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Nanotechnologies — Characterization of carbon nanotube and carbon nanofibre aerosols to be used in inhalation toxicity tests

Nanotechnologies — Caractérisation des aérosols de nanotubes de carbone et de nanofibres de carbone à utiliser dans les tests de toxicité par inhalation

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Foreword

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This document was prepared by Technical Committee ISO/TC 229, *Nanotechnologies*.

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Introduction

Inhalation is the primary route of exposure to aerosolised carbon nanotubes (CNTs) and carbon nanofibres (CNFs). Exposure to CNTs or CNFs can occur in consumer settings as well as in occupational settings. Occupational exposure to CNTs or CNFs can occur at all phases of the manufacturing, handling, and formulation of the material into final products[1,2]. Consumers are potentially exposed to CNTs or CNFs released as products of degradation, weathering, or mechanical processes (e.g. grinding or polishing) from consumer products that contain CNT or CNF embedded into a matrix[3,4].

Similar to other nanomaterials, the physicochemical properties of CNTs or CNFs are greatly diverse in terms of diameter, length, shape, arrangement of carbon atoms, surface chemistry, defects, and impurities. Their different physicochemical characteristics are responsible for different functional properties such as mechanical, electrical, optical, and thermal properties. Many previous inhalation toxicity studies of CNT and CNF aerosols reported various hazards from acute inflammation to carcinogenicity and the toxicological responses to CNT and CNF aerosols vary depending on their physicochemical characteristics[5].

Among the various physicochemical characteristics, morphological factors such as length and rigidity have been suggested as key parameters related to the toxicity of CNT and CNF aerosols[6,7]. CNT and CNF aerosols can consist of individual primary fibres in the nanoscale[8] and aggregated or agglomerated structures, including those with diameters larger than 100 nm[9]. Among various types of CNT and CNF, the asbestos-like pathogenicity has been observed only in long (>5 µm) and rigid fibres, but not in short or tangled CNT[6]. Thus, a better understanding of the characteristics of generated CNT or CNF aerosols in relation to toxicity end points is key for risk assessment and safer-by-design approaches.

The framework for material characterization for inhalation studies consists of (1) characterization of as-produced (pristine) or supplied material, (2) characterization of administered material, (3) characterization of material following administration, and (4) human exposure characterization[10]. This document focuses on the first two characterization needs, which include physicochemical properties (e.g. size, size distribution, aggregation/agglomeration, and shape) and measurement of concentration (e.g. mass, number, surface area, and volume). These parameters can be measured by direct (online) or indirect (off-line) methods and each technique needs specific sampling procedures. However, the limited technologies in the generation and characterization of nanofibres make it difficult to perform inhalation toxicity studies, although the inhalation exposure to CNT and CNF is highly likely in the workplace[9,11], and research facilities[8], where they are in use. In this regard, this document provides the current status of CNT and CNF aerosol characterization used in the inhalation toxicity tests as well as the physicochemical properties of CNTs and CNFs and their relationship with toxicity end points.

This document complements the work of other international organizations including the Organization for Economic Co-operation and Development (OECD) which has published guidelines and guidance on the performance of inhalation toxicity studies[12,13]. ISO 10808 describes the characterization of nanoparticles in inhalation exposure chambers for inhalation toxicity testing. This document is different from ISO 10808 and focuses on different types of nanomaterials (nanotubes and nanofibres opposed to nanoparticles) because many characterization methods and important physicochemical parameters related to the toxicity of CNT and CNF are different from those of nanoparticles. Recommendations and guidelines to assist investigators in making appropriate choices for the characterization of CNT and CNF aerosols to be studied are presented in this document.

Nanotechnologies — Characterization of carbon nanotube and carbon nanofibre aerosols to be used in inhalation toxicity tests

1 Scope

This document reviews characterization of CNT and CNF aerosols for inhalation exposure studies. The document also provides useful information on appropriate characterization of CNT and CNF, which is required to evaluate and understand the inhalation toxicity of CNT and CNF aerosols. This document neither provides guidance on aerosol characterization for other carbon nanomaterials, nor provides guidance for characterization of carbon nanotube and nanofibre aerosols in the workplace or ambient air.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 80004 (all parts), *Nanotechnologies — Vocabulary*

3 Terms and definitions

For the purposes of this document, the terms and definitions given ISO 80004 (all parts), and the following apply.

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

3.1
carbon nanotube
nanotube composed of carbon

Note 1 to entry: Carbon nanotubes usually consist of curved graphene layers, including single-wall carbon nanotubes and multiwall carbon nanotubes.

[SOURCE: ISO/TS 80004-3:2020, 3.3.3]

3.2
multiwall carbon nanotube
MWCNT
multi-walled *carbon nanotube* (3.1) composed of nested, concentric or near-concentric graphene sheets with interlayer distances similar to those of graphite

Note 1 to entry: The structure is normally considered to be many single-wall carbon nanotubes nesting each other, and would be cylindrical for small diameters but tends to have a polygonal cross-section as the diameter increases.

[SOURCE: ISO/TS 80004-3:2020, 3.3.6]

3.3
single-wall carbon nanotube
SWCNT
carbon nanotube (3.1) consisting of a single cylindrical graphene layer

Note 1 to entry: The structure can be visualized as a graphene sheet rolled into a cylindrical honeycomb structure.

[SOURCE: ISO/TS 80004-3:2020, 3.3.4]

3.4
carbon nanofibre
CNF
nanofibre (3.5) composed of carbon

[SOURCE: ISO/TS 80004-3:2020, 3.3.1]

3.5
nanofibre
nano-object (3.28) with two similar external dimensions in the nanoscale and the third dimension significantly larger

Note 1 to entry: A *nanofibre* (3.5) can be flexible or rigid.

Note 2 to entry: The two similar external dimensions are considered to differ in size by less than three times and the significantly larger external dimension is considered to differ from the other two by more than three times.

Note 3 to entry: The largest external dimension is not necessarily in the nanoscale.

[SOURCE: ISO/TS 80004-2:2015, 4.5]

3.6
aerosol
metastable suspension of solid or liquid particles in a gas

[SOURCE: ISO TR 27628:2007, 2.3]

3.7
inhalation chamber system
system prepared to expose experimental animals to an inhaled test substance of predetermined duration and dose by either nose-only or whole-body method

Note 1 to entry: This system consists of chamber, head-only and nose-only.

Note 2 to entry: The term "nose-only" includes head-only, nose-only, or snout-only.

Note 3 to entry: [SOURCE: OECD TG 403[18], 412[12], 413[13]]

3.8
nanoparticle generation system
device to make nanoparticle aerosol with controlled size distribution and concentration

[SOURCE: ISO 10808:2010, 3.3]

3.9
aspect ratio
ratio of length to width of a particle

[SOURCE: ISO 10312:2019, 3.8]

3.10
rigidity
inability to be to bent or forced out of shape or ability of a material to resist deformation

Note 1 to entry: This term applies to CNT or CNF.

Note 2 to entry: Asbestos fibres and MWNT-7 are examples of rigid structures.

3.11
aggregate
particle comprising strongly bonded or fused particles where the resulting external surface area is significantly smaller than the sum of calculated surface areas of the individual components

Note 1 to entry: The forces holding an aggregate together are strong forces, for example, covalent bonds, or those resulting from sintering or complex physical entanglement, or otherwise combined former primary particles.

Note 2 to entry: Aggregates are also termed secondary particles and the original source particles are termed primary particles.

[SOURCE: ISO 26824:2013, 1.3]

3.12
agglomerate
collection of weakly or medium strongly bound particles where the resulting external surface area is similar to the sum of the surface areas of the individual components

Note 1 to entry: The forces holding an agglomerate together are weak forces, for example van der Waals forces or simple physical entanglement.

Note 2 to entry: Agglomerates are also termed secondary particles and the original source particles are termed primary particles.

[SOURCE: ISO/TS 80004-2:2015, 3.4]

3.13
biodurability
ability of a material to resist *dissolution* (3.14) and mechanical disintegration from chemical and physical clearance mechanisms

[SOURCE: ISO/TR 19057:2017, 3.3]

3.14
dissolution
process of obtaining a solution containing the analyte of interest

Note 1 to entry: Dissolution is the act of dissolving and the resulting species may be molecular or ionic.

[SOURCE: ISO/TR 19057:2017, 3.6]

3.15
aerodynamic diameter
diameter of a sphere of 1 g cm^{-3} density with the same terminal settling velocity in calm air as the particle, under the prevailing conditions of temperature, pressure and relative humidity

Note 1 to entry: The particle aerodynamic diameter depends on the size, density and shape of the particle.

Note 2 to entry: Aerodynamic diameter is related to the inertial properties of aerosol particles.

[SOURCE: ISO 4225:2020, 3.1.5.13]

3.16
differential mobility analysing system
DMAS
system to measure the size distribution of submicrometer aerosol particles consisting of a *DEMC* (3.19), a particle charge conditioner, flow meters, a particle detector, interconnecting plumbing, a computer, and suitable software

[SOURCE: ISO 15900: 2020, 3.12]

3.17
geometric mean diameter
GMD
measure of the central tendency of particle size distribution using the logarithm of particle diameters

Note 1 to entry: The GMD is normally computed from particle counts and when noted may be based on surface area or particle volume with appropriate weighting, as:

$$\ln(\mathrm{GMD}) = \frac{\sum_{i=m}^{n} \Delta N_i \ln(d_i)}{N}$$

where

d_i is the midpoint diameter for the size channel, i

N is the total concentration

ΔN_i is the concentration within the size channel, i

m is the first channel

n is the last channel

[SOURCE: ISO 10808:2010, 3.5]

3.18
geometric standard deviation
GSD
measure of the width or spread of particle sizes, computed for the *DMAS* (3.16) by

$$\ln(\mathrm{GSD}) = \sqrt{\frac{\sum_{i=m}^{n} N_i \left[\ln d_i - \ln(\mathrm{GMD})\right]^2}{N-1}}$$

[SOURCE: ISO 10808:2010, 3.6]

3.19
differential electrical mobility classifier
DEMC
classifier that is able to select aerosol particle sizes from a distribution that enters it and pass only selected sizes to the exit

Note 1 to entry: A DEMC is sometimes called a Differential Electrical Mobility Spectrometer (DEMS). A DEMC classified aerosol particle sizes by balancing the electrical force on each particle in an electrical field with its aerodynamic drag force. Classified particles have different sizes due to their number of electrical charges and a narrow range of electrical mobility determined by the operating conditions and physical dimensions of the DEMC.

[SOURCE: ISO 10801: 2010, 3.2]

3.20
count median diameter
CMD
diameter equal to *GMD* (3.17) for particle counts assuming a logarithmic normal distribution

Note 1 to entry: The general form of the relationship as described in ISO 9276-5:2005 is

$$CMD = x_{50,r} = x_{50,p} e^{(r-p)s^2}$$

where

e is the base of natural logarithms, e = 2,718 28;

p is the dimensionality (type of quantity) of a distribution

$p = 0$ is the number,

$p = 1$ is the length,

$p = 2$ is the area, and

$p = 3$ is the volume or mass;

r is the dimensionality (type of quantity) of a distribution, where

$r = 0$ is the number,

$r = 1$ is the length,

$r = 2$ is the area, and

$r = 3$ is the volume or mass;

s is the standard deviation of the density distribution

$x_{50,r}$ is the median particle size of a cumulative distribution of dimensionality, r.

[SOURCE: ISO 10808:2010, 3.7]

3.21
mass median aerodynamic diameter
MMAD
calculated aerodynamic diameter which divides the particles of an aerosol in half based on mass of the particles

Note 1 to entry: Fifty percent of the particles by mass will be larger than the median diameter and 50 per cent of the particles will be smaller than the median.

[SOURCE: EPA IRIS Glossary; ISO 15779:2011, 3.30]

3.22
mobility diameter
diameter of a spherical particle that has the same mobility as the particle under consideration

Note 1 to entry: Mobility diameter is generally used to describe particles smaller than approximately 500 nm, and is independent of the density of the particle

[SOURCE: ISO/TR 27628:2007, 2.10]

3.23
particle density
ratio obtained by dividing the mass of a sample of aggregate particles by the volume, including both permeable and impermeable pores within the particles (but not including the voids between the particles)

Note 1 to entry: It is expressed as mass per unit volume, i.e. kilograms per cubic meter (kg/m^3)

[SOURCE: ISO 20290-1, 3.2]

3.24
specific surface area
surface area per unit mass of a particle or material

[SOURCE: ISO/TR 27628:2007, 2.19]

3.25
respirable fraction
mass fraction of inhaled particles which penetrate to the unciliated airways

[SOURCE: ISO 7708:1995, 2.11]

3.26
inhalable fraction
fraction of total airborne particles of given particle size inhaled through the nose and mouth

Note 1 to entry: Adapted from ISO 7708:1995, 2.3.

Note 2 to entry: The fractions specified in 3.3 to 3.8, as defined at specific particle size (characterized by thermodynamic and aerodynamic diameters), are independent of the basis of measurement, e.g. mass, area or particle count.

Note 3 to entry: A significant portion of the inhaled particles may be exhaled, but since these are smaller particles their effect on the mass deposited may be minimal.

[SOURCE: ISO 13138:2012, 3.3]

3.27
nanomaterial
material with any external dimension in the nanoscale or having internal structure or surface structure in the nanoscale

Note 1 to entry: This generic term is inclusive of nano-object and nanostructured material.

Note 2 to entry: See also engineered nanomaterial, manufactured nanomaterial and incidental nanomaterial.

[SOURCE: ISO/TS 80004-1:2015, 2.4]

3.28
nano-object
discrete piece of material with one, two or three external dimensions in the nanoscale

Note 1 to entry: The second and third external dimensions are orthogonal to the first dimension and to each other.

[SOURCE: ISO/TS 80004-2:2015, 2.2]

3.29
nanoparticle
nano-object (3.28) with all external dimensions in the nanoscale where the lengths of the longest and the shortest axes of the nano-object do not differ significantly

Note 1 to entry: If the dimensions differ significantly (typically by more than 3 times), terms such as *nanofibre* (3.5) or *nanoplate* (3.30) may be preferred to the term nanoparticle.

Note 2 to entry: Ultrafine particles may be nanoparticles.

[SOURCE: ISO/TS 80004-2:2015, 4.4]

3.30
nanoplate
nano-object (3.28) with one external dimension in the nanoscale and the two other external dimensions significantly larger

Note 1 to entry: The larger external dimensions are not necessarily in the nanoscale.

[SOURCE: ISO/TS 80004-2:2015, 4.6]

3.31
nanotube
hollow *nanofibre* (3.5)

[SOURCE: ISO/TS 80004-2:2015, 4.8]

3.32
particle
minute piece of matter with defined physical boundaries

Note 1 to entry: A physical boundary can also be described as an interface.

Note 2 to entry: A particle can move as a unit.

Note 3 to entry: This general definition applies to particle nano-objects.

[SOURCE: ISO 26824:2013, 1.1]

3.33
primary particle
original source *particle* (3.32) of *agglomerates* (3.12) or *aggregates* (3.11) or mixtures of the two

Note 1 to entry: Constituent particles of agglomerates or aggregates at a certain actual state may be primary particles, but often the constituents are aggregates.

Note 2 to entry: Agglomerates and aggregates are also termed secondary particles.

[SOURCE: ISO 26824:2013, 1.4]

3.34
hazard
source with a potential to cause injury and ill health

Note 1 to entry: Hazards can include sources with the potential to cause harm or hazardous situations, or circumstances with the potential for exposure leading to injury and ill health.

[SOURCE: ISO 45001:2018, 3.19]

4 Abbreviated terms

APM	aerosol particle mass analyser
APS	aerodynamic particle sizer
CMD	count median diameter
CML	count median length
DCFH-DA	2'-7'dichlorofluorescein diacetate
DMAS	differential mobility analysing system
EDX	energy dispersive X-ray analyser
EM	electron microscopy
GC-MS	gas chromatography–mass spectrometry
ICP-MS	inductively coupled plasma mass spectrometry
NOAA	nano-objects and their aggregates and agglomerates
OECD	organization for economic co-operation and development
OPC	optical particle counter
SEM	scanning electron microscope
TEM	transmission electron microscope
TEOM	tapered element oscillating microbalance
TG	test guideline
TGA	thermogravimetric analysis
XRD	X-ray diffraction

5 Considerations in CNT and CNF inhalation studies

5.1 General

Inhalation toxicity studies are important for hazard evaluation and as a first step to understanding the potential health risks to workers and the general population if exposed to aerosolised CNTs and CNFs. In designing an inhalation study for CNTs and CNFs as well as the interpretation of results obtained from the inhalation study, physicochemical characterization of CNT and nanofibres before aerosol generation and in aerosols as generated is critical for the evaluation of the potential health risks of CNTs and CNFs following inhalation exposure.

5.2 Workplace exposure scenario

When conducting an inhalation toxicity study for CNTs and CNFs, actual workplace exposure scenarios need to be considered. The generation of CNT and CNF aerosols needs to simulate actual workplace CNT and CNF exposures in terms of concentration (mass if known), shape, size, size distribution, frequency of exposure, and handling and manufacturing conditions of the CNTs and CNFs. However, the maximum concentration needs to be less than 5 mg (total mass)/L for aerosols and particle aerosol higher than 2 mg (total mass)/L can only be attempted if a respirable particle size can be maintained[12,13]. However, achieving 2 mg/l (2 g/m^3) of CNT and CNF for such low-density material is practically not feasible for

subacute and subchronic studies. Most of the inhalation studies were performed at a dose level less than 5 mg/m^3, which produced inflammation and carcinogenic effect on the lung[34,35]. For high dose levels, the high amount of fibres deposited in the alveoli can produce lung overload in rats (mostly used experimental animals in inhalation testing), which is believed to trigger the cascade of events leading to stasis of clearance. Consequently, high enough CNT doses and lung overload can trigger sustained pulmonary inflammation[36]. Because nanofibres have a high surface area or number to mass ratio, the conventional loading guidelines to avoid lung overload can not be applied to CNTs and CNFs.

The starting materials for the generation of CNT and CNF aerosols can be a powder, a liquid of well or poorly suspended CNTs or CNFs, or a solid-state material. The generation of CNT and CNF aerosols is difficult because of their hydrophobic nature and agglomeration propensity, although it can be improved by the use of dispersion media such as phospholipids[37]. Thus, the diversity and difficulties in the generation of fibres can limit the simulation in an inhalation toxicity study with respect to the actual workplace exposures. Various methods of CNT and CNF generation could be adopted as described in ISO/TR 19601[38].

5.3 Existing inhalation toxicity testing guidelines

The generation of regulatory hazard data are based on existing inhalation test guidelines (TGs) published by the OECD or equivalent national and international bodies. The OECD TGs for an inhalation toxicity study include TGs 436, 412[12], 413[13], and Guidance Document (GD) 39. Among them, TGs 412 and 413 were recently revised to include the testing of nanomaterials. These TGs highlighted that the MMAD needs to be less than 2 µm with a GSD up to 3[12,13]. Also, GD 39 has been revised to include issues specific to nanomaterials testing.

6 Physicochemical parameters related to the toxicity of CNTs and CNFs

6.1 General

Because the toxicity of nanomaterials is closely related with their physicochemical properties, a thorough investigation of the relationship between the physicochemical properties and toxicity end points is important for a better understanding of the mechanism of toxicity and safer-by-design approaches of CNTs and CNFs (see Annex A). Furthermore, this information can be very useful for risk assessment and regulatory purposes as inhalation toxicity studies for every CNT and CNF cannot be performed. Furthermore, it should be noted that physicochemical properties can modulate each other. For example, the state of aggregation/agglomeration can be influenced by various factors including surface charge, surface chemistry, and defects. Furthermore, the surface charge can be influenced by various factors including surface chemistry and impurities. Besides the intrinsic properties of CNTs and CNFs, various extrinsic factors, such as dispersion media and biological fluids where fibres are in contact with, can also modulate the physicochemical properties of CNTs and CNFs. Since the physicochemical properties can be changed by the aerosol generation processes, the measurement of physicochemical parameters of CNTs and CNFs before and after the generation of aerosols is needed[10].

The information about the correlation between aerosol properties and toxicity end points are limited because of the complexity and difficulties of inhalation studies. As an alternative study, the direct administration of CNFs and CNFs into the lung, pleural space, and peritoneal space, or in vitro studies have been extensively reported and demonstrated several physicochemical parameters related to the toxicity end points. Thus, the information from these alternative studies can be very useful to understand the results of inhalation toxicity studies and their relationship with the physicochemical properties of the generated aerosols.

6.2 Aerodynamic properties of aerosols for deposition of fibres

A characterization of the aerodynamic properties of aerosols is critical to understand the pulmonary deposition and penetration, and resultant toxicity of CNTs and CNFs because the deposition dose of CNT and CNF aerosols is the biologically effective dose. The aerosols can deposit in the lung by various respiratory deposition mechanisms such as inertial impaction, Brownian diffusion, gravitational

sedimentation, interception, and electrostatic effects. The deposition of particles in the respiratory tract is influenced by several factors including their size, shape, and density.

The size of the aerosolised CNTs and CNFs in an inhalation study is important for pulmonary deposition and penetration in the alveoli where the clearance is relatively limited. On the basis of information from other aerosols, it is known that the size of airborne particles from 10 nm to 100 nm has about 20 % to 50 % deposition rate in alveoli[10]. Whereas, as the size of the aerosolised particles increases from about 100 nm, more particles can deposit in the airways where the clearance is efficient via a mucociliary clearance mechanism[39,40]. The range of aerosol sizes for deposition in the alveoli are variable depending on the agents (e.g. size, shape, and density) as well as hosts (e.g. sex, strain, species, and disease state). A first evaluation of the pulmonary deposition of the inhaled nanomaterials is usually undertaken using in silico lung deposition estimation models. Various models are available to estimate the total and regional lung deposition of aerosolised nanomaterials. Examples include the Human Respiratory Tract Model (HRTM)[41] and the Multiple-Path Particle Dosimetry Model (MPPD)[42]. However, these models have been developed for roundish particles, not fibres, thus careful attention is needed when applying them to fibres and plates. For the application of models to estimate deposition efficiency, there are also been some issues in the reporting of the specific input parameter values used within the model, which are necessary to reproduce results. These values are frequently missing, incomplete or unclear, especially in older publications, although the importance of reporting these values is increasingly being recognized. Uncertainties in the measurements of aerosol parameters used as input to deposition models, and their implications for deposition efficiency calculations and ultimately dose are also generally ignored[43].

The shape of aerosols including the state of aggregation/agglomeration can influence the pulmonary deposition and clearance[44]. Although the aerosols are highly agglomerated, the CNTs and CNFs deposited in the lungs can deagglomerate, which can produce fibre pathogenicity[44]. In contrast, aerosols composed of singlet fibres can re-agglomerate in the lungs, which can exhibit a changed clearance and extrapulmonary translocation[45]. Therefore, deagglomeration or re-agglomeration of aerosols in contact with biological fluids can be considered in the characterization of their physicochemical properties.

The density or specific gravity of CNTs and CNFs is important because this property is one of the main factors that influence the aerodynamic behaviour and deposition fractions in the lungs. The aerodynamic properties for deposition of CNT and CNF aerosols larger than 0,3 µm are especially influenced by the density, whereas particles less than 100 nm are not influenced by the density[44]. Because the density is highly correlated with the volume, the volume per unit mass increases as the density decreases. This can induce volumetric overload in cells, especially in phagocytic cells. Nanomaterials that exceed 6 % of normal cell volume can induce volumetric overload, which subsequently impairs cell function; therefore, the density of CNTs and CNFs can influence the toxicity of nanofibres[44]. The density is a key parameter in estimating the deposition of nanomaterials in the lung using in silico estimation models such as MPPD.

6.3 Size and shape (including length, width, aspect ratio, state of aggregation/agglomeration, and rigidity)

Size and shape are key factors in the fibre pathogenicity, but these factors are closely related to each other. For example, as the diameter of CNT and CNF increases, the rigidity increases, and the increased rigidity can provide better dispersion and reduce aggregation/agglomeration[7,46]. Comparative toxicity studies of MWCNTs with different thickness showed that rigid thin MWCNTs have higher toxicity in vivo and in vitro compared to rigid thick CNTs because thin fibres have a higher potential for disruption of membrane integrity[6,46,47]. There is a threshold thickness of MWCNTs to produce piercing and frustrated phagocytosis to mesothelial cells in vitro as rigid thick (diameter: 150 nm) MWCNTs showed much less toxicity than that of rigid thin (diameter: 50 nm) MWCNT[46]. Furthermore, threshold rigidity values for asbestos-like pathogenicity of MWCNTs in a mouse pleural inflammation model were proposed as a bending ratio of 0,66 and static bending persistence length of 0,87[6]. The length and width of fibres are important for the pathogenicity of CNTs and CNFs because the respective factors are critical for frustrated phagocytosis and cell penetration. The lower threshold length of fibres, which can result in frustrated phagocytosis, has been suggested to be 4 µm to 5 µm[48,49]. Intraperitoneal injection

of CNTs demonstrated that the long fibres are more potent to produce toxicity and mesothelioma than short fibres[50-52]. Furthermore, the range of the width of fibres could also influence cell penetration. It has been reported that thin fibres with some ranges (e.g. 9,4 nm and 50 nm) are more pathogenic than thick fibres with some ranges (e.g. 70 nm and 150 nm)[46,47].

The aggregation/agglomeration status of CNT and CNF is a result of the complex physicochemical properties (e.g. diameter, surface charge, defects, and hydrophobicity) and condition of the liquid medium (e.g. pH, salt, and dispersant) when CNTs and CNFs are dispersed from a liquid suspension. Unlike nanoparticles, it is more difficult to differentiate aggregation, agglomeration, and tangled forms of nanofibres. In addition, an evaluation of the impact of the aggregation/agglomeration status on the toxicity end points has been limited because the aggregation/agglomeration state can vary with experimental conditions. According to current knowledge, the agglomerated and/or tangled CNTs can be less toxic than well-dispersed and/or rigid CNTs. If the diameter of the CNTs is too large to be phagocytosed by reticuloendothelial cells, the CNTs are less potent to produce frustrated phagocytosis and inflammasome activation[46]. In addition, if the diameter of tangled CNTs is small enough to allow for phagocytosis, the toxic potential is also less than well-dispersed CNTs because complete phagocytosis is possible[53]. Although some studies have reported that the intratracheal instillation of agglomerated CNTs can produce severe pulmonary toxicity, such as granulomatous inflammation with discrete granulomas often surrounded by hypertrophied epithelioid macrophages[54], it could not be reproduced in an inhalation toxicity study in terms of inhalability and deposition in the lung[55].

The length and diameter can also be expressed as an aspect ratio (length/diameter), and an aspect ratio more than 3:1 is well known as a parameter for the asbestos-like toxicity such as granulomas, fibrosis, and cancer[34,56,57]. When occurring together, a high aspect ratio (>3:1), thin diameter (<50 nm), long length (>5 µm), high rigidity (>0,66 bending ratio and >0,87 static bending persistence length), minimal aggregation/agglomeration, and high durability are believed to be major pathogenic factors of CNT and CNF.

6.4 Specific surface area

The specific surface areas or surface-to-mass ratios of CNTs and CNFs are relatively high compared to metal-based nanomaterials due to the low density/mass. Because the specific surface area value originates mainly from size and shape, the impact of surface area on the toxic potential of CNTs and CNFs remains unclear. On the other hand, this value is an important dose metric for comparison between nanomaterials. However, the surface area metric can not be pertinent for CNTs and CNFs, although the surface area has been suggested as a dose metric for nanoparticles[58,59]. For example, a CNT which has been classified by IARC as highly toxic has a low surface-to-mass ratio, while many of MWCNTs exhibiting a high surface-to-mass ratio show less toxicity[6,45].

6.5 Crystalline structure and defects

There is little information regarding the impact of crystalline structure and defects on the toxicity of CNTs and CNFs. CNTs and CNFs without defects are composed of hexagonal rings of sp^2-hybridized carbons, and the purification and functionalization processes can induce structural defects[60]. Defects can affect the shape and functionality of MWCNTs, and the most common defects of CNTs and CNFs include vacancies, heptagon-pentagon pairs of transformations, doping, and interstitials, edges, and adatoms[60]. CNTs and CNFs can have the potential to generate or quench free radicals, and defects can increase both properties[61]. However, more studies are needed to evaluate the role of defects and their effect on the toxicity of CNTs and CNFs.

6.6 Surface chemistry, functionalization, surface charge, impurities, and radical generation/scavenging potential

There are many parameters related to surface chemistry, but surface functionalization, surface charge, and free radical generation/scavenging potentials have been suggested to be the main parameters related to the toxicity of CNTs and CNFs. Surface functionalization is closely related to the aggregation/agglomeration, surface charge, and radical generation/scavenging potential. Oxidized CNTs have shown higher cytotoxicity than pristine CNT in human lymphocytes[62], human neuroblastoma cells[63], and

mouse embryonic stem cells[64]. The fibrogenic potential of cationic CNT modified by polyetherimide was higher than pristine CNTs, while anionic CNT modified by COOH-PEG shows less potential than pristine CNTs[65].

The effect of surface charge on toxicity is complicated because the surface charge of CNTs can be modified by various factors including dispersion medium, surface functionalization, and protein corona formation[65]. In addition, these factors in combination can influence the dispersibility of CNTs and CNFs, which can contribute to the toxicity. In addition, the formation of protein corona to CNTs and CNFs can modulate the biological effects of nanomaterials. In general, a protein-corona formation of CNTs and CNFs can mitigate the cytotoxicity[66], but it can increase the possibility for opsonisation by innate immune cells and induce immunological responses such as neo-antigen formation and antigen presentation to immune cells[67].

The purity of CNTs and CNFs can be variable. The lowest purity can be less than 50 % for arc discharge synthesis and the highest purity can be 99,9 % depending on the purification processes. The composition of CNTs and CNFs can be categorised by trace organic (e.g. amorphous carbon) and inorganic elements (metals)[68]. The levels of trace metals can be measured by ICP-MS, and high levels of Al, Fe, Co, Ni, and Mo have been detected in workplaces that manufacture CNTs[69]. Among various metals, the levels of Fe are positively correlated with the radical generation potential measured by electron spin resonance[70]. Carbon-based nanomaterials have both a radical scavenging effect and a radical generating effect. Impurities and defects of CNTs can increase the potential for both radical generating and scavenging effects[61]. Thus, compared to pristine CNTs, CNTs after the elimination of their defects and impurities by heating at 2 400 °C reduced pulmonary toxicity[61,71]. However, the elimination of defects and impurities did not result in any differences in their carcinogenic potential compared to pristine CNTs, which could be because the main parameters of the carcinogenic potential of CNTs are size and shape[71]. In addition, the levels of radical generation by CNTs were reported to be closely correlated with the Fe content in CNTs because of the Fenton reaction by Fe[70]. Although the various chemical properties, such as surface chemistry, functionalization, surface charge, impurities, and radical generation/scavenging potential, can modulate the toxicity, they are considered minor modulators for the toxicity of CNT and CNF; the major modulators of toxicity (i.e. granulomas, fibrosis, and cancer) are the physicochemical parameters related to the shape of the CNTs and CNFs[6,71,72].

6.7 Biodurability

Different nanomaterials are reported to be biopersistent as they resist breakage or dissolution and clearance which in turn lead to their accumulation in an organ. It is therefore of great relevance to assess this property of nanomaterials and determine their degradation half-lives in addition to their other properties. Biodurable nanomaterials maintain their particulate state which can increase the potential for their bioaccumulation[73]. The release of ions from soluble nanomaterials has also been shown to be strongly associated with their toxicity[74]. It is also of great relevance to study the impact of surface coating and functional groups on biodurability in biological and environmental surroundings. The surface modification of nanomaterials can reduce biodurability and minimize health risks. For example, accumulation in cellular lysosomes for SWCNTs showed an enzymatic degradation by lysosomal R-glucosidase, horseradish peroxidase, myeloperoxidase, and heme oxygenase-1[75]. Similar studies with CNTs have indicated that carboxylated CNTs are more susceptible to biodegradation compared to other CNTs[76].

7 Issues for the characterization of CNT and CNF aerosols

7.1 General

When the physicochemical properties, such as size, shape, and surface chemistry, of CNTs and CNFs before generation are changed by aerosol generation processes, the inhalation toxicity studies do not reflect human exposure conditions. In addition, the aerosolisation technique used can change the characteristics of aerosol in a number of ways during the process of aerosolisation. Thus the size distribution of the aerosol can be different from the original material. For this reason, a characterization of the CNT and CNF test materials prior to aerosol generation needs to be conducted first for the

inhalation toxicity of aerosols. This information can then be compared to that obtained in the second stage of characterization of the generated aerosols, which is crucial in understanding and evaluating the toxicity of CNT and CNF. Further information on the characterization of CNT and CNF before aerosol generation and aerosols as generated is provided in Tables 1 and 2.

7.2 Characterization of physicochemical properties of CNT and CNF prior to aerosol generation

7.2.1 General

The physicochemical characterization of test CNT and CNF as manufactured or pristine can be performed before the generation of CNT and CNF aerosols or in situ generation. CNTs and CNFs are manufactured using diverse synthesis procedures that impart those unique properties designed for specific applications. As a result, the materials have a complex structure, including impurities, and have different surface properties (coatings or other modifications). Because physicochemical properties influence the toxicity of CNT and CNF, the thorough characterization is essential to understand the toxicity data. Useful information on physicochemical properties includes, but is not limited to, particle size, size distribution, shape, aggregation/agglomeration, surface characteristics such as surface area and charge, crystalline structure, dustiness, composition, and purity (Table 1).

Table 1 — Physicochemical properties to be characterized prior to aerosol generation

Properties	Apparatus	Reference
Size and size distribution	TEM, SEM	ISO/TS 10797; ISO/TS 10798
Shape	TEM, SEM	ISO/TS 10797; ISO/TS 10798, ISO/TS 11888
Aggregation/agglomeration[a]	TEM, SEM	ISO/TS 10797, ISO/TS 10798
Specific surface area	BET	ISO 9277
Surface charge	Zeta potential meter	ISO 13099-2
Crystalline structure	XRD, Raman	ISO 22262-3
Composition, purity, and impurity	TGA, GC-MS, TEM-EDX; SEM-EDX, ICP-OES, ICP-MS	ISO/TS 11308, ISO/TS 11251, ISO/TS 10798, ISO/TS 13278
Biodurability	EC/OC measurement, UV-Vis, TEM, SEM	ISO/TR 19057
Dustiness	Rotating drum method	EN 17199-2:2019[86]
	Continuous drop method	EN 17199-3:2019[87]
	Small rotating drum method	EN 17199-4:2019[88]
	Vortex shaker method	EN 17199-5:2019[89]

[a] Agglomeration/aggregation is not relevant for a powder form used for dry dispersion aerosol generation.

7.2.2 Size and size distribution

The size and size distribution of CNTs and CNFs can be evaluated in two stages: primary fibres and fibres in a medium for liquid dispersion. The size and size distribution of primary nanotubes and nanofibres can be measured by TEM and SEM. The size of primary nanotubes or nanofibres is composed of its width (or diameter) and length. TEM can be used to more accurately measure the diameter of fibres compared to SEM, while SEM can be used to more accurately measure the length of fibres compared to TEM. The measurement of length and diameter is needed in pair for each counted fibre particle, and both ends of the fibre particle need to be clearly seen to measure. Thus, the length of fibres is difficult to measure for tangled fibres. The width (or diameter) of fibres can be regulated by the number of walls and the use of metal catalysts and influences the rigidity of fibres.

When generating aerosols from a liquid medium with dispersed CNTs and CNFs, the evaluation of size and size distribution is needed before the generation of aerosols to ensure that high-quality aerosols can be generated. The hydrodynamic size by the dynamic light scattering method cannot provide accurate size information of fibre-shaped nanomaterials because the scattering of a laser can be changed by the direction of fibres. However, it can provide information about dispersion status because strongly agglomerated fibres have larger sizes than less agglomerated fibres[90]. Another method to evaluate the size distribution is measuring the length of fibres under a light microscope by preparation of glass slides by mixing the dispersed samples with glycerol at a 1:1 (v/v) ratio because glycerol can minimize the movement of samples[91]. With this method, the percentage of fibres having specific ranges of length distribution can be provided (e.g. less than 5 µm, 5 µm to 10 µm, and 10 µm to 15 µm). However, this method cannot provide an accurate size or size distribution of fibres in a liquid medium and only agglomerated and aggregated CNTs and CNFs will be visible due to the resolution of light microscopy. Fibres less than 100 nm width are unlikely to be visible using conventional light microscopy. Other novel methods based on lasers offer lower resolution and visibility of CNTs[92].

7.2.3 Shape (rigidity and agglomeration/aggregation)

The shape factors of CNTs and CNFs are diverse and include the number of walls, the arrangement of graphite layers, and rigidity. The shape of CNTs and CNFs can be measured by TEM and/or SEM. TEM is useful to evaluate the number of walls, the arrangement of graphite layers, and the rigidity of CNTs and CNFs. The standard measurement method of rigidity has been published as ISO/TS 11888. The bending ratio and static bending persistence length are major parameters describing the rigidity of CNTs and CNFs, which is calculated by the length from end to end and along the axis of the individual strands of nanofibres[79]. The new measurement methods have been developed such as measurement of the flexural rigidity by dynamic SEM[93]. The degree of aggregation/agglomeration can be evaluated by the morphological characteristics using TEM or SEM according to ISO/TS 10797 and ISO/TS 10798. In liquids, CNTs and CNFs generally have a high aggregation/agglomeration status; therefore, the confirmation of fibres dispersion in the liquid with minimum aggregation/agglomeration is needed before the generation of aerosols.

7.2.4 Surface area

The specific surface area of CNTs and CNFs samples is routinely determined by the measurement of N_2 gas adsorption and calculations using the BET isotherm. Also, the specific surface area of CNTs and CNFs can be calculated as a function of the characteristics such as the number of walls and diameter[95]. Test samples for BET measurement can be prepared in a contamination-free environment and requires 200 mg or more as a dry powder for the general purpose of BET analysis[96].

7.2.5 Crystalline structures

The crystalline structures and defects of CNTs and CNFs can be measured by XRD and Raman spectroscopy. Because CNTs and CNFs are composed of crystalline graphite structures, the observation of crystalline structure is essential for a confirmation of the basic physicochemical properties of CNTs and CNFs.

7.2.6 Surface chemistry, functionalization, surface charge, and radical generation/scavenging potential

The identification of surface functional groups can be performed by X-ray photoelectron spectroscopy and Boehm titration. The surface charge of CNTs and CNFs can be determined via a zeta potential measurement according to the ISO 13099-2. The potential for radical generating effects of CNTs and CNFs can be measured by a DCFH-DA assay or electron spin resonance (ISO/TS 18827), while the potential for radical scavenging effects can be measured by a superoxide dismutase assay.

7.2.7 Composition, purity, and impurities

The overall purity and impurities of CNTs and CNFs can be measured by TGA according to the ISO/TS 11308. TGA is typically used to quantify the level of non-volatile impurities present in the CNT

and CNF. TGA is also useful for measuring the thermal stability of CNTs and CNFs. However, TGA cannot provide the relative fractions of differently structured carbon materials (e.g. amorphous, sp2, and sp3), and the thermal stability is different among carbonaceous materials. The volatile components of CNTs and CNFs can be measured by GC-MS according to ISO/TS 11251.

The elemental compositions of CNTs and CNFs can be measured by an EDX technique and inductively coupled plasma technique. EDX is an option for electron microscopy accompanied by TEM or SEM, which is incorporated with TEM or SEM (i.e. TEM-EDS and SEM-EDS). The EDS technique is useful to characterize the elemental composition of catalysts and other inorganic impurities in the material. The inductively coupled plasma technique can be incorporated with the mass spectrometry (ICP-MS) or optical emission spectrometry (ICP-OES). The method of ICP-MS for CNT is published as ISO/TS 13278.

7.2.8 Biodurability (in vivo and in vitro tests)

Adaptation of traditional dissolution rate studies to mimic the physical and chemical conditions encountered in biological tissues and organs allows investigators to estimate biodurability through a calculation of residence times based on the chemical dissolution mechanism. The dissolution rates, a measure of their biodurability, can be determined using in vivo and in vitro tests. The in vivo tests are based on intratracheal or inhalation exposures and measures the retained fibres in an organ at a certain time-point[98], while in vitro tests can be performed using cellular in vitro systems or acellular in vitro testing systems. Cellular in vitro systems involves the treatment of cells with fibres followed by measurement of morphological changes or elemental content[98]. Macrophages such as alveolar macrophage and monocyte-derived macrophage are commonly used for the cellular in vitro system. Acellular in vitro system involves the determination of the degree of dissolution of fibres using simulated biological fluids[22]. Numerous simulated biological fluids including simulated lung extracellular fluid and phagolysosomal simulant fluid can be used for the biodurability study in the inhalation setting[22]. The evaluation of dissolution rates in acellular in vitro systems can be assessed by the changes in the sample mass of fibres (e.g. EC/OC, UV-Vis spectroscopy), the concentration of released ions (e.g. ICP-MS for metal-doped fibres), or a physicochemical characteristic (e.g. TEM or SEM)[98].

7.3 CNT and CNF aerosol characterization (sampling and measurement)

7.3.1 General

Based on several workplace surveys or exposure assessment studies, CNT aerosols can exist as dispersed form[8] or as airborne agglomerate forms of primary CNTs and CNFs, which were found in most workplace exposures[9]. In addition, the recently revised OECD test guidelines 412 (subacute) and 413 (subchronic) have requested aerosol characterization[12,13]. During the process of aerosol generation, the physicochemical properties of CNTs and CNFs can be modified by dispersion and generation methods including grinding, brushing, micronising, and atomising. Thus, it needs to be noted that generated aerosols have similar characteristics with the reality (at the workplace or during further life cycle when it is embedded in a polymer matrix material). To characterize CNT and CNF aerosols in the inhalation chamber, the selection and use of appropriate aerosol samplers and particle size measurement devices are important. The characterization of CNT and CNF aerosols represent some of the most difficult physical measurements not experienced from other particles. Therefore, it is necessary to consider the distinct features of sampling and measuring devices. Because there are no sampling and measurement devices unique to CNT and CNF, devices used in nanoparticle sampling and measurement devices can be adopted in the CNT and CNF sampling and measurement. Some of the instruments used for sampling and measuring CNT and CNF are included in Table B.1. Because the dosimetry for inhalation study remains controversial, it is good practice to measure concentrations in all three matrices (mass, surface area, and number) for a better understanding of the toxicological outcome.

Table 2 — Characterization of CNT and CNF aerosols as generated

Properties	Apparatus	Reference
Size and size distribution		
— MMAD	Cascade impactor, MOUDI[a], ELPI®[b]	OECD 412 , OECD 413[12,13] ISO/TS 21361 (ELPI®[a])
— CMD	APS, TEM, SEM, DMAS, OPC	References [100] and [101] ISO 28439 (DMAS) ISO 21501-1 (OPC)
— CML	TEM, SEM	References [100] and [101]
— Tube median diameter	TEM, SEM	References [100] and [101]
— Tube median length	TEM, SEM	References [100] and [101]
Shape		
— Aspect ratio — Agglomeration/aggregation — Rigidity	TEM, SEM TEM, SEM TEM, SEM	ISO/TS 10797; ISO/TS 10798 ISO/TS 11888; Reference [93]
Crystalline structure and defects	XRD, Raman, TEM	References [104] and [105]
Composition, purity, and impurity	TGA, GC-MS, ICP-MS, ICP-OES, TEM-EDX; SEM-EDX	ISO/TS 11308; ISO/TS 11251; ISO/TS 10798 ISO/TS 13278
Density	DEMC, APM	References [94], [106] and [107]
Concentration (mass)	Gravimetric measurements (sampling on filter and mass balance, TEOM), EC/OC	NIOSH 0500 (Mass balance)[108] NIOSH 0600 (Mass balance)[109] TEOM[100] NIOSH 5040 (EC/OC)[110]
Concentration (number)	DMAS, CPC, OPC	ISO 28439 (DMAS) ISO 27891 (CPC) ISO 21501-1 (OPC)

[a] MOUDI is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

[b] ELPI® is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

7.3.2 Size and size distribution of CNT and CNF aerosols

7.3.2.1 General

Since the particle size determines the lung deposition of particles, knowledge on the particle size of CNTs and CNFs will allow for the selection of ideal testing approaches and for setting up starting conditions. Obtaining a stable particle size distribution is critical to the success of inhalation studies.

7.3.2.2 Mass median aerodynamic diameter

MMAD and GSD have been frequently measured in inhalation toxicity studies of CNT and CNF. MMAD less than 2 µm ensures sufficient exposure to the low respiratory tract of rodents[12,13]. The inhalation study of MWNT-7 (Mitsui Co.) reported that the MMAD of fibres was 1,5 µm with a GSD of 1,69 and CMD of 0,42 µm[112]. The MMAD of aerosolised MWCNT (Helix Material Solutions) ranged from 164 nm to 209 nm with a GSD less than 2[113]. Another study with MWCNT from the same company showed an

MMAD of 714 nm ± 328 nm and GSD of 2[114]. A 90 d inhalation study of Graphistrength C100 MWCNT (Arkema) reported 1,62 µm MMAD and 4,67 GSD for the medium dose, and 2,3 µm MMAD and 2,47 GSD for the high dose[35]. A 90 d inhalation study of Baytube (Bayer) reported 2,74 µm to 3,42 µm MMAD and 1,98 to 2,14 GSD, which were measured by a critical orifice cascade impactor, while those parameters by an aerodynamic particle sizer were 167 µm to 2,19 µm MMAD and 1,67 to 1,76 GSD[100]. A 14 d inhalation study of MWCNT (Shenzhen Nanotech Port Co.) reported 0,7 µm to 1 µm MMAD and 2 GSD for 0,3 mg/m^3 to 1 mg/m^3, and 1,8 µm MMAD and 2,5 GSD for 5 mg/m^3[115].

The aggregated/agglomerated CNTs such as Baytube[100] tend to have larger MMADs in comparison with a well-dispersed CNT such as MWNT-7[116]. Some studies have shown that MMAD and GSD were within the range prescribed by OECD test guidelines (MMAD ≤ 2 µm; GSD 1-3), but in some studies, they were outside of the recommended range. In those cases, an explanation of why the MMAD and GSD range was not obtained is required in the document. MOUDI[1)] (micro-orifice uniform deposit impactor) or a cascade impactor can be used for all materials including fibrous materials for the determination of exposure concentrations in terms of mass and size distribution[12,13]. The MMAD of CNF can also be determined by an impactor with a constant flow air sampler.

The MOUDI[1)] cascade impactor is a frequently adopted method for the measurement of size distribution based on MMAD[12,13,107]. It collects size-fractionated particle samples in the 0,056 µm to 10 µm aerodynamic diameter range for gravimetric and/or chemical analyses. Other cascade impactors including the ELPI® (Electrical Low Pressure Impactor) and ELPI®+[2)] can also be used if the size range is appropriate and enough mass concentration is collected[117]. The ELPI® and ELPI®+ measures in real-time the particle size distribution and number concentration in the size range of 6 nm to 10 µm. For accurate quantitative measurements on particle number concentrations using the ELPI® and ELPI®+, the density information of the airborne agglomerates is needed, which can be very challenging. CNT or CNF deposited on the stages are collected on substrates, which can be analysed for mass, or via chemical analysis or microscopy. However, to limit particle rebound and inaccuracy in measurements, the substrates are greased. The grease coating used to prevent particle rebound, can interfere or prevent chemical microscopy analysis. However, every technique for size measurement of aerosols has an error for high aspect ratio nanomaterials such as CNTs and CNFs[94].

7.3.2.3 Count median diameter

The CMDs of aerosolised CNTs and CNFs have been frequently measured by DMAS or OPC[65,73]. DMAS in combination with APS (e.g. TSI 3321) or OPC can measure a wide range of the particle size distribution for most CNT aerosols. The mobility diameter of CNTs cannot always be measured using a DMAS due to arching or corona discharges within the classifier[74]. The DMAS can also show anomalous responses above certain voltages when characterizing aggregates of airborne CNTs or CNFs[118]. CMD values obtained with an OPC and a DMAS are usually different because the instruments cover different particle size ranges. The DMASs measure a mobility diameter while OPCs measure an optical diameter. In addition, CMD can be measured by TEM or SEM[100,101]. DMAS can also provide data on the stability of CNT or CNF concentrations during aerosol generation by monitoring number concentrations in the test chamber in real-time.

7.3.2.4 Count median length

CMLs of aerosolised CNTs have been reported in inhalation studies[57,101,116,119]. The measurement of CNT diameter and length has usually been conducted by TEM or SEM. The diameters of the CNTs or CNFs usually have small variations, while the length can give great variations. This data can be used as an aspect ratio (length/diameter) of the aerosolised CNTs or CNFs. The length of CNTs and CNFs can be plotted against the cumulative numbers of fibres to obtain a CML and GSD[101]. However, measuring the length of fibres can be very challenging if most of the airborne particles are agglomerates.

1) MOUDI is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

2) ELPI® and ELPI®+ are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products.

7.3.3 The shape of CNT and CNF aerosols

7.3.3.1 General

CNTs and CNFs can have a variety of shapes with the same composition depending on their manufacturing methods as well as use and handling after manufacturing. Furthermore, the shape of primary fibres can be highly altered by the aerosol generation method. There are several methods of sampling of CNT or CNF such as by electrostatic precipitation[118,120], by thermophoretic precipitation[120,121] and by filteration. Electrostatic and thermophoretic precipitators collect particles directly onto a TEM grid for direct TEM analysis. For the sampling of airborne particles by filteration, the filter is placed in a sampler (open face or conductive cowl sampler) and connected to a pump for static or breathing zone sampling. Soo et al. have investigated the collection efficiency of commercially available porous filters for the sampling of airborne nanoparticles[122]. Polycarbonate or mixed cellulose filter can be used for subsequent TEM analysis. Preparation of the filter (to transfer the particles collected on a filter to a TEM grid) is required before performing TEM analysis[8,123,124]. Gold coated filters are suggested for direct SEM analysis. No preparation of the filter is required prior to SEM analysis.

7.3.3.2 Aspect ratio

CNTs and CNFs aspect ratios of the generated aerosols show different values compared to bulk nanofibres before generation. The aspect ratio of CVD manufactured MWNT-7 powder was greater than 100, while aerosolised MWNT-7 was nearly 60[34].

7.3.3.3 Aggregation/agglomeration in the aerosol state

The aggregates of CNT and CNF can be formed by strong chemical bonding forces (e.g. covalent bonds) or by sintering or complex physical entanglement. The agglomerates of CNT and CNF can be formed by weak forces (e.g. van der Waals forces) or simple physical entanglement. The agglomeration state is a description of the agglomerate at a given moment in time; changes of the agglomeration state are indicative of the dynamic equilibrium, which depends on the nanoparticle generation system, dust concentration within the test chamber, time after generation, and environmental conditions (ISO / TR 13014). The aggregation and agglomeration state of aerosols can be analysed by SEM or TEM image[94].

7.3.3.4 Rigidity

ISO/TS 11888 can be used for the measurement of rigidity. It suggests two parameters for the rigidity as bending ratio and static bending persistence length, which can be obtained by a TEM image analysis. Furthermore, new measurement methods of rigidity are also available such as measurement of the flexural rigidity using a dynamic SEM and measurement of bending force by atomic force microscopy[93].

7.3.4 Crystalline structure and defects

If the CNT or CNF aerosol generation process involves a harsh mechanical or chemical process to generate the CNT or CNF, the structural defects can be made compared to that before aerosol generation. The crystal structure of CNT and CNF can be evaluated by X-Ray diffraction, Raman spectroscopy, or other appropriate methods. Raman spectroscopy can provide detailed information regarding both the purity of the sample as well as structural information, including diameter distribution, electronic structure, and chirality, which determines either metallic or semiconducting properties and bandgap energy as well as defects in the sp^2 graphene layer[105]. TEM with electron diffraction can determine some crystal structure[104].

7.3.5 Surface chemistry

The surface properties of CNTs and CNFs can be modulated by their production method, the application of post-synthesis modifications (purification), and/or covalent functionalization of their external surface. Acidic purification, mechanical purification (e.g. heating), or functionalization of CNT could damage the surface structure of CNTs and CNFs. In addition, aerosol generation processes involving

micronisation, milling, grinding, and atomisation could mechanically damage the surface structures of CNTs and CNFs. The airborne CNTs or CNFs collected on a filter can be analysed by Raman spectrometry, which is one of the appropriate methods for characterization of surface chemistry and crystal structure[105].

7.3.6 Composition analysis

The airborne CNTs or CNFs collected on a filter during aerosol generation can be analysed for chemical composition based on ISO/TS 11308, ISO/TS 11251, ISO/TS 10798, NIOSH 7300[126], or equivalent methods. The measurement technique is the same as in 7.2.7.

7.3.7 Fibre density

The effective density of a particle less than 1 000 nm in diameter can be defined as the particle mass divided by the particle volume based on mobility diameter, and this can be obtained by the combination of a tandem differential mobility analyser and aerosol particle mass analyser in series (tandem DEMC-APM system)[94,106,107]. Thus, this method enables direct (real-time) measurement of the effective density of CNT and CNF. However, rigid and single fibres have limitations in the measurement of DEMC-APM system because the size that the DEMC selects depends on the tendency of the fibres to align and the settling of the DEMC (affecting the voltage over the DEMC).

7.3.8 Concentration

7.3.8.1 General

The purpose of inhalation toxicity testing is to characterize the toxicity of certain materials via the inhalation route for a certain duration and to provide robust data for quantitative inhalation risk assessment. A concentration-response evaluation is a step in the risk assessment process that examines the relationship between the magnitude of exposure and the response of the test system. Although conventional inhalation toxicity testing is based on the mass of the material (mg/m^3), recent revised OECD inhalation testing guidelines recommended including the surface area concentration, and number concentration in addition to mass concentration[12,13]. It is also desirable to maintain a stable airborne CNT or CNF concentration in the test chamber of no more than ±20 % deviation.

The evaluation and characterization of airborne CNTs and CNFs is challenging. Direct reading instruments enable to differentiate between compact clusters, fractal clusters, particles and fibres morphologies. They report an equivalent diameter rather than the diameter and length traditionally reported for fibres. In addition, traditionally fibres have been counted and reported as fibres/ml according to the World Health Organization (WHO), which defines fibre of a length greater than 5 µm, a diameter less than 3 µm, and an aspect ratio larger than 3:1. However, there are currently no counting rules for CNTs and the counting methods for airborne asbestos and micron-sized fibres cannot be directly applied to CNTs. However, a number of studies have been published and authors have been suggested rules for semiquantitative or quantitative measurement[124,127,128].

7.3.8.2 Mass

CNT and CNF aerosol concentrations are expressed as a mass per volume metric, such as mg/l or mg/m^3[107,117], where the mass concentration is related to the test chemical. The mass concentration of CNT and CNF is the material concentration sampled in the breathing zone of the animals in the inhalation chamber, which can be analysed by gravimetric analysis of the filter before and after sampling. Most studies determined their CNT and CNF aerosol mass concentrations principally with a gravimetric method. The elemental carbon mass of CNT and CNF aerosols can be compared with the exposure limit values (e.g. US NIOSH REL for CNT/CNF 1 µg/m^3 elemental carbon as a respirable mass 8 h time-weighted average concentration according to NIOSH method 5040[110]). For real-time monitoring of CNT and CNF at low concentrations, a TEOM can be used[100].

7.3.8.3 Number

It is desirable to measure the number concentration of CNT or CNF for additional dose metrics because the most appropriate dose metric for nanomaterials is disputable in various situations[129]. Real-time instruments such as CPC, DMAS, and OPC can measure number concentration. CPC and DMAS can provide number concentrations of particles over the size range from few nm to one or few µm and in general OPC from 0,3 µm to 15 µm or greater. Many CNT and CNF studies used these real-time number concentration monitors as supplementary concentration measurement devices[34,100,130]. These devices can also monitor the stability of the number concentration in the inhalation chamber.

7.3.8.4 Surface area

Since an appropriate dose metric for nanomaterials is not well understood for nanofibres, a measurement of the surface area concentration of CNT or CNF can be used for additional dose metrics. Aerosol diffusion charging has been shown to provide a measure of the surface area online where the charging rate is low[131]. These devices have been shown to measure aerosol surface area well for particles smaller than 100 nm in diameter[132]. At larger diameters, the measured surface area is progressively underestimated. In particular, the surface area of porous particle structures, as well as that of highly aggregated particles, will generally not be determined, while online aerosol surface area measurements are desirable during inhalation exposure studies, uncertainties associated with current techniques suggest caution when interpreting such measurements[44]. There are real-time instruments [e.g. Nanoparticle Surface Area Monitor (NSAM), DiscMini, and Partector], which measure the human lung-deposited surface area (LDSA) of particles (reported as $\mu m^2/cm^3$) corresponding to the tracheobronchial and alveolar regions of the lung. They can provide a simple and fast solution for estimating the surface area-equivalent dose in the lungs. The off-line surface area measurement of CNT/CNF aerosols can be performed by a BET method after collecting of aerosols by membrane filters[133].

7.4 Direct and indirect measurement

7.4.1 Direct measurement

Table 3 summarizes the direct (or online) measurement method for the concentration of CNT and CNF aerosols. The number concentration of CNT aerosol in the inhalation chamber system is frequently directly measured by APS, OPC, CPC, and DMAS. Although these instruments provide information on number concentration, unlike other nanoparticles, the number concentration of CNTs and CNFs measured by APS, OPC, and DMAS can give inaccurate information of concentration stability in terms of number concentration because of the light scattering area (APS and OPC) or dipole moment (DMAS). However, CPC can accurately count the number of particles[127]. The estimation of mass concentration from the CNT or CNF number concentration is not possible.

Table 3 — Direct (online) measurement method for the concentration of CNT and CNF aerosols

Method	Principle	Metrics and range	Use in an inhalation study
APS	Spectrometer measuring the aerodynamic size of particles. Time-of-flight aerodynamic sizing determines the particle's behaviour while airborne and is unaffected by the index of refraction or Mie scattering.	Particle size distribution and number concentration. Aerodynamic particle diameter size range: 0,5 µm to 20 µm	Can measure in real-time the particle size distribution of aerosols.
OPC	Measuring light scattering with a laser. Particle size and number concentration are obtained from the intensity and count of scattered light, respectively.	Particle size distribution and number concentration. Optical based diameter. Size range: 0,3 µm to 10 µm	Can measure the number concentration of aggregated/agglomerated CNTs or CNFs.

Table 3 *(continued)*

Method	Principle	Metrics and range	Use in an inhalation study
CPC	Particles grow larger with condensation when passing a supersaturated atmosphere of butanol or water. Particles smaller than 0,3 µm – 0,5 µm can be measured.	Number concentration. Size range: 0,01 µm to 1 µm	Can count number concentration of small CNT and CNF.
DMAS	Particles are selectively classified with respect to size by a change in intensity of an electrical field and are counted using a CPC.	Particle size distribution and number concentration. Electrical mobility diameter. Size range: 5 nm up to 1 000 nm	Can count the number concentration of small CNT and CNF with mobility size information. Not suitable for larger particles.
TEOM	The oscillation frequency of the microbalance changes with the mass of particles collected on the filter.	Mass concentration; 0 g/m^3 to 5 g/m^3	When the concentration is high, a more frequent filter change is needed for accurate measurement.

7.4.2 Indirect measurement

Table 4 summarizes the indirect measurement methods used to determine the concentrations of CNT and CNF aerosols. In most CNT and CNF inhalation studies, the mass concentrations were presented in terms of mg/m^3 by using gravimetric analysis after sampling on a filter. Sampling respirable airborne CNTs or CNFs onto a using a cyclone has also been performed to quantify the amount of elemental carbon and for comparison with the NIOSH recommended exposure limit (REL). Trace metals used for the synthesis of CNTs or CNFs can be quantified by ICP-MS or OES after sampling of CNT or CNF aerosols.

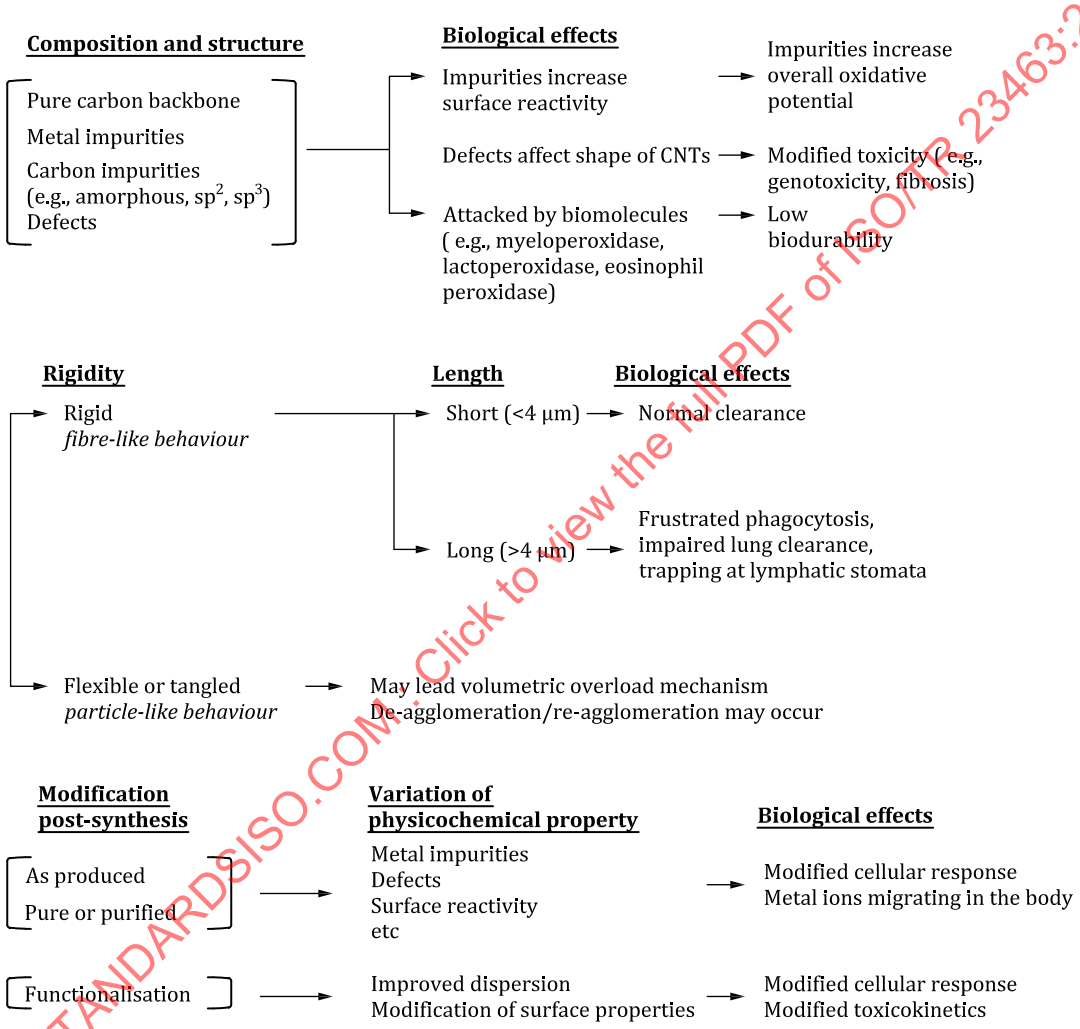
Table 4 — Indirect (off-line) measurement method for the concentration of CNT and CNF aerosols

Method	Principle	Use in an inhalation study	References
Gravimetric analysis	CNTs and CNFs are collected on a filter and weighed with a balance (e.g. NIOSH NMAM 0500; 0600).	Most studies used this method.	[107,117]
Elemental carbon (EC) analysis	CNTs and CNFs that are made of elemental carbon are collected onto a quartz filter, which is combusted to measure elemental carbon quantitatively (e.g. NIOSH NMAM 5040).	Method separates EC from OC (organic carbon) and allows the mass concentration of EC to be obtained.	[119,134,135]
Trace metal analysis	CNTs and CNFs containing catalytic metals as impurities are chemically quantified using analytical methods such as ICP-MS, OES, or qualitatively by EDX.	If metal content is known and high, it can be used as a surrogate marker.	[101,119]
TEM/SEM	CNTs and CNFs sampled on a filter can be counted using an EM.	Optimal surface density of 0,008–0,10 particles/µm^2 (filter area 420 mm^2).	[107,117,136]

Annex A
(informative)

Physicochemical properties of CNT associated with biological activity

Figure A.1 presents a flowchart of physical and chemical properties associated with the biological activity of CNT.



SOURCE: Reference [45], Figure 4, reproduced with the permission of the authors.

Figure A.1 — Flowchart of physical and chemical properties associated with the biological activity of CNT

Annex B
(informative)

CNT and CNF aerosol monitoring instruments

Table B.1 presents information about the CNT and CNF aerosol monitoring instruments.

Table B.1 — Information about the CNT and CNF aerosol monitoring instruments

Metric	Devices/ techniques	Principles	Remarks	Use in CNT/CNF	References
Mass directly	Size-selective static sampler	Serial impaction stages collect sequentially smaller particles based on aerodynamic size.	The only devices offering a cut point around 100 nm are cascade impactors (Berner-type low-pressure impactors, or Microorifice impactors). Allows gravimetric and chemical analysis of samples on stages below 100 nm.	MOUDI[a] can be used, as mentioned in OECD TG 412 and 413	[12, 13, 34, 137]
	TEOM	The oscillation frequency of the microbalance changes with the mass of particles collected on the filter.	Sensitive real-time monitors such as a TEOM are useful to measure nano aerosol mass concentration online, with a suitable size-selective inlet.	TEOM was used to measure CNT 0,1 mg/m^3	[100]
Mass by calculation	Electrical Low Pressure Impactor (ELPI®)[b]	Charged unipolar particles are collected by a low-pressure cascade impactor that is charged and connected to an electrometer.	Real-time size-selective (aerodynamic diameter) detection of active surface-area concentration giving aerosol size distribution. Mass concentration of aerosols can be calculated, only if particle charge and density are assumed or known. The ELPI®[b] can detect airborne particles between 6 nm – 10 µm. Size-selected samples can be further analysed off-line (as above).	not used for inhalation toxicity test	[107]
	DMAS	Charged particles were selectively deposited with respect to size by the change in intensity of a collecting electrical field.	Real-time size-selective (mobility diameter) detection of number concentration, giving aerosol size distribution. Mass concentration of aerosols can be calculated, only if particle shape and density are known or assumed.	Not used for mass estimation of CNT and CNF	[107]

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[a] MOUDI is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

[b] ELPI® is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

Table B.1 *(continued)*

Metric	Devices/ techniques	Principles	Remarks	Use in CNT/CNF	References
Number directly	CPC	Supersaturated vapour causes particle growth and particle number determined by intensity reduction by a light beam of air.	CPCs provide real-time number concentration measurements between their particle diameter detection limits. Without a nanoparticle pre-separator, they are not specific to the nanometer size range. Generally, different types of CPCs can detect airborne particles between 5,5 nm to 9 µm. P-Trak has diffusion screen to limit top size to 1 µm. Particle number concentration.	Count concentration of CNT particles	[130]
	DMAS	Particles were selectively deposited with respect to size by the change in intensity of a collecting electrical field.	Real-time size-selective (mobility diameter) detection of number concentration, giving number-based size distribution.	Count concentration of CNT particles	[107, 116, 130]
	Electron microscopy	Projected image formation using electron beam and magnetic optics.	Off-line analysis of electron microscope samples can provide information on size-specific aerosol number concentration.		[116]
Surface area directly Number by calculation	Diffusion charger	The aerosol is drawn through small channel air flow rates; measurements of penetrating fractions lead to the size distribution.	Real-time measurement of aerosol active surface area. The active surface area does not scale directly with the geometric surface area for particles larger than 100 nm. Note that not all commercially available diffusion chargers have a response that scales with particle active surface area for particles smaller than 100 nm. Diffusion chargers are only specific to nanoparticles if used with an appropriate inlet pre-separator.		
	Electrical Low-Pressure Impactor (ELPI®)[b]	Charged unipolar particles are collected by a low-pressure cascade impactor that is charged and connected to an electrometer.	Real-time size-selective (aerodynamic diameter) detection of active surface-area concentration. The active surface area does not scale directly with the geometric surface area for particles larger than 100 nm.		

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Table B.1 *(continued)*

Metric	Devices/ techniques	Principles	Remarks	Use in CNT/CNF	References
	Electron microscopy	Projected image formation using electron beam and magnetic optics.	Off-line analysis of electron microscope samples can provide information on particle surface area with respect to size. TEM analysis provides direct information on the projected area of collected particles, which are related to the geometric area for some particle shapes.		
Surface area by calculation	DMAS	Particles were selectively deposited with respect to size by a change in intensity of a collecting electrical field.	Real-time size-selective (mobility diameter) detection of number concentration. Data are interpreted in terms of aerosol surface area under certain circumstances. For instance, the mobility diameter of open agglomerates has been shown to correlate well with a projected surface area.		
	DMAS and ELPI®[b] used in parallel	DMAS+ELPI [b]	Differences in measured aerodynamic and mobility can be used to infer the particle fractal dimension, which can be further used to estimate surface area.		

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[b] ELPI® is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

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