupational settings can the potential adverse effects of CNCs be determined. In the case of CNCs derived from cotton, the basis for an in-depth risk assessment of inhalatory exposure is now possible due to the establishment of nebulising these high aspect ratio nanomaterials with the existing ALICE system. For the first time the fulfilment of functioning cornerstones of the ALICE such as spatially uniform deposition, as well as reliability and controllability of the deposited dose have been experimentally verified, which facilitates the investigation of the potential adverse effects of inhaled CNCs with *in vitro* assays.

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