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Nanotechnologies — Considerations for performing toxicokinetic studies with nanomaterials

Nanotechnologies - Considérations pour réaliser des études toxico cinétiques de nanomatériaux

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

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Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

Nanomaterials (NMs) are a family of chemicals that, like any other chemicals, can exert a range of toxicities. Toxicokinetics can support the safety evaluation of compounds including NMs by identifying potential target organs, and especially for NMs, the potential for persistence in organs (including cellular uptake and compartmentalization). Also, toxicokinetic information can be used to evaluate if a NM behaves differently from a similar NM or bulk material with the same chemical composition, e.g. with regard to barrier penetration. As for all studies with NMs, a proper characterization of the NM dispersions or aerosols used in the toxicokinetic studies is essential.

Importance of toxicokinetic information for risk assessment (of nanomaterials)

Toxicokinetics describes the absorption, distribution, metabolism and excretion (ADME) of foreign compounds in the body with time. It links the external exposure with the internal dose and is thus a key aspect for toxicity. If a NM is absorbed by the body through any of the potential exposure routes (oral, respiratory, dermal) it can enter into the blood or lymph circulation. Subsequent distribution to internal organs determines potential target tissues and potential toxicity. Alternatively, NMs can be intravenously administered (e.g. as nanomedicine) thus directly entering the blood circulation, potentially resulting in wide spread tissue distribution. Toxicokinetics therefore aids in the design of targeted toxicity studies and in identifying potential target organs and can thus also provide relevant information for justification or waiving of toxicity studies. In addition, toxicokinetic information can be useful as basis for grouping and read-across of NMs. Risk assessments based on internal concentrations, determined using toxicokinetic information, can be more realistic than risk assessments based on external doses, as nanoparticles (NPs) can show specific tissue distribution and accumulation. Toxicokinetic studies can be used to build toxicokinetic models, especially physiologically based pharmacokinetic (PBPK) models, which then can be used to extrapolate experimental toxicity data to other species, tissues, exposure routes, exposure durations and doses. Due to the accumulation of some NPs, the ability to extrapolate to longer exposure durations is of special importance for NMs.

Why a technical report specifically for nanomaterials?

A considerable body of published literature, including many national and international guidelines, exists on the use of toxicokinetic methods to study the fate of chemicals in the body. In addition, OECD Test Guideline (TG) 417 on Toxicokinetics (latest update dated 2010) gives an extensive description for evaluation of the toxicokinetic profile of chemicals but excludes NMs specifically. ISO 10993-16:2017 *Biological evaluation of medical devices — Part 16: Toxicokinetic study design for degradation products and leachables*, provides an overview for toxicokinetic studies for leachables of medical devices. Furthermore, the European Medicines Agency's ICH S3A (Toxicokinetics: A Guidance for Assessing Systemic Exposure in Toxicology Studies) and ICH S3B (Pharmacokinetics: Repeated Dose Tissue Distribution Studies) give guidance on the design and conduct of toxicokinetic studies to assist in the development of new drugs.

Guidelines also exist on toxicokinetic modelling, especially the development and application of physiologically-based pharmacokinetic (PBPK) models. For example, the United States Food and Drug Administration's Draft Physiologically Based Pharmacokinetic Analyses — Format and Content Guidance for Industry, provides the standard content and format of PBPK study reports while the United States Environmental Protection Agency's Approaches for the Application of Physiologically Based Pharmacokinetic (PBPK) Models and Supporting Data in Risk Assessment, addresses the application and evaluation of PBPK models for risk assessment purposes. The European Medicines Agency (EMA) has published a "Guideline on the qualification and reporting of physiologically based pharmacokinetic (PBPK) modelling and simulation" in 2016[1]. WHO has published the "Characterization and application of physiologically based pharmacokinetic models in risk assessment"[2].

As stated, the current OECD toxicokinetics TG 417 explicitly states that the guideline is not intended for the testing of NMs[3], as the toxicokinetics of NMs are different from dissolved ions/molecules and large particles. This was confirmed in a report on preliminary review of OECD Test Guidelines for their applicability to NMs[4]. Additionally, the PBPK models described in the current and mentioned guidance documents are not suitable for NMs, as the processes governing the distribution of NPs is different from

those of the dissolved (molecular/ionic) substances addressed by the current guidance documents (e.g. Reference [5]).

New guidelines or specific additions to existing guidelines about the case of NMs are thus necessary. A review of the current knowledge on the specific toxicokinetic characteristics of NMs and the issues around toxicokinetic testing is a practical preparative step to ensure the best possible understanding of testing needed to obtain relevant information on toxicokinetics of NMs.

How are nanomaterials different from dissolved ions/molecules and large particles?

Nanomaterials (NMs) present a unique family of chemicals that, by their particulate nature and reduction in size, acquire specific physical chemical properties not present for their bulk or soluble counterparts, that might or might not be accompanied by specific toxicity as discussed previously in many reports (e.g. References [6], [7], [8], [9], [10]).

Toxicokinetics of NPs is of special interest because, in comparison to larger sized particles, the small size of NPs could enable an increased rate of translocation beyond the portal of entry to the lymphatic fluid and blood circulation, from where they can reach potentially all internal organs[11]. In addition, smaller sized NPs can show a more widespread organ distribution than larger sized particles[12]. For the same reason, transport across barriers such as the blood-brain barrier and placenta can occur (e.g. References [13] and [14]).

Other notable differences between the toxicokinetic behaviour of dissolved molecular/ionic substances and NMs can be understood within the context of the principles that govern the absorption, distribution, metabolism and excretion (ADME) of a substance. For dissolved molecular/ionic substances, toxicokinetics is driven by 1) passive transport, which includes simple diffusion and filtration or 2) special transport, which includes active transport, carrier-mediated transporter systems and facilitated diffusion through cellular membranes, enzymatic metabolism and passive or active excretion. For NMs, toxicokinetics involves aggregation, agglomeration, protein corona formation, active cellular uptake, distribution through macrophages, and for certain NMs degradation, and excretion[15]. In addition, the surface chemistry/composition affects the toxicokinetics of NPs by its potential of binding a variety of biomolecules on the surface (also designated the “protein” corona). As excretion is often limited, bioaccumulation can occur similar to other poorly metabolized molecules. Thus, the requirements for the testing and modelling of the toxicokinetics of NMs can differ significantly from those identified for dissolved substances. In this respect, especially the potential for accumulation and persistence in organs needs to be evaluated, for example in repeated dose and prolonged toxicokinetic studies.

Nanotechnologies — Considerations for performing toxicokinetic studies with nanomaterials

1 Scope

This document describes the background and principles for toxicokinetic studies relevant for nanomaterials.

Annex A shows the definitions for terminology with respect to toxicokinetics as used in OECD TG 417:2010.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

For the purposes of this document, the terms and definitions given in the ISO 80004 series Nanotechnologies Vocabulary and the following apply.

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

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

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

3.1
agglomerate
collection of weakly or medium strongly bound *particles* (3.12) 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 26824:2013, 1.2]

3.2
aggregate
particle (3.12) comprising strongly bonded or fused particles where the resulting external surface area is significantly smaller than the sum of surface areas of the individual components

Note 1 to entry: The forces holding an aggregate together are strong forces, for example covalent or ionic 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, modified — Note 1 adapted.]

3.3
nanoscale
length range approximately from 1 nm to 100 nm

Note 1 to entry: Properties that are not extrapolations from larger sizes are predominantly exhibited in this length range.

[SOURCE: ISO/TS 80004-1: 2015, 2.1]

3.4
nanotechnology
application of scientific knowledge to manipulate and control matter predominantly in the *nanoscale* (3.3) to make use of size- and structure-dependent properties and phenomena distinct from those associated with individual atoms or molecules, or extrapolation from larger sizes of the same material

Note 1 to entry: Manipulation and control includes material synthesis.

[SOURCE: ISO/TS 80004-1: 2015, 2.3]

3.5
nanomaterial
material with any external dimension in the *nanoscale* (3.3) or having internal structure or surface structure in the nanoscale

Note 1 to entry: This generic term is inclusive of *nano-object* (3.6) and *nanostructured material* (3.8).

Note 2 to entry: See also 3.6 to 3.11.

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

3.6
nano-object
discrete piece of material with one, two or three external dimensions in the *nanoscale* (3.3)

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

[SOURCE: ISO/TS 80004-1: 2015, 2.5]

3.7
nanostructure
composition of inter-related constituent parts in which one or more of those parts is a *nanoscale* (3.3) region

Note 1 to entry: A region is defined by a boundary representing a discontinuity in properties.

[SOURCE: ISO/TS 80004-1: 2015, 2.6]

3.8
nanostructured material
material having internal *nanostructure* (3.7) or surface nanostructure

Note 1 to entry: This definition does not exclude the possibility for a *nano-object* (3.6) to have internal structure or surface structure. If external dimension(s) are in the *nanoscale* (3.3), the term nano-object is recommended.

[SOURCE: ISO/TS 80004-1: 2015, 2.7]

3.9
nanoparticle
nano-object (3.6) with all external dimensions in the *nanoscale* (3.3) 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* (ISO/TS 80004-2:2017, 4.5) or *nanoplate* (ISO/TS 80004-2:2017 4.6) may be preferred to the term nanoparticle.

[SOURCE: ISO/TS 80004-2:2017, 4.4, modified — Note 1 to entry has been changed for clarification.]

3.12
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 particle definition applies to *nano-objects* (3.6).

[SOURCE: ISO 26824:2013, 1.1]

3.13
substance
single chemical element or compound, or a complex structure of compounds

[SOURCE: ISO 10993-9:2009, 3.6]

4 Abbreviations

AAS	Atomic Absorption Spectrometry
ADME	Absorption, Distribution, Metabolism, Excretion
AUC	Area under the Curve
BALF	Bronchoalveolar lavage fluid
ICP-MS	Inductively Coupled Plasma – Mass Spectrometry
IV	Intravenous
IVIVE	in vitro in vivo extrapolation
MPS	mononuclear phagocytic system
MWCNT	Multi Walled Carbon Nanotubes
NM(s)	Nanomaterial(s)
NP(s)	Nanoparticle(s)
PBPK	Physiologically Based Pharmacokinetic (model)
SSA	Specific Surface Area
TG	Test Guideline

5 Importance of toxicokinetic information for risk assessment of nanomaterials

5.1 General

Toxicokinetic studies are important to obtain insight in the toxicologically relevant target organs that can be considered more closely in the safety evaluation and risk assessment of NMs and/or NPs. Furthermore, information might be obtained on relevant exposure durations (e.g. acute, chronic) to be applied in toxicity studies based on the persistence of the NP over time. Finally, such information is essential to enable more reliable extrapolations over species, time and exposure routes and can be used for grouping, read-across and waiving.

5.2 Possible use of toxicokinetic information

For dissolved substances, legislation differs in the requirement for providing kinetic information, also between countries, but most often this information is not required by legislation[16]. However, toxicokinetic knowledge is essential for various purposes in the current risk assessment approach based on animal tests:

— to predict systemic exposure and internal tissue dose (correlate given dose with target dose);

— to know whether a test, such as a genotoxicity test in bone marrow or sperm, is relevant (does the substance reach these tissues?);

— to perform route-to-route extrapolation (see e.g. Reference [17]);

— to perform high-to-low-dose extrapolation or to select appropriate doses (see e.g. Reference [18] and [19]);

— to verify human relevance of test results from animals (i.e. perform interspecies extrapolation; e.g. Reference [20]);

— to enable extrapolation in time for accumulating substances, as animal tests do not cover an entire human lifetime, while accumulation can lead to increases in concentration in a tissue that continues lifelong (e.g. Reference [21]).

When avoiding animal tests as much as possible and performing a risk assessment based mostly on *in vitro* test results, as envisioned by the 3Rs principle[22], kinetic information becomes even more essential. *In vitro* tests do not provide for the totality of the toxicokinetics of a whole body, as animals do: the absorption in the intestines, for example, is not included in an *in vitro* test with liver cells. Thus, *in vitro* test results need to be supplemented with kinetic information using kinetic models, in a process named *in vitro in vivo* extrapolation (IVIVE).

In addition, toxicokinetic information provides insight into potential target organs and organ burden that might ultimately result in toxicity. This allows for improved selection and design of hazard studies, e.g. waiving a certain systemic study if absorption and accumulation of the substances are known not to occur, or adding additional analyses to a study that are relevant to identified target organs.

These considerations are valid for both NMs and soluble substances. Specific for non-degradable NMs is that there is a higher potential for accumulation. In the case of accumulation, determination of the kinetics is of greater importance for the correct estimation of a health risk, as an extrapolation in time needs to be made. This is valid for accumulating NMs just as much as it is for accumulating substances. Internal (or target tissue) concentrations are therefore better dose metrics for risk assessment purposes than external doses.

Specific for NMs is also that they have a distinct distribution pattern, with high proportions in organs of the mononuclear phagocytic system (MPS) notably in the liver and spleen. Such information can, for example, warrant special attention for potential effects on liver and spleen cell populations[21][23].

Due to the many forms in which NMs can occur or be produced, of which testing all would require a large amount of resources, grouping is of high interest for NMs. Recent papers on possibilities for grouping of NMs describe kinetic parameters as essential pieces of information on which to base group formation and justification: degradation (including dissolution), distribution and potential bioaccumulation or persistence and distribution[24][25][26]. Dissolution is actually a physico-chemical parameter that also is dependent of the local environment (e.g. water, buffer or (simulated) body fluids), but can also be seen as a kinetic parameter. The rate of dissolution/degradation provides insight in the toxicokinetic behaviour of a NM. Until dissolution occurs, the kinetics of NMs are governed by the particulate nature of the NMs, whereas after dissolution the (dissolved) ions or molecules determine the toxicokinetics. Distribution studies are needed to assess if and to which extent the different NMs show distribution to the same target organs, as part of a scientific justification for grouping, and to assess if the same hazards can be considered. Accumulation is a kinetic parameter, which is not measured directly, but is determined by all other (more basic) kinetic parameters, i.e. absorption, distribution, and elimination.

5.3 Key toxicokinetic issues for nanomaterials

The kinetic properties of a compound include the biodistribution, biodegradation and biopersistence and can be described by the time course for absorption, distribution, metabolism and excretion (ADME) of a compound in the body with time. Absorption, distribution, (metabolism), and excretion can be described as potentially sequential processes. The basic principles that are described in OECD 417[3] and ISO 10993-16:2017 provide a framework how to perform toxicokinetic studies. An OECD Expert meeting, Toxicokinetics of Manufactured Nanomaterials, identified issues for toxicokinetics for NMs and discussed how to address them[27].

The absorption of current NMs/nano-objects after oral exposure is commonly very low, in the order of 1 % and less[17][28].

Another major difference between the toxicokinetics of dissolved substances and NMs is that the tissue distribution for dissolved substances is concentration dependent (i.e. the difference in concentration in the circulation/blood and the organ determines the organ uptake), and that an equilibrium is generally obtained between blood and organ concentration. In contrast, NMs/NPs are rapidly removed from the systemic circulation by cells of the mononuclear phagocytic system (MPS) as indicated by the observed distribution of a major fraction of an injected dose into spleen and liver[12][17][29]. However, also granulocytes are able to take up NPs[30]. This implies that plasma is usually not a suitable media to monitor NP exposure and plasma kinetic parameters such as plasma area under the curve (AUC) are generally not relevant. In addition, PBPK-models for NPs need to be based on blood flow and the uptake of the NPs by macrophages (e.g. References [5], [31] and [32]) or need to consider specific targeting by ligands as components of an NM for drug targeting[33][34], instead of equilibrium partitioning.

Regarding the metabolism, biotransformation or degradation might be a more appropriate term given the uncertainty associated with the occurrence of enzymatic metabolism for many NMs (e.g. for inorganic NMs such as the metal and metal oxides). However, organic NMs can be metabolized. The dissolution of a NM can also be seen as a more general process that transforms NMs, and is thereby similar to metabolism.

Excretion of systemically available NMs is possible through breast milk[35], urine and bile[13], but seemingly not for all types of NPs. For some NPs (e.g. TiO_2), the only elimination route (besides breast milk) seems to be dissolution, which renders insoluble NMs very persistent and accumulative.

Thus, even though kinetic information in general is just as important for molecular substances as for NMs (see 5.1), the type of kinetic information that is necessary differs and other issues arise when testing for toxicokinetic properties. Key kinetic parameters for NMs are:

— degradation, which is determined mostly by the dissolution rate in the various physiologically relevant surroundings (incl. in macrophages) (elaborated on in 6.1);

— absorption (i.e. translocation over the external barriers, dependent on the exposure route) (elaborated on in chapter 9);

— uptake by macrophages/granulocytes or by monocytes in tissues (as a very new parameter, feasibility yet unknown);

— elimination rate from the tissues (elaborated on in chapter 12).

The latter can, together with physiological information on macrophage content of tissues, help determine the potential uptake rate into tissues. Ultimately these key parameters determine the tissue distribution of the NM and indicate the target organs potentially at risk for toxic effects.

6 Factors influencing the toxicokinetics of nanomaterials

6.1 Dissolution rate

A major factor for the induction of an adverse (toxic) effect by NMs is considered to be related to the presence or release of free nano-objects, ions, molecules or components from the individual NM. In

this respect, the dissolution, or rather the dissolution rate, of the NM can be considered crucial for risk assessment. If a NM has been completely dissolved before absorption, the classical risk assessment of the dissolved chemical/molecules can be applied (i.e. no special NM considerations are applicable[36][37]).

NM dissolution rates have been found to be extremely sensitive to variables of the experimental testing protocol, e.g. NM dispersion procedure, primary and agglomerate/aggregate size distributions, temperature, pH, composition of the test medium, hydrodynamic conditions (stirring, etc.). This sensitivity is significantly larger than with dissolved substances. Furthermore, there is still no consensus on which is the most suitable combination of solid-liquid separation step (ultrafiltration, ultracentrifugation, etc.) and elemental analysis technique (atomic spectrometry, voltammetry, etc.), nor which dissolved fraction (free ions, low MW dissolved complexes, metal bound to macromolecules, etc.) is the most relevant for toxicology purposes. Therefore, NM dissolution rate in physiologically relevant media seems to still be an ill-defined endpoint from a regulatory point of view. Further development and standardization of test methods for dissolution rate is therefore highly necessary (ISO/TR 19057)[38].

The dissolution rate of a given NM in humans varies with type of body fluid e.g. through differences in pH of these fluids. It is therefore relevant to determine the dissolution rate in a representative set of media, which mimic the relevant body fluids. Relevant body fluids are not only saliva, lung mucus, gastric juice, intestinal fluid and plasma, but also lysosomal fluid, as NMs are known to end up in lysosomes of macrophages[37]. As an example, NiO nanowire-like particles were 100 % dissolved within 24 h when mixed with artificial lysosomal fluid, while they dissolved only minimally (3,5 % to 6,5 %) in water, saline and artificial interstitial lung fluid (Gamble's solution) at 216 h. Spherical NiO NPs were only 12 % and 35 % dissolved after 216 h when mixed with artificial lysosomal fluid, and the largest, irregular-shaped NiO NPs hardly dissolved in any solution indicating an effect of shape[39]. In this case, the nanowire like particles are eliminated within 24 h. Both in the case of the nanowirelike particles and the nanospheres, NPs and ions can be present during the first 24 h, but at a different ratio (that is changing in time), impacting the risk assessment.

6.2 Physical chemical properties determinant for toxicokinetic behavior

Several distinct factors influence the kinetics of ENM (apart from those that also influence the kinetics of molecular substances)[13][40][41][42][43]:

— the size (primary particle and agglomeration/aggregation) of NM;

— the surface charge of NM;

— the morphology/shape (e.g. the aspect ratio in case of fibres);

— protein binding to NM;

— surface chemistry (e.g. coatings, hydrophobicity).

Both the size and surface charge have shown to affect the composition and density of proteins attached to NPs[44].

As for the dissolution rate, these physical-chemical properties might change in different environments, e.g. as pristine material, in dosing medium, body fluids, and in tissues. Therefore, physical-chemical characterization may need to be determined at various stages of the toxicokinetic testing.

It is still difficult to analyse the relationship between phys-chem properties of NMs and their toxicokinetic behaviour, as there are few studies systematically studying these relationships by varying one property at a time. In addition, the quality of the studies is not always sufficient, especially those from the beginning of the research on NMs, when there was still too little knowledge to ensure certain quality.

For every study performed with NMs, including toxicokinetic studies, knowledge on the physicochemical parameters (e.g. size, agglomeration/aggregation, morphology, degradation/dissolution, surface charge, surface chemistry) of the NM dispersion or aerosol evaluated needs to be available. For information on

the determination of various physicochemical parameters a number of ISO documents are available (for overview see ISO TR 18196:2016).

Size

In many studies, it was observed that the smaller sized NM resulted in a more widespread biodistribution, i.e. to other tissues, compared with larger-sized NM. For example, when comparing the size of Au-NPs, it was reported that the smallest NPs (i.e. 10 nm) showed the most widespread organ distribution after intravenous administration[12]. Poly(amidoamine) PAMAM dendrimers of 5 nm sized particles showed a more favourable distribution to tumors in mice compared to the 11 nm and 22 nm particles, which was suggested to be due to less immune recognition and less organ-specific binding[45]. Conflicting results have been obtained on the effect of size on the pattern of distribution: For Ag-NPs, the 20 nm particles distributed mainly to liver after IV injection, followed by kidneys and spleen, whereas the larger particles (80 nm and 110 nm) distributed mainly to spleen followed by liver and lung. In the other organs evaluated, no major differences between the sizes were observed[29]. In another study, however, also for Ag-NPs after intravenous administration, all particle sizes investigated (10 nm, 40 nm, and 100 nm), regardless of their coating, showed the highest silver concentrations in the spleen and liver, followed by lung, kidney, and brain[46].

There are also reports that smaller NM lead to higher organ concentrations, indicating higher tissue specific absorption. For example, silver concentrations were significantly higher in the spleen, lung, kidney, brain, and blood of mice treated with 10 nm Ag NPs than those treated with larger particles. This finding correlated with relevant adverse effects (midzonal hepatocellular necrosis, gall bladder haemorrhage) observed in the mice treated with 10 nm Ag NPs, while lesions observed in mice treated with 40 nm and 100 nm Ag NPs, lesions were milder or negligible, respectively[46]. Following inhalation exposure, there was no size dependent retention observed in the lung. All sizes of NPs investigated (10 nm, 15 nm, 35 nm and 75 nm of iridium-192 NPs) showed similar retention times[47]. There was a slow long-term clearance of iridium from the rat lung (i.e. retention half-times of several hundred days). There was a low translocation in the body (maximum 0,4 % of the lung burden). However, organ distribution to the liver and spleen did show a marked difference between the 10 nm and 15 nm size on the one hand and 35 nm and 75 nm on the other hand with high levels for the smaller NPs. Kreyling et al[48] also demonstrated that smaller iridium and carbon NPs showed a higher translocation into the body following inhalation[48].

A complicating factor in systematic evaluation of the influence of the NP size on toxicokinetics, is that the other properties (e.g. shape, surface composition etc.) need to be kept similar among the tested particles.

There seem to be optimal size ranges for tissue uptake, which differ with the organ and the cell type *in vitro*, respectively. For agglomerated NPs (>0,5 µm), uptake by macrophages is expected to be the major internalization pathway while smaller particles are primarily processed by endocytotic pathways[13]. For gold NPs, it has been found that the optimum size for uptake in human breast cancer SK-BR-3 cells is 25 nm to 50 nm[49].

Another aspect of size is the fact that NPs can form superstructures based on the primary particles as potential building blocks in the formation of agglomerates (i.e. structures that are formed by weak Van Der Waals forces) and aggregates (i.e. structures formed by strong molecular binding forces). For Au NPs with a primary particle size of 5 nm to 8 nm, these structures can have sizes ranging from 40 nm up to 2 000 nm[50]. A confounding effect was noted with the intravenous dosing of the Au nanostructures with a considerable amount of aggregates remaining in the administration syringes. Although such losses to labware have been reported more often and might not be specific for aggregates, it is possible that aggregate losses occur more readily due to their faster sedimentation. Aggregation can change the potential to accumulate, leading to size-dependent differences in distribution. The difference in distribution is seen most dramatically in the lung with the aggregates showing significantly higher accumulation compared with the primary particles. Aggregates also accumulated significantly more in the heart[50].

Surface properties, including charge

The surface and especially the coating of the NPs can also have an effect on NP distribution, partly through the modification of the surface charge. As different cells can have a different membrane charge, which is dependent on their redox state, NPs with a certain surface charge will not be taken up by all cells with equal efficiency[51]. For dendrimer-coated NPs, in addition to the effect of their size as described above, the organ distribution pattern was also affected by the surface charge, as positively charged dendrimers showed higher distribution to the kidney, whereas neutral non-charged and negatively charged 5 nm dendrimers tended to be preferentially distributed to liver and spleen[45]. Similar observations were made for gold/dendrimer composite nanodevices[52]. For quantum dots (QDs) biodistribution studies demonstrated that negative and neutral CdSe/ZnS QDs preferentially distributed in the liver and the spleen, whereas positive QDs mainly deposited in the kidney and in the brain[53]. From these studies it might be concluded that a positive surface charge would favour migration to the kidneys.

It is also well known that coating NPs or nanorods with polyethyleneglycol (PEG) affects the half-life in the blood[31][54][55]. This is probably more a steric effect than a charge effect. This has been demonstrated for example for the PEGylation of Au nanorods, Au NPs and organic nanotubes[55][56][57][58]. Higher PEG grafting levels were advantageous for MPS avoidance, for enhancing permeability and retention (EPR) in tumor sites, and for suppression of aggregation of the gold nanorods in the circulation[56]. For the PEG-Au-NPs, intravenous injection of 4 nm and 13 nm NPs showed a prolonged circulation time up to 7 d[57]. These kinetic trends were well correlated with tissue distribution patterns, particularly in liver, spleen, and mesenteric lymph node. PEGylated particles bind very few proteins, avoid uptake by the MPS, and therefore circulate longer in the blood[59][60]. Also for citrate-ligand-capped 10 nm Au NPs and PEGylated 10 nm Au NPs a difference was observed for distribution to breast milk after intravenous administration to lactating mice with the PEGylated Au NPs showing a higher and more prolonged presence in breast milk[61].

For three different Ag NP sizes (10 nm, 40 nm, 100 nm) there was no difference in the distribution between Ag NPs coated with citrate-or polyvinylpyrrolidone[46].

Several ISO documents are available describing methods for the surface characterization of NMs and NPs, including ISO/TR 13014:2012, ISO/TS 14101:2012, ISO/TR 14187:2011, and ISO 20579-4:2018. For consistent surface chemistry also issues regarding reproducibility of the NPs themselves are important to consider due to their inherent variability. An appropriate set of characterization methods consistently applied at critical times can be useful tools to assess and identify reproducibility and sources of variability[62].

Protein corona

After contact with any biological environment the NPs will immediately be coated with biomolecules as present in the surrounding biological matrix[59]. This layer of mostly proteins is generally considered as the “protein corona”. With as many as 3 700 proteins expected in the complete plasma proteome, and even other proteins present in other fluids, it is clear that the composition of the protein corona around a NP can vary enormously and does affect toxicokinetics of NP and the recognition by cells[59][63][64][65]. Even serum might not be the perfect model for the *in vivo* blood environment, because it misses the blood coagulation factors[44].

An important identified effect of the protein corona, which depends on its composition is, for example, the stabilization of a NP. Albumin in water or Dulbecco’s Modified Eagle Medium (DMEM) has shown to stabilize many NMs, protecting against agglomeration. Bronchoalveolar lavage fluid induces the opposite effect, increasing agglomeration.

A second important effect of the protein corona is that some proteins (the opsonins) lead to recognition by macrophages. Walkey et al.[44] found that the most active promotors of cell association (which can probably be regarded as opsonins) were proteins known to be involved in binding to hyaluronan, a major component of cell surfaces. Opsonisation therefore often causes fast uptake by macrophages, leading to a quick decrease of NPs in the circulation[59][66], fast transfer to and concentration in organs of the MPS [previously called the reticuloendothelial system (RES)], such as the liver and spleen. In contrast, dysopsonins (albumin, apolipoprotein A-I, A-IV, C-III, and H) reduce the affinity of NMs to the MPS. As opposed to the action of albumin, its fetal analog fetuin led to uptake of 50 nm polystyrene nanospheres by liver macrophages (Kupffer cells)[13].

Walkey et al.[44] found that the proportions of the serum proteins in the corona does not reflect the relative abundance of the proteins in serum itself; the properties of the NMs thus influence the corona composition. It might be clear that the surface characteristics of NPs which favor or disfavor opsonization profoundly influence their toxicokinetics. A hydrophilic and neutral surface of NMs disfavors binding of opsonins. Walkley et al.[44] found that the cationic gold NPs gave a higher cell association than the anionic and neutral gold NPs of the same size (all including their protein coronas). In contrast, there were few differences in the corona between several particles incubated with BALF (bronchoalveolar lavage fluid), although possibly also some cellular and serum proteins were detected due to agitation of the lung tissue due to BALF harvesting[67]. The BALF used was obtained from patients with pulmonary alveolar proteinosis which is rich in lipids and surfactant associated proteins.

It has also been found that protein coronas are dynamic, changing in composition with time. At first contact, the proteins present most abundantly in the particle environment bind to the particle, which are replaced in time by proteins for which the bond is more favourable energetically[68][69]. The most strongly bound proteins form the so called "hard" corona, while the proteins that have a dynamic exchange with their environment are designated the "soft" corona[68]. The effect of the protein corona on biological processes is yet unknown. However, the protein corona itself is composed of endogenous biomolecules that could contain binding motifs which can be recognised by cells[65].

The relative importance of the protein corona composition was shown by modelling work of Walkley et al.[44] The protein corona fingerprint of a library of 105 surface modified gold NPs was more predictive of (alveolar) A549 cell association than a model combining the information on core size (measured by TEM), dielectric environment (measured by AS), hydrodynamic diameter (measured by DLS) and the zeta potential. Also, the fingerprint was more predictive than the total amount of protein absorbed, indicating the importance of the protein corona composition. Interestingly, the model relating the protein corona fingerprint of gold NPs to their cell association could not predict the cell association from the protein corona fingerprint of silver NPs with some of the same surface ligands as used for the gold NPs. Clearly, the NP core itself has some unknown effect on the cell association. It was found that the core material (silver or gold) had a larger effect on the protein corona composition (larger difference in composition) than the ligands attached to the NP surface did. Probably, the properties of the core NP determine the density, arrangement, and orientation of the ligands, influencing the attachment of proteins[44].

Aggarwal et al.[59] provide a good overview on the various aspects of the protein corona and coating of NPs including the effects of the protein corona on biodistribution of the NPs.

This effect of protein corona composition raises the question whether this composition is different in the typical species used in animal tests, as for example rats are known to have higher levels of alpha-fetoprotein in serum protein than humans[70]. Possibly, this could lead to interspecies differences in toxicokinetics of NPs. When performing *in vitro* tests, the protein corona composition might be even further distant from the composition in humans, as culture medium has a different composition than the different body fluids. Some reassurance that the composition of the environment does not matter too much is obtained from the finding of Walkley et al.[44] that the corona composition does not reflect the proportions in the serum to which the NPs were exposed. Nevertheless, until certainty is provided that the protein composition of the surroundings does not influence the composition of the corona on the NP, the protein corona needs to be considered when performing *in vitro* studies and for interspecies extrapolation. Alternatively, NPs could first be exposed to a physiologically-mimicking fluid before being added to the *in vitro* test.

Shape

For gold NPs, it has been found that spherical NPs were taken up more readily by MCF-7cells than rod-shaped particles[51]. In addition, a smaller amount of longer rod-shaped gold NPs were taken up than shorter gold NPs with similar surface charges. The lower uptake of (longer) rod-shaped particles seems to lead to a lower elimination from cells, as well, as a higher accumulation has been reported. When using antibody targeted to adhesion molecules and neovascular expression markers on endothelial cells, Au coated nanorods showed a higher specific and lower non-specific accumulation under microfluidic flow conditions that mimic the vasculature, when compared to their spherical counterparts[71]. Similar

observations were made in both an *in vitro* flow system of a synthetic microvascular network and *in vivo* in mice[71].

Through the resulting curvature of spherical particle, size has an effect on shape, which was observed to affect the binding of biomolecules to the surface of Au NPs[69].

As indicated in 6.1, shape also influences dissolution rates which can affect uptake as the toxicokinetics are then partly governed by the dissolved substances.

7 Analytical challenges

7.1 General

In contrast to hazard studies, toxicokinetic studies need quantitative chemical analysis of the test substance in various complex matrices including tissues. In order to confirm the accuracy of the measured values, a mass balance needs to be performed in which the levels of test substance in all matrices are totalled; if the total amount measured reaches ≥90 % of the total amount administered, the distribution in all matrices are considered to be accurate. In the case of significant losses compared to the external dose, the determined absorbed dose is less certain. Toxicokinetic studies can thus lead to analytical challenges regarding detection limits and complex biological samples, with specific additional challenges for the quantification of NMs. Furthermore, any analytical method needs to be specific, sensitive, and reproducible and produce data that show linearity over the range of expected NP or elemental concentrations. ISO TR 18196:2016 catalogues techniques for the characterization and quantitation of NMs. Table B.1 in Annex B provides information on quantitation methods including their advantages and limitations..

Samples of matrices may need to be prepared for analysis to remove interfering materials or to improve quantitation. For example, residual blood in samples of organs for which a low distribution might be suspected, the organs may need to be perfused to remove the blood from the vascular system before these organ samples are analyzed. Alternatively, the data might be corrected for the presence of residual blood[28][72][73]. However, according to the current knowledge on toxicokinetics of NMs, they are very quickly removed from the blood, thus any contamination via residual blood is probably very low.

7.2 Analysis of element

There are few quantitative analytical methods to determine NM concentration from tissues. Metal NPs (silver, gold, TiO_2, iron, and Cu, etc.) are easier to analyse than other NMs because combustion of the tissue, one of the forms of sample preparation, does not destroy the particle. For metal and metal oxides, such as Au, TiO_2 and SiO_2 NPs, the elemental content of Au or Ti can be determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS)[74]. However, after the complete dissolution of the test sample in an acid environment elemental analysis by ICP-MS does not demonstrate the presence of the (nano)particles themselves. But especially for NPs that do not show degradation and/or dissolution, the presence of the elemental metal indicates the presence of the NPs. For NPs that dissolve in an aqueous environment such as silver, measurement of the element might overestimate the concentration of silver NPs, as the Ag ions are detected as well. So, this needs to be considered during the analysis.

One of the challenges with ICP-MS is overcoming spectral interferences from components in the matrix such as organic components, minerals, and digestion acids which can compromise the results[75]. Laser desorption/ionization mass spectrometry (LDI-MS) in an imaging format was developed to investigate surface chemistry dictated intra-organ distribution of NPs with a capability to detect and image at to mole levels of NPs with almost no interferences from biomolecules[76].

For carbon based NPs, the chemical analyses are much more challenging because sample preparation can involve combustion of the sample, and detecting carbon-based NPs in a carbon environment will be difficult. Few studies have directly investigated the clearance and kinetics of pristine MWCNTs because these analyses are problematic. To this end, catalytic cobalt analysis was performed by Pauluhn[77]. However, catalytic metal ions could separate from the CNTs and independently translocate to other organs within the body[78]. ^{14}C analysis of ^{14}C-taurine-MWCNTs was performed by Deng et

al.[79] and Czarny et al.[80] Czarny et al.[80] demonstrated that the observed ^{14}C in the organs was indeed accompanied by the presence of MWCNT in several organs. As molecular tagging can alter physicochemical and biological properties[81][82][83], the kinetics and toxicity of tagged CNTs can differ from those of pristine CNTs.[84] In addition, the clearance could be accelerated or delayed for well-dispersed particles, as the degree of agglomeration affected the cellular sensitivity (i.e. cytotoxicity) of the carbon nanotube[40]. Shinohara[85] therefore applied a method developed by Tamura et al.[83] involving non-dispersive infrared (NDIR) analysis of the CO_2 generated from the decomposition of pristine MWCNTs that was separated from tissues by acid digestion and heat pretreatment. This was combined with optical microscopy and transmission electron microscopy (TEM) to observe the MWCNTs particles in the tissue.

Atomic Absorption Spectrometry (AAS) can also be used for the detection of elements. For example Ag mass distribution was verified by determination of the Ag concentration with the graphite furnace atomic absorption spectrometry[86] and AAS was used for determination of ZnO:EU (europium-doped-zinc oxide) NPs in tissues after oral administration[87]. Electrothermal atomic absorption spectrometry is applied for the identification of environmental pollutants such as various heavy metals for which MWCNT were used as absorbant[88] and lead (Pb) contamination in food products could be extracted with nanoTiO_2[89]. AAS was also used for determination of the platinum content of a nanomedicine drug delivery system for diamine(dichloro)platinum[90]. The use of AAS for biodistribution studies is limited, as for metal and metal oxides mainly ICP-MS or variants thereof (e.g. ICP-OES, inductively coupled plasma optical emission spectrometry) are used for elemental analysis.

7.3 Analysis of element radiolabel or fluorescence label

Due to the property of NPs to translocate whatever the exposure conditions, studies tracking the distribution of NMs *in vivo* at realistic exposure scenarios will be necessary, but will also be technically challenging. The labelling of NMs offers good possibilities for such studies but can be associated with certain experimental pitfalls.

A specific labelling of NMs to follow their fate *in vivo* can be done by using radioactive isotopes as radiolabels or fluorescent dyes. This can be done by adding a label to the NM by specific binding, or by using a radio-isotope of the NM itself. A disadvantage of the specific labelling is that the label can detach from the NM[91][92]. In that case the label is measured while it is detached from the NMs so incorrect results are obtained on the distribution of the NPs. So, when using any specific labelling technique a careful evaluation of the integrity of the label-nanoparticle combination is necessary. Use of radiolabeled NPs allow easy and fast determination of total amounts of parent substance and metabolites in a tissue at the time of sampling[93].

Alternatively radioactive isotopes can be used that are isotopes of a metal being part of the NM (e.g. gold or silver). For example, for nanogold specifically radiolabeled Au NPs were obtained by radio-activation (i.e. by neutron beam activation in a research nuclear reactor) of the Au metal component, and the radioactive decline of the activated (^{197}Au (n, γ) ^{198}Au) Au atoms was determined[94]. With this approach, there is some certainty that the NPs themselves are detected, although for silver NPs there is still uncertainty regarding the release of silver ions. In addition, natural stable isotopes can be used to demonstrate uptake from the application site[95]. By using ZnO NPs enriched for the natural isotope ^{68}Zn it was demonstrated that Zn present as ZnO NPs in sunscreen formulations was taken up from the skin application site[95]. The ^{68}Zn could be detected in blood and urine of the human volunteers. As the ^{68}Zn was evaluated using ICP-MS measurements it is not known whether ^{68}Zn has been absorbed as ZnO particles or soluble Zn or both. Also in internal organs of mice including the liver, the presence of ^{68}Zn was observed but here also penetration of the ZnO NPs themselves could not be established[96]. The Zn homeostasis was largely maintained and the presence of ZnO particles in the sunscreen did not elicit and adverse biological effect[96].

As mentioned above, one main issue associated with ADME studies with labelled NMs is ensuring that the label remains with the NPs following administration and entry into the body. Another major issue is ensuring that the label does not alter the behaviour of the NPs[81][82][83], particularly since the changes in surface chemistry of the NP can significantly influence the physicochemical properties of the NPs and, consequently, the toxicokinetic profile and toxicity of the NPs[4]. Furthermore, labelling of NMs is limited due to availability and the safety issues of radiolabelled NMs. Especially for inhalation toxicity

tests, the use of radiolabelled NMs poses problems, due to the resulting contamination of expensive equipment.

7.4 Determination of particles

NPs can be composed of a varying number of molecules depending on the actual size of an NP. However, via the protein corona or coating on a NP it is the whole particle that interacts with a receptor on a cell or organ and not the individual molecules composing the NP. So, depending on actual size (i.e the number of molecules in a NP) a cell is exposed to a relative low or high molecular dose. These molecules do contribute to the total dose administered especially if that dose is expressed in mass units. However, information on the dose in only mass units is not sufficient. Also information on the dose expressed as number of particles and/or surface area needs to be considered.

A wide range of techniques have been used for visualising NPs in tissues. Electron microscopy, in particular, is widely used for this purpose, although other microscopy techniques including spectral imaging and raman microspectroscopy are also being developed (e.g. see review of Ostrowski et al.[97] It is possible to use these techniques to estimate 'particle number doses' in cells (e.g. Reference [98]) and tissues (e.g. Mercer et al.[99] extrapolated the amount of CNT detected in five scored slides to whole organ), however, they are currently labour intensive, especially at low doses, and the accuracy of the results vary dependent on details of the technique and scoring approach. It is therefore unlikely that they could be routinely adopted for determining particle number doses for toxicokinetic studies without significant development of automated operations. However, approaches combining mass measurements (e.g. ICP-MS) with limited targeted visualisation techniques can be of use in this area, especially if combined with appropriate particle analysis techniques to confirm the elemental characteristics of the particles identified (e.g. energy-dispersive X-ray spectroscopy). Advanced techniques such as micro-computed X-ray tomography[100] can enable the derivation of total particle doses in tissues but require specialist equipment and are thus unlikely to be widely used for toxicokinetic studies. Currently the most promising approach for the direct quantification of tissue particle doses seems to be single particle ICP-MS (sp-ICP-MS).

Single particles of metals and metal oxides can be measured using sp-ICP-MS, even in matrices such as food and human tissues (e.g. References [101] and [102]). Sp-ICP-MS is considered a promising analytical approach for the detection and characterization of NPs at low concentrations, as it allows simultaneous determination of particle size distribution and number while simultaneously distinguishing between dissolved and particulate analytes[101][103][104][105]. However, it might not be used for imaging of NP size distribution in tissues since its mode of sample introduction restricts the imaging analysis of solid samples. It might also not be used for quantitative imaging for intracellular and tissue NPs. Laser ablation (LA) coupled to sp-ICP-MS was therefore developed to provide quantitative imaging of intracellular NPs[106]. Use of the sp-ICP-MS in epidemiological studies might also provide insight in possible long term disease effects due to accumulation of NMs in organs. Presence of various particles was reported in various organs including lung, blood stream, liver and spleen[102][107][108].

An international interlaboratory study has been performed to determine repeatability and reproducibility of the determination of the median particle size and particle number concentration of Ag NPs in chicken meat[109]. The particle size determination showed standard deviations of 2 % and 5 % for repeatability, and reproducibility standard deviations were 15 % and 25 %, respectively, for two meat homogenates spiked with polyvinylpyrrolidone (PVP)-stabilized Ag NPs. However, determination of particle number in the test samples showed more variation with repeatability standard deviations of 7 % and 18 % and reproducibility standard deviations of 70 % and 90 %[109]. It was concluded that the sp-ICP-MS method showed promise for the determination of the mean particle diameter with acceptable standards deviation up to 25 % which is not uncommon for such measurements. The determination of mass fraction and particle number concentrations was concluded currently not to be reproducible nor true enough for application in complex real world samples[109]. This might be NM dependent as for TiO_2 NPs reproducible results were obtained for both the TiO_2 NP number and Ti mass content[102].

Techniques for measuring carbon nanotubes and other carbon based NMs in tissues are also being developed but the sensitivity of many techniques, including Raman spectroscopy and near-infrared fluorescence, are currently low[110].

7.5 Limit of detection

The limit of detection (LOD) and limit of quantification (LOQ) are important determinants in the toxicokinetic studies. They determine the lowest amount of NM that can be reliably detected for NM or NM components (e.g. elemental metal) in tissues and/or blood. Generally the LOD is the mean plus 3 to 5 times the standard deviation of the blank control samples and the LOQ the mean plus 10 times the standard deviation. The LOD/LOQ is not the same for all test samples as different organs may have a different LOD and LOQ. Ideally, the LOD/LOQ can be determined for each organ independently by using blank organ samples[111][112]. A sensitive detection method with a low LOD is especially important for studying uptake of NM over biological barriers, as the usually low absorption of NMs can be missed in case of relatively high LODs.

Inductively coupled plasma-optical emission spectroscopy (ICP-OES) has been employed with limits of detection reported in the µg/L range, orders of magnitude higher than those possible with ICP-MS analysis[113][114]. In contrast, sp-ICP-MS is able to directly quantify particle size, mass and number distributions at ng/L concentrations using chemical digestion with strong base tetramethylammonium hydroxide (TMAH)[115].

8 Issues relevant for dosing conditions

8.1 General

As known for dissolved substances, the dosing conditions have a high impact on the ultimate outcome of toxicokinetic studies with NMs. Firstly, the dispersion state of the test material in the dosing matrix affects the extent of aggregation and agglomeration of the NPs. This size will impact the uptake of the material by cells and any paracellular transport (i.e. in-between cells) as already discussed in 6.2. For inhalation studies, the aerosol dispersion and aggregate/agglomerate particle sizes also determine whether the alveolar regions can be reached in the lung, impacting the possibility of systemic absorption. Strict dispersion protocols including proper characterization thus need to be included in any guideline for toxicokinetic studies with NMs. In addition, part of an applied dose can be lost due to adsorption to the used labware[72]. So, a control on the actual administered dose needs to be included in toxicokinetic studies.

Secondly, the chosen doses or test concentrations can have an effect on the final outcome of a toxicokinetic study. For dissolved substances, enzyme or transporter saturation can lead to different uptake, excretion or metabolite formation at higher doses than at lower doses. In the case of NMs, high doses or concentrations can lead to overloading of macrophages (comparable to enzyme saturation), gelation in the intestines (probably related to agglomeration of the particles) which decreases the availability for intestinal absorption[116], and formation of nanoparticle layers on epithelial barriers, blocking all transport of any substance. Koeneman et al.[117] found that the percentage of TiO_2 NPs (<40 nm) that translocated over a Caco-2 monolayer (with M-cells or with goblet cells) was dependent on the dose; higher doses gave a lower percentage of translocation. This could have been due to gelation/aggregation/agglomeration, but can also have been caused by saturation of the pathway leading to translocation, which is most likely transcytosis.

The overloading of macrophages in lungs (commonly termed “lung overload”) has led to the proposal by industry that doses leading to such conditions can not be tested in hazard studies as they would not be representative for real life exposures[26][118][119]. Similarly, for dissolved substances, for reasons of animal welfare, it has been proposed to maximize the administered dose at the inflection point of dose-AUC relationships, as this can be regarded as the kinetically derived maximum dose (e.g. Reference [18]). However, the doses in hazard tests needs to cover both realistic as well as high doses to be able to detect any potential hazard. Testing according to the benchmark approach with more doses with fewer animals can be an option[120]. To be able to interpret these results well, the doses in kinetic studies preferably also cover this range of realistic and high doses, where any changes in kinetics at higher doses can then be uncovered. As not all effects of dose level on kinetics of a new NP will be known initially, and these effects can both lead to under- and overestimation of the hazard at lower doses, it is not possible to select a worst case dose as a means to enable conservative risk assessment with minimal testing.

A third dosing issue that affects toxicokinetic results is the choice for applying a single dose or repeated dosing. For NMs that are not cleared from the lung within 24 h or that do not fully dissolve within 24 h when within the internal tissues, daily repeated dosing will lead to accumulation of the material in the lung and/or internal tissues, respectively. This would result in higher tissue levels found in repeated dose studies than in single dose studies at the same dose level. The time required to come to steady state tissue concentrations can be particularly long for low solubility NMs. Redistribution of NPs from liver to spleen has been found to occur after intravenous administration (e.g. Reference [17]), but as this can occur during a recovery time after a single dose, too, repeated dose testing is not necessary to be able to include any redistribution. More important in this respect is the observation time after the NP administration.

Exposure to NPs has been reported to affect the tight junctions of gut epithelial cells[121], potentially enhancing paracellular translocation and thus total systemic absorption of NPs at later exposures. Koenemann et al.[117] also found that repeated exposures (10 d) caused a decrease in the Caco-2 cell layer integrity (measured by the TEER value), while a single exposure did not. In such cases, repeated dose studies might lead to higher absorption values than found in single dose studies. Thus, when the goal of the toxicokinetic information derived from a study is to aid the risk assessment of chronic exposures, repeated dosing kinetic studies provide better information.

In animal studies, for practical reasons, the substance dose is generally administered as a bolus, i.e. the whole daily dose in one single, small volume of liquid (usually) at one time point in the day. This is not reflective of human exposures through e.g. the diet, inhaled air at the workplace or on the skin through applied cosmetics, where the exposure is spread over the day. At the same total daily dose, a bolus dose will thus cause a higher peak exposure (though during a short time) instead of a continuous dose, which can potentially lead to the effects described above for higher doses. Continuous dosing can be seen as repeated dosing over the day, leading to inclusion of the effects described above for repeated dosing.

In the case of lung exposures, bolus doses, applied as intratracheal instillations, have additional disadvantages. Firstly, by placing a small volume of liquid with the NPs in the trachea does not necessarily lead to the exposure of the deeper, alveolar parts of the lung. Secondly, intratracheal dosage of NPs has shown to lead to an uneven distribution of NPs over the two lung lobes[85][122]. Taking one lung lobe only for e.g. histopathology and the other for determining the lung load, can then lead to errors in the results. Multiple-dose administrations do not remarkably change the uneven pulmonary microdistribution of intratracheally instilled NPs, but multiple-dose administrations did reduce variations in the TiO_2 content for each lung lobe[123]. The choice of intratracheal dosing device (gavage needle or microsprayer) does not appear to affect the distribution between the lung lobes: the right lobe contains more than the left for both devices[124]. There was also no difference between these devices in the NP content reaching the lung lobes, while a smaller variation was achieved when using a microsprayer[124].

In the case of oral exposures, inclusion of the NPs in the diet can affect the dispersion of and protein corona on the NPs, through the diet components. As described above the dispersion and protein corona can affect the resulting toxicokinetics. As it can be difficult to analyse the properties of the NM, including protein corona, in the diet, this constrains the information on the dose.

In short, when the goal of the toxicokinetic information derived from a study is to aid the risk assessment of continuous exposures, preferably continuous exposures through diet, inhaled air or skin applications are applied in the study. However, the advantage of single parenteral dosing (by gavage, instillation, or intravenously) is that the dose applied is well known.

8.2 Dose metrics

The common dose metric in toxicology is mass substance per mass or volume of exposure medium, tissue or body weight (e.g. mg/kg diet, ng/m^3 air or mg/kg bodyweight per day). For dissolved substances, this dose metric is also used for toxicokinetics (e.g. mg/g liver, ng/mL blood) or sometimes the number of moles (e.g. mmol/g enzyme, nmol/mL blood), especially in case of enzymatic conversions. As the different properties of NMs in comparison to larger particles can arise from their higher surface area, the surface area can also be the most relevant metric for some of the toxicokinetic behaviour and toxicity. In these cases, the total administered surface area would theoretically be a better dose metric

than mass of the NM. Indeed, Braakhuis et al.[125] found that the surface area was a better dose metric than mass for describing the dose-response relationship for pulmonary toxicity of silver NPs. When using surface area, the effect of the size of the different NPs tested on the dose-response curve nearly completely disappeared.

As the interaction with tissues and/or cells is occurring with particles themselves, irrespective of their size or number of molecules present in a particle, the number of particles can also be considered as dose metric. However, with the discussion on the most suitable dose metrics still ongoing, it is generally recommended to gather enough information on the NM formulation to enable expressing the dose in mass, surface area and number of particles.

9 Absorption of nanomaterials

9.1 General

There are three major routes of exposure to NMs: via the skin (dermal exposure), via the gastro-intestinal tract (oral exposure), and via the respiratory tract (inhalation exposure). For nanomedicines also the parenteral route of administration (e.g. intravenous, intramuscular and others) needs to be considered, which bypass the crossing of the natural barriers for foreign invaders of the skin, respiratory and gastro-intestinal tract. Absorption of chemicals and drugs used in the conventional toxicokinetic studies are concentration gradient dependent, while the absorption of NMs cannot be gradient dependent but barrier dependent. In many cases, aggregation and agglomeration of NMs can hinder passage across biological barriers.

In *in vitro* models of the skin, lung, and intestine, the absorption can be studied more mechanistically than in *in vivo* tests. Although an individual *in vitro* test can have less resemblance to the complex *in vivo* situation, a combination of non-animal studies can be used to find the relevant information. Furthermore, regulations in many jurisdictions require the use of animals only as a last resort. Gordon et al.[126] provides an overview of non-animal models of various epithelial barriers, including skin, intestine and lung, used currently. Yet, the challenges for *in vitro* testing with NMs needs to be taken into account.

In animal studies, absorption can be determined by analysing all tissues for the level of the studied NM and comparing the total amount present in the organs to the external (applied) dose through a mass balance. Alternatively, the absorption can be determined by comparing organ levels obtained after intravenous dosing to those organ levels obtained after dosing through the exposure route of interest. It needs then to be considered that, depending on the route of administration, differences in the obtained protein corona can occur which could have an effect on the relative organ levels.

In the following paragraphs the current knowledge on the absorption of NPs is summarized for each exposure route, as well as the known advantages and disadvantages of test systems to determine the absorption.

9.2 Skin

Test models

Dermal penetration can be assessed by evaluating skin penetration *in vitro* for which the skin of many mammalian species, including humans, can be used. *In vitro* models for skin absorption are described in TG OECD 428, whereas *in vivo* skin absorption studies are described in TG OECD 427. However, these test guidelines were developed for chemicals and were not designed for NPs. Non-animal models comprise excised skin from human, rat and/or pig, reconstructed human skin models, artificial skin surrogates, and *in silico* models[126]. NPs quantitation remains a problem in these studies especially in view of the fact that dermal penetration of NPs is generally considered to be low or absent[127][128][129][130].

Current knowledge on absorption of NPs through the skin

In general, NP penetration of the skin is limited to the first cell layers of the stratum corneum[127]. TiO_2 NPs were concluded not to be absorbed dermally at all by the European Scientific Committee

for Consumer Safety (SCCS) after evaluation of a range of studies but presence in hair follicles might occur[131]. However, for some NMs, there seems to be limited systemic uptake via the dermal route. For example, when ZnO NMs (20 nm) was applied on the skin in a sunscreen formulation, the Zn was observed in the blood, originating from the ZnO in the sunscreen[95]. Although it was not demonstrated that ZnO particle absorption occurred, it was clearly demonstrated by using the natural ^{68}Zn isotope that the detected Zn was originating from the ZnO NPs. In addition, Nabeshi et al.[132] reported dermal translocation of 70 nm silica NPs and quantum dots in mice after 28 d of repeated application, with detection of these particles in the skin at the application site (ear), in regional lymph nodes, in the liver and in the brain. It was not reported, however, whether the mice were able to lick the site of application and thus enable oral exposure to the particles which might lead to liver exposure more easily.

For skin penetration and absorption, the quality of the skin in terms of skin damage, like abrasions and UVB damage (sunburns), mechanical stressors (skin flexing), and the effects of solvents and vehicles used can affect the skin penetration[128][133]. For example, in an *in vitro* system using human skin exposed to Ag NPs, a low translocation into the receptor fluid was found, which was increased fivefold in damaged skin[134]. However, the method of detection used (electrothermal atomic absorption spectroscopy) cannot discriminate between silver ions and silver particles, so translocation of the NPs themselves was not demonstrated. In burn patients treated with nanocrystalline silver-containing wound dressings, increased blood silver serum levels were observed, indicating Ag uptake[135]. The silver levels in blood were considered to be non-toxic to the patients[135]. Similar observations were made for human skin in which no penetration into the viable epidermis of Ag NPs was observed after application to intact skin, while Ag NPs were reaching the viable epidermis in human skin with a partial skin burn[133].

On the other side, the physical-chemical properties of the NM can influence the absorption. In a recent review it was shown that for certain (rather) small NPs, skin penetration might be possible especially for metal and metal oxide NPs[136]. A size dependent skin penetration can be distinguished: NPs ≤4 nm can penetrate and permeate intact skin, NPs size between 4 nm and 20 nm can potentially permeate intact and damaged skin, NPs size between 21 nm and 45 nm can penetrate and permeate only damaged skin, NPs size >45 nm cannot penetrate nor permeate the skin. This contrasts with the results of Nabeshi et al.[132], described above, for 70 nm silica NPs.

In addition, dissolution also needs to be considered as dissolved ions can be taken up and cause local and systemic effects. On hairless mouse skin, the shape of the silver NPs was shown to determine the level of skin penetration[137]. Filon et al proposes for skin absorption a decision tree to evaluate the potential risk for consumers and workers exposed to NPs[136].

9.3 Gastrointestinal (GI) tract

Test models

Also for the GI tract, *in vitro* models are available to study migration over intestinal cells, such as the commonly used Transwell® cell culture systems using Caco-2 cells[36][138][139][140][141]. These *in vitro* cellular models can indicate low, medium or high translocation, although especially low translocation can be difficult to determine in view of limitations of the sensitivity of the measurement technique. Quantitative absorption determination, such as the translocation percentages reported to vary between 1,6 % and 12,3 % of the added dose of polystyrene NPs[138][142], are not reliable from these simple gut models. Caco-2 cells are colon cells, which can be expected to have other functionalities than cells of the small intestine.

To define a proper intestinal *in vitro* model for NP absorption studies, the physiological architecture of the gut needs to be considered, as was well described by [121]: "Gut epithelium is composed of enterocytes, responsible for nutrient absorption, and up to 24 % mucus-secreting Goblet cells[143]. The mucus is cytoprotective and represents an efficient physical barrier against pathogens[144]. The most distal part of the ileum presents Peyer's patches, responsible for gut immunity. This part is also called follicle-associated epithelium (FAE); it is composed of enterocytes and microfold cells (M-cells). M-cells are specialized in the absorption and translocation of large molecules, bacteria and viruses from the intestinal lumen to immune cells. The uptake of mineral microparticles (i.e. >100 nm) in the gut mainly occurs through M-cells, while nanoparticles (i.e. <100 nm) are also taken up through

enterocytes and goblet cells[145][146][147][148][149][150]. Indeed the apical plasma membrane of mature enterocyte is essentially unable to undergo endocytosis; microvilli morphology per se sterically prevents the invagination of large endocytic vesicles. Consequently mature enterocytes are not able to accumulate and transfer micro- or macro-particles by transcytosis[151]. In rare cases of endocytic events in enterocyte layers, i.e. in the microcrypt areas between neighbouring microvilli, endosomes are usually retained in the apical cytoplasm, just beneath microvilli[151]. Goblet cells are still able to undergo endocytosis, and consequently would accumulate nano- and microparticles, but their ability to transfer microparticles by transcytosis has not been reported." The addition of M-cells to Caco-2 cultures is observed more and more[36][121][152] and their inclusion in an *in vitro* gut model can clearly be defined as essential for nanoparticle absorption studies[153].

Three-dimensional gut tissues have been introduced, produced for example by creating medium flow along Caco-2 cells, which then polarize and grow into a villi-like structure by themselves[154]. The added value of such cell cultures and *in vitro* setups has yet to be shown for the study of gut absorption of NPs.

Another important feature of intestinal models is the presence of tight junctions between the epithelial cells. These play a role in the barrier function of the monolayer and limit transfer of materials between the apical and basolateral sides via paracellular routes. Caco-2 monolayers also have adherens junctions, which consist of cell adhesion molecules such as cadherin[141]. Adherens junctions play an important role in maintaining tight junctions by regulating the expression of claudin[155]. In Caco-2 monolayers, tight junctions are located near the apical side of the intercellular spaces, and adherens junctions are located more basolaterally in the intercellular spaces[156][157]. Therefore, when the apical side of the Caco-2 monolayers is exposed, it would be difficult for the NMs to interact with cadherin, because tight junctions would limit the entry of NMs into the intercellular space. In contrast, when the basolateral side of the monolayers is exposed, it would be possible for NMs to interact with cadherin directly. However, it has been reported that exposure to NPs disrupts the tight junctions (e.g. References [117], [121], and [141]) so the NPs can gain access to the cadherin receptor on the basolateral side.

The properties of the membrane (e.g. material, pore size) can influence the NPs translocation rate across the polarized cells[158][159]. Membrane inserts with larger pores allow for increased translocation compared with smaller pore sizes. On the other hand, decreased translocation due to NPs adsorption on the membrane can also occur[140]. Therefore, for a reliable measurement of the transepithelial passage it is crucial to conduct cell-free experiments to assess whether the NPs are not withheld by the insert membrane itself.

Especially for the GI tract, the potential interaction that might occur with all components and gastrointestinal fluids could warrant a more dedicated evaluation of these interactions[36]. How NM behave in the various GI-tract fluids of mouth, stomach, small and large intestines can be investigated in a model. Various *in vitro* non cellular models are available[160][161][162][163][164]. Some of these models are static without passage but also dynamic models are available each with their own limitations[36].

Current knowledge on absorption of NPs in the GI tract

There is still a lack of knowledge to what extent single particles, small aggregates/agglomerates and larger aggregates/agglomerates can be translocated across the GI tract epithelium. Also the potential interaction of the NM with various food components and the processing of food is an additional confounding factor. Contact with the food components can result in changes of the protein corona of NMs[36]. Another issue can be the generation of various nanostructures. It also needs to be realised that food itself contains a multitude of nanostructures (e.g. micelles, fat droplets and others).

Uptake from the GI tract was demonstrated for several NMs (e.g.References [28], [145], [148], [165], [166], and [167]) but a lack of uptake of NPs was also observed (e.g. References [168], [169], and [170]). Closer study of these conflicting reports for TiO_2 NPs (Suppl. Inf. of [21]), concluded that the low absorption reported by some (<1 %) might not have been detectable for others due to higher backgrounds from the feed used[171] or cage enrichers[172], less sensitive analytical methods and/or the application of a single dose instead of repeated, accumulating doses[21]. Furthermore, the application of high doses might have decreased the absorption, due to gelation or other processes, as discussed in chapter 7. In addition, the dose suspensions might not all have been stable (leading to agglomeration and thus more difficult uptake) or part of the applied dose could have been lost due to adsorption to the

labware[72]). Overall, Heringa et al.[21] found the weight of evidence to point to absorption of TiO_2 by the gut, be it very low. This was confirmed by the findings with radiolabelled TiO_2 NPs[28].

In a human volunteer study nanosilver (size around 60 nm) was orally dosed up to 14 d[173]. Serum silver concentration was detected in 42 % of subjects receiving 100 µg/d and in 92 % of subjects receiving 480 µg/d, but was undetectable in the urine[173]. The silver concentrations were determined by ICP-MS and it was presumed that the majority of the silver was present as silver ions, so uptake of NPs could not be established. This example shows the challenges of studies with NPs that readily dissolve.

In general, smaller particles have been found to have a higher uptake, shown for TiO_2 *in vivo*[148], Ag *in vivo*[167], and Ag *in vitro*[141]. Nevertheless, large TiO_2 particles (>100 nm) can still be absorbed[145]. In contrast, larger Au NPs were found to be internalized by Caco-2 cells to a greater extent than smaller Au NPs[141]. However, all sizes of Au NPs transferred to the basolateral side to similar extent. These results suggest uptake by gut epithelial cells does not automatically lead to translocation over the epithelial barrier and that size-dependence of the uptake differs per NP core composition. As it has been shown Au NPs collect a different protein corona than Ag NPs[44], which can explain these observed differences. Polystyrene NPs (PSNPs) were found to translocate across *in vitro* intestinal barrier models and this was increased by a polyethylene glycol derivative coating on the particles[138][139], indicating that surface modifications can also have an effect on the absorption.

A difference in intestinal absorption between adult mice and pups was reported for nanosilver particles as well as dosed silver ions[61]. This result seems plausible given that human infants are known to have higher intestinal permeability than adults[174][175], but caution is in place around these results, as the information given in this publication does not enable exclusion that silver ions were measured instead of silver NPs.

9.4 Respiratory tract

Test models

Lung absorption can be studied using animal models or *in vitro* models. OECD TG 417 does not describe in detail how exposure by inhalation needs to be performed, it focusses on kinetics assessment by an oral exposure study. Instead, dosing considerations from inhalation study guidelines such as OECD TG 412 and 413 can be followed. As stated earlier, true inhalation is preferred over dosing by intratracheal instillation (see 8.1). True inhalation exposure can be performed by nose-only exposure and by whole body exposure, where nose-only is preferred as whole body exposure can lead to simultaneous oral exposure by deposition of the particles on the fur, which is licked.

Different immortalized cell lines are available for the lung, of which most originate from the central airways and only one (A549) from an alveolar epithelial adenocarcinoma, as reviewed by Gordon et al.[126] For lung absorption studies, alveolar cultures would be preferred, as this the physiological area where most absorption takes place. However, the A549 cell line suffers leakiness as it does not form tight junctions, which makes it unsuitable for absorption studies. Any *in vitro* absorption model applied needs to have a barrier function similar to the *in vivo* situation in humans. Alternatively, primary cells can be used, which are not so readily acquired, or tissues from primary cells may be bought from commercial suppliers[126]. For the latter, however, no alveolar tissue is yet available, though being in development.

Current knowledge on absorption of NPs after inhalation

In the lung, the translocation of NPs occurs primarily in the deep lung and depends on the deposition of the particles in the alveoli of the respiratory part of the lung. This deposition depends on the aerodynamic size of the aerosols or particles and can be calculated (modelled) by the International Commission on Radiological Protection (ICRP) and/or the Multiple-Path Particle Dosimetry (MPPD) models[176][177]. The ICRP model is a relatively simple model that indicates areas of fractional deposition based on particles size[176][178], whereas the MPPD model can estimate the fraction of particle deposition per lung lobe[177]. More recently, the ICRP 1994 model[176] has been updated into a more complex multicompartment model[178]. The alveolar deposition fractions are greater for NPs than for larger particles (e.g. 0,2-0,3 for 10 nm to 100-nm C_{60} fullerene particles and 0,05 to 0,2 for 100 nm to 1 000 nm particles in rats[179]).

The fractions not deposited in the alveoli are either exhaled again or deposited in the upper and middle airways. From here, the NPs can be swallowed into GI tract, increasing levels in faeces and potentially leading to absorption into systemic circulation. These upper and middle parts of the airways (nasal and trancheal/bronchial epithelium) are ciliated, allowing transport of the deposited particles by the cilia to the oesophagus, where the particles are finally swallowed. Particles deposited on the bronchi and bronchioles can be cleared by the bronchial mucociliary escalator within 5 min because the bronchial length (throat to terminal bronchiole) in rats is approximately 53 mm[180] and ciliary motion rates are 7,5 mm/min to 13,6 mm/min[181]. As a significant fraction of the inhaled NPs can thus become available for the oral route (e.g. Reference [182]), systemic findings from inhalation studies need always be considered as possibly arising from the oral exposure (too).

For the NPs deposited in the alveoli, not only the fraction, which is taken up by the lung epithelium and passed to the circulation, is of toxicological relevance (as it is for other exposure routes). Also particles that redistribute to the interstitium or end up in the draining lymph nodes of the lungs can cause local effects. In addition, the fraction that is retained in the alveoli can also cause toxic effects, depending on the persistence. Persistence of any type of particles, including NPs can lead to chronic stress-induced damage, possibly leading ultimately to lung fibrosis and thoracic tumours[85]. Toxicokinetic information from inhalation studies can thus include translocation to systemic circulation as well as translocation to interstitium and local lymph nodes, and the persistence of retained particles in the lung.

For lung exposure also the potential of the NPs to migrate along the olfactory nerve from the nose into the brain (olfactory bulb) needs to be considered[183][184].

Persistence in the lung

The main clearance pathway of particulate matter deposited on the alveolar surface is phagocytosis by the alveolar macrophages, which can be followed by transport to the larynx[185][186][187] and subsequent GI tract exposure. These macrophages can also migrate to the local lymph nodes, providing another lung clearance pathway through macrophages. A pathway from the lung outside of the macrophages is by penetration of the epithelial cell barrier, entrance in the epithelial cells, penetration of the pulmonary interstitium and subsequent clearance via the lymph nodes or entry into the systemic circulation through the endothelial cell layer. This second main pathway (epithelial barrier passage) is regarded as considerably more slow than the first (macrophage uptake and subsequent transport out of the lung[188]. The process of phagocytosis of deposited particles takes place within 6 h to 12 h after deposition of the NM, however, the subsequent clearance is much slower ([189] and references therein).

The clearance from the lung, and conversely, the persistence in the lung, can be determined in an *in vivo* study by measuring the amount of NMs in the lung at different time points after the last administration. When using analytical methods that do not discriminate between particles and dissolved substance, this amount in the lung can consist of both forms. In that case, knowing the dissolution rate in artificial lysosomal fluid (pH 4,5) or Gamble's solution (pH 7,4) can help estimating the fraction of NPs in the measured amount.

To subsequently determine the clearance rate from the measured lung burdens in an *in vivo* study, kinetic modelling can be applied. One-compartment models have often been used for the evaluation of pulmonary clearance[190][191]. The first order clearance rate constants derived from such models for highly persistent substances often decrease as the observation period increases. Therefore, first order clearance rate constants estimated by using a 1-compartment model over different observation periods cannot be compared with each other. In addition, a 1-compartment model does not fit the measured burden closely. The clearance rate (k) from the lung can be determined from the measurement of lung burdens and lymph node burdens[39].

A two-compartment model was reported to provide a better fit to the measured burden and can be applied to evaluate two clearance pathways: a fast clearance after deposition on the lung surface and a slow clearance after retention in the epithelium[188]. In such a two- compartment model for pulmonary elimination, the alveolar surface and the interstitium, respectively form the two compartments. A similar two compartment model was developed by Gregoratto et al.[192] as improvement for the 1994 ICRP model. In this model, the clearance pathways from the alveolar surface and alveolar interstitium have been considered to be the bronchial mucociliary escalator via the bronchi, and translocation to lung-associated lymph nodes via the interstitium, respectively[193][194][195]. For humans, the two-

compartment ICRP model showed that approximately 40 % of the alveolar deposit was sequestered in the interstitium whereas the remaining fraction was cleared via the ciliated airways with a half-time of about 300 d[195]. However, it was suggested that clearances both by the bronchial mucociliary escalator via the bronchi after macrophage phagocytosis and translocation to the thoracic lymph nodes can be described as clearance from the alveolar surface area[196]. Then, the alveolar interstitium can be considered as a lung compartment where particles accumulate, rather than as an intermediate compartment for slow particle clearance. Accumulation in the alveolar interstitium might be physiologically related to macrophages which have phagocytosed NPs and have subsequently been sequestered within the interstitium although epithelial passage of non-phagocytized NPs cannot be excluded[197].

There are species differences in the alveolar retention of particles. For rats, the models indicate deposition in the alveoli. When evaluating particle retention in rats and cynomolgus monkeys, rats retained a greater portion in the alveolar lumen, whereas in the primates a greater portion was retained in the lung interstitium[198]. A similar difference was noted for rats exposed to diesel exhaust particles (82 % to 85 % retained in alveolar compartment) and workers chronically exposed to particulate material (57 % to 91 % found in intersitium)[199]. The percentage of particles in the interstitium of humans was found to increase with increased dose (exposure concentration, years of exposure, and/or lung burden)[199].

Poorly soluble (nano)particles appear to be very persistent in the lung. Elimination half-times from the lung for both fine and ultrafine (nano)particles in rats of 29 d to 202 d have been reported when based on particle content of the lung[200][201]. In a study with seven different types of TiO_2 particles[202], the lungs retained 9,0 % to 18 % of the administered TiO_2 without coating after 91 d for doses of 0,375 mg/kg bw to 2,0 mg/kg bw and 21 % to 37 % for doses of 3,0 mg/kg bw to 6,0 mg/kg bw. Higher doses thus led to higher retentions, as has also been seen by Shinohara et al. in other studies and Pauluhn et al.[39][196][201][202] The primary particle size (6 nm to a few hundred nanometres), surface area (6 m^2/g to 300 m^2/g), agglomerate size (69 nm to 400 nm), shape (spherical, spindle-shape, needle-like), and crystalline form (rutile and anatase) did not appear to affect the pulmonary clearance of TiO_2 particles. The absence of an effect of crystalline form is in contrast with what was found by [203]. who reported that amorphous TiO_2 and anatase TiO_2 can penetrate into cells, whereas rutile TiO_2 cannot and explained this by the finding that amorphous TiO_2 and anatase TiO_2 are likely to agglomerate into smaller particles than rutile TiO_2 (amorphous TiO_2: 200 nm, anatase TiO_2: 40 nm to 90 nm, rutile TiO_2: 1 200 nm). However, Shinohara et al.[202] found that the crystalline form did not dictate the agglomerate sizes of their TiO_2 materials and suggested that the differences in clearance found by Ismagilov et al.[203] were rather due to agglomerate size differences, which happened to coincide with crystalline form differences in their case.

Also an effect of shape was found as fibre-like particles were cleared more slowly compared to spherical particle[188][202][204][205]. In contrast, pulmonary clearance rate constants were much higher for NiO nanoparticles with a wire-shape than for the spherical and irregular-shaped NiO nanoparticles[39].

In contrast to Shinohara et al.[202], other studies in rats show that clearance by macrophages is much less efficient for NP compared with larger particles. For example, only about 0,1 % of TiO_2 NPs were internalized by macrophages within 24 h after aerosol inhalation, compared with more than 100-fold more for microparticles already within 1 h[206]. This slower removal of NPs by alveolar macrophages (resulting in mucociliary transport) can increase the probability of their uptake by epithelial cells and translocation to the systemic circulation or lymph nodes[91]. A longer lung retention time of smaller TiO_2 NPs (21 nm) than larger particles (250 nm) has been found, coupled with a preferential translocation of smaller particles into the interstitium[207][208], which was also observed with carbon black particles[209]. The different results of Shinohara et al.[202], while using similar particle sizes (6 nm to a few hundred nm) might be caused by their use of a different exposure method (intratracheal instillation vs inhalation) and the different peak lung burden they obtained (0,09 to 1,5 mg/lung vs. 5,2 mg/lung).

The half-life of intratracheally instilled fullerene C_{60} (18 nm to 29 nm) was 15 d to 28 d[188], which was shorter than the half-life of carbon black particles in rat lungs reported in [209] (GM, 14 nm to 70 nm; 64 d). On the basis of the two pathway clearance model simulation, more than 90 % of the instilled C_{60} particles were estimated to have been eliminated via the rapid clearance pathway, i.e.

via phagocytosis by macrophages, migration of the macrophages to the trachea and bronchi, and subsequent tracheobronchial clearance (i.e. mucociliary escalator). At 6 mo after instillation, more than 99 % of the lung burden was estimated to be in the alveolar epithelial cells for prolonged periods because C_{60} NPs were not observed in the pulmonary interstitium, but in the cytoplasm of alveolar epithelial cells using TEM.

For MWCNTs, it has been reported that 90 % of what was found in the lung 1 d after inhalation exposure was still present 6 mo later[77] or even 364 d later after lung instillation[85], but also that the lung burden decreased from 78 % initially after intratracheal instillation to 28 % after 28 d[79] Spherical C_{60} NPs were cleared from the lungs much faster than MWCNTs (half-life <1 mo[188]) although C_{60} and CNTs are both carbon allotropes, and both have a graphene structure. For other poorly soluble fibres, the pulmonary clearance was similarly slow to those of MWCNTs, e.g. crocidolite and amosite half-life >13 mo[210]; silicon carbide whisker, half-life 16 mo[211].

In the case of fibre-like forms, high aspect ratios (i.e. high fibre length compared to the width) are known to hamper phagocytosis by macrophages in the lung (as e.g. in the case of asbestos), resulting in lung persistence. Some studies have indicated that fibres of >15 µm length could not be fully phagocytised, inducing cytotoxicity[212][213]. This cannot explain the long retention seen for 364 d with MWCNTs by [85], however, as these had a shorter length (average 2,4 µm, with only 1,1 % of the tubes being >15 µm). In addition, their TEM analyses showed that alveolar macrophages internalized the instilled MWCNTs and no MWCNTs were piercing the macrophage cell wall. These macrophages remained in the lung for at least 364 d. Interestingly, the MWCNTs were not found in the nuclei or other intracellular organelles.

Another cause for long lung retention, particularly when observed in rats, can be lung overload: alveolar doses that are so high that the capacity of macrophages is saturated and therefore the clearance is significantly delayed, resulting in prolonged retention of particles at such higher doses as compared to lower doses. The threshold for lung overload in the rat has been suggested to occur at particle doses higher than 1,0 mg/g lung[200][214]. For tangled MWCNTs minimal lung overload was observed at 0,1 µL/g to 0,3 µL/g_per lung (0,1 mg/m^3) and complete lung overload occurred at 1,2 µL/g to 12 µL/g per lung (1,5 mg to 6,0 mg/m^3)[77]. Shinohara et al.[85] calculated that their administered pristine MWCNTs only reached a dose of 0,12 µL/g per lung, while they still found long retention times (>364 d). This difference indicates that the threshold for the occurrence of lung overload might not be that clear-cut and instead may depend on particle characteristics (size, shape, etc.) and/or administration method (as this can result in different local doses). In addition, the calculation models to estimate the alveolar dose may not be sufficiently accurate, or at least not for all types of particles.

Translocation of NPs through the lung

Translocation of NPs from the alveoli to the lymph nodes or systemic circulation and then further to other organs, has been reported for many NMs: TiO_2 (e.g. Reference [73]), Au (References [94][215]), and MWCNTs (e.g. Reference [85]). Ag NPs were also identified and quantitated in systemic tissues (lung, liver, spleen, and placenta) after inhalation, by transmission electron microscopy coupled with energy-dispersive X-ray spectroscopy and single-particle inductively coupled plasma mass spectrometry, suggesting these are translocated as particles, not as silver ions. However, only 0,02 % of the Ag amount in the placenta was present as NPs [216]. These NPs might thus also have formed anew from soluble Ag ions, as observed by Van Der Zande[217]. Thus, it is not clear whether Ag NPs are absorbed as particles.

The translocated fractions are very small, though, typically <1 % by mass, and the majority of the NPs remains in the lung. The translocated fractions might be too small to be detectable for some methods, e.g. <0,2 % (liver) and 0,02 % (brain) of the instillation dose of C_{60} fullerenes[188].

A dose-dependent increase has been observed in translocation rate suggesting that the translocation to thoracic lymph nodes was enhanced at higher nanoparticle doses unlike pulmonary clearance[196]. Therefore, they considered pulmonary overload not to be associated with the thoracic lymph node clearance route.

In a study with seven TiO_2 NMs, differing mostly in size and shape, with only one having a coating, no significant differences were seen in the amounts translocated to the thoracic lymph nodes after intratracheal administration to rats, except for the coated particle[202]. This seems to indicate that size and shape do not influence translocation from the lung. Translocation of NiO NPs from the lungs

to the thoracic lymph nodes increased in a time- and dose-dependent manner for three spherical and irregular-shaped NiO NPs, but not for the wire-like NiO NPs[39], indicating shape can have an effect nonetheless. However, the lack of translocation was due to the relative fast dissolution of these wirelike NiO NPs.

In contrast to Shinohara et al.[202], other studies indicated that both primary particle size and agglomerate size affect translocation in the lung. In rats, smaller (21 nm) anatase TiO_2 NP were found to penetrate more easily into the pulmonary interstitial space than the larger (250 nm) particles at equivalent masses, both after 12 w inhalation and after intratracheal instillation[207]. In a study with 20 nm vs. 80 nm aggregates comprising the same primary size (2 nm to 4 nm) of iridium NPs, greater translocation from the lungs, wider distribution and higher accumulation in secondary organs was found for the smaller than the larger aggregates.[48] In a study with inhalation exposure to airborne agglomerates of gold NPs (Au NPs) of similar size distribution and number concentration, but different primary diameters (7 nm or 20 nm), the agglomerates containing 7 nm AuNPs resulted in highest deposition by mass concentration in the lungs, followed by brain regions including the olfactory bulb, hippocampus, striatum, frontal cortex, entorhinal cortex, septum, cerebellum; aorta, oesophagus, and kidney[184]. However, it is considered that the amounts found in the secondary organs in these studies could have originated from the uptake through the GI tract or olfactory nerve, instead of uptake through the lung.

As stated above, the interstitial translocation across the alveolar epithelium is more prominent in larger species (dogs, nonhuman primates) than in rodents, both in case of fine particles[218] and ultrafine (i.e. nano-) particles[198][199]. It is therefore reasonable to assume that the high translocation of NP observed in rats might also occur in humans[189]. This might depend on the NP used of course, and be different for other types of NP. In addition, differences in lung translocation may occur due to sex differences and in compromised lungs (e.g. susceptibility models).

10 Distribution

10.1 General

Following translocation across the portal of entry, distribution of certain NPs to different organs and tissues can occur through the blood or the lymphatic system. When systemically available, generally a rapid sequestration of NPs by cells of the mononuclear phagocytic system (MPS) occurs as indicated by high levels of IV administered NPs into the liver and the spleen[12][17][29][219]. The NPs are actively and quasi-irreversibly removed from the blood by the phagocytizing cells of the MPS, which is not concentration dependent. In addition to MPS cells also granulocytes were observed to take up NPs. Depending on the mouse strain (Th1 or Th2 responder) granulocytes may even show a higher uptake compared to macrophages[30]. Blood concentrations after IV exposure rapidly decline, e.g. to <1 % of the administered dose in 15 min[219]. Therefore, the concentration in blood or plasma generally has little value for internal dose estimation, as the concentration in organs does not depend on the blood concentration. Thus, the plasma concentration of an NP does not represent the body concentration of a NP, as is often the case the traditional toxicokinetics. Traditional toxicokinetic parameters such as blood AUC, V_d, C_{max}, etc are subsequently of no practical value in NM toxicokinetics.

10.2 Organ distribution

The rapid removal from the blood is caused by the phagocytic uptake by monocytes (macrophages) in the organs rich in this type of cells (i.e. the MPS): liver, spleen, bone marrow, lymph nodes and lung. Among these organs, NPs have been found mostly in liver and spleen[13][59][165][220][221][222][223][224][225], including for MWCNTs[80][99]. Also in human liver and spleen, TiO_2 NPs have been reported[102]. Nonetheless, they have also been found in brain, kidneys, testes, and heart as well[116][166][217][219].

The distribution can depend on the exposure route: following inhalation or intratracheal administration of TiO_2 NPs, Ti has been detected in the lungs and lung-associated lymph nodes but remained below the detection limit in other organs, including liver, spleen, kidney and brain[190][191][222][226]. Only when using a highly sensitive method, Ti was also detected in the liver[73][196] and other organ[80][99], however, the amount translocated was low, much lower than to the lung-associated lymph nodes. After

oral exposure to TiO_2 NPs, Ti has been detected liver and spleen, and a small amount translocated to the lungs[221][222][223]. For NiO NPs, a similar pattern has been observed after intratracheal instillation as for TiO_2 NPs: some increase in liver burden, but no significant increases in the NiO levels of the kidney, spleen, and brain[39]. At 90 d after intravenous administration indications for redistribution were observed for TiO_2 NPs with a decrease in liver and increase in spleen levels[17], while for MWCNT a continuous decrease in the lung was noted and continued increase in various organs at d 360 after pharyngeal aspiration[80].

Soluble NPs can show a different distribution than non-soluble NPs. For nanosilver a dose dependent increase of Ag content was present in brain, kidneys, liver, lungs, stomach and testes after oral administration[166] [217]. The translocation of Ag from the GI tract could be mainly attributed to migration of Ag+ ions [217]. The oral administration of Ag-NP resulted in a widespread presence of Ag in various organs which was mainly cleared from the organs at 8 w with the exception of brain and testis. The organ Ag content was highly correlated with the presence of Ag+ ions in the Ag-NP suspension indicating that Ag+ ions and to a lesser extent Ag NP passed the intestines. Remarkably, Ag NPs could be demonstrated in animals treated with $AgNO_3$ solutions, indicating the new formation of NPs from Ag+ *in vivo*. It was concluded that exposure to silver NPs appears to give similar internal exposure as exposure to silver salts[217]. Thus, the distribution of Ag and other soluble NPs can reflect the distribution of its ions, rather than that of the NPs.

The ability to measure the internal dose of an NP in tissues is essential in identifying potential target organs for toxicity testing and in the construction of an appropriate dose–response relationship. This is especially true for NPs that can accumulate in tissues over time. Liver and spleen TiO_2 burdens hardly appeared to decrease after cessation of exposure, while the TiO_2 burden in the lung, kidney, heart and blood did decrease over time[219]. Also others have found steady liver and spleen levels of TiO_2 and even increasing spleen levels in time indicating redistribution of NM[17]. This presents a new toxicological paradigm, particularly for organs that are normally protected against the entry of larger particulate materials. This also makes it essential that dose–response relationships for NPs are determined for multiple target organs, including some that may not be a first tier consideration in the risk assessment of dissolved substances.

Oral and intravenous exposure to gold NPs of different sizes resulted in an increased organ distribution in mice and rats with decreasing particle size[12][227].

Mercer et al.[99] found that distant tissues only contained singlet MWCNTs, whereas agglomerates accounted for approximately 54 % of lung burden (after inhalation exposure), suggesting the MWCNTs are translocated and distributed in their singlet form. Heringa et al.[102] reported that the TiO_2 they detected in human liver and spleen was visible in agglomerate/aggregate form with microscopy, but the detected particles sizes with sp-ICP-MS (85 nm to 720 nm) indicated single particles can be present as well.

10.3 Transport across the placenta, BBB and to reproductive organs

In a recent review, Hougaard et al.[14] evaluated the possibilities for migration of NPs into the placenta and fetus. In addition to animals (using intravenous administration) also in vitro and ex vivo placental models are available both from animal and human origin. The migration of NPs into the placenta and pups was considered possible, while it is generally known that high-molecular-weight species (1 000 Da) do not penetrate the placenta by passive diffusion[228]. For various types of NPs (e.g. TiO_2, SiO_2, Ag, C_{60} fullerenes) the migration into the placenta and pups was demonstrated, be it to a low extent[14][216] [229]. It has been speculated that NPs either directly injure the blood-placenta barrier or are actively transported through it, to explain their transplacental transfer.

The transport across the placenta of particles does appear to have a size boundary: nSP70 (silica particles of 70 nm) NPs were found in placental trophoblasts, fetal liver, and fetal brain, while no particles were seen in these locations after exposure to nSP300 (silica particles of 300 nm) or mSP1 000 (silica particles of 1 000 nm)[228]. Surface modification of the nSP70 particles with COOH or NH_2 functional groups did not change their ability to transfer across the placenta and reach the fetus but the modified particles did not affect uterine weight, fetal weight and fetal resorption rate.

NPs of silica (70 nm) have been detected in male germ cells in the testis[230], indicating such NPs can cross the blood-testis barrier. Transcytosis is one mechanism by which silica NPs, after uptake by cells, could be transferred across the blood–testis barrier[229]. As it has been reported that titanium dioxide nanoparticle pass through and loosen gap junctions by acting on the structural proteins of adherens junctions between cells, such as vascular endothelial cadherin[231], silica NPs can migrate to the testes by increasing the permeability of the epithelium, too[229].

Passage into nucleus olfactorius of the brain can occur after inhalation and migration along the olfactory nerve (see above 9.4 Translocation of NPs through the lung). Thus, increases in brain nanoparticle levels in studies with exposure by inhalation or intratracheal or intranasal instillation are not indicative of blood-brain barrier (BBB) passage. However, BBB passage cannot be completely excluded after uptake in the lung in view of gastro-intestinal uptake after clearance from the lung by the mucociliary cascade. Some oral and IV studies have reported increased levels in brain, suggesting passage over the BBB, such as for Ag[166], TiO_2[17] and Au NPs[232], where the latter showed a higher percentage of the dose in the brain with smaller NP size. In fact, encapsulating drugs in NPs is currently seen as a method to deliver drugs specifically to the brain. According to the review of Zhou et al.[233] this can be accomplished through different mechanisms. Ag NPs are thought to disrupt the tight junction between the endothelial cells by causing an inflammatory response, in which the produced cytokines increase the microvascular permeability. Uncoated Au NPs were found to be transported by ion channels (as ion channel blockers decreased their uptake into the brain). Coated Au NPs, as well as conjugated carbon quantum dots, liposomes and polymer NPs, can be taken up through receptor-mediated endocytosis when a protein or other ligand is used for coating or conjugating, which is recognized by receptors in the BBB, such as transferrin or PEG. Surface chemistry and charge (endothelial cell membranes are negatively charged[233]) are thus important properties governing BBB passage.

11 Metabolism/degradation

Conventional metabolism involves enzymatic degradation including phase I and II biotransformation. Similar enzymatic degradation has been shown to be occurring for carbon nanotubes. So far, there is little evidence on such biotransformation for other poorly soluble NMs such as SiO_2, TiO_2, gold NPs, etc. Generally, enzymatic metabolism of metal and metal oxide NPs is considered to be minimal or absent. The mechanisms by which NPs can be degraded by biotransformation are not fully understood, but other mechanisms than enzymatic degradation can be involved. For example, gold surfaces can release gold ions after incubation with macrophages[234] and in acidic fluids[235]. See for dissolution also 6.1. Various aspects of possible degradation and dissolution of particles are presented in ISO/TR 19057:2017.

Carbon nanotubes have been known to be degraded by neutrophil myeloperoxidase or horse radish peroxidase[51][236]. C_{60} exposure lead to the formation of three additional peaks in lung tissue extract, of which one was identified to belong to $C_{60}O$ and the others could not be identified[188]. Hamano et al.[237] reported that C_{60} was oxidized by the synthetic cytochrome P-450 chemical model system; however, Bullard-Dillard et al.[224] did not detect $C_{60}O$ in rat tissues. If C_{60} is metabolized to $C_{60}O$ and/or to the unidentified compounds, the amount metabolized is probably small[188].

12 Excretion

After oral administration/exposure, and even after inhalation exposure (due to the mucociliary escalator), excretion of non-absorbed NPs occurs via the faeces from the GI tract (e.g. Reference [182]). No significantly increased level was found in faeces and urine after IV exposure to TiO_2 P25 NPs, indicating no considerable elimination through kidney and bile[219]. However, NiO NPs have been shown to be excreted by urine, with wire-like particles excreted to a larger extent in the first 24 h post administration than spherical or irregularly shaped particles[39]. The wire-like particles did have a higher SSA and a higher dissolution rate than the other particle forms. Also for 20 nm and 80 nm silica NPs excretion via the urine has been observed after intravenous administration of the silica NPs[238].

Distribution to, and subsequent excretion by breast milk has been reported regularly, e.g. for TiO_2 NPs[122], fullerenes[239], Ag[240] and Au[35]. The amount of IV-administered citrate-ligand-capped Ag NPs (10 nm, 50 nm and 100 nm) distributed to breast milk seemingly increased with decreasing particle

size, but when taking into account the blood concentration, larger Ag were more readily distributed to breast milk than smaller NPs and Ag+[35]. Probably, both the blood concentrations and transfer to breast milk were actually caused by silver ions, and not by silver NPs, as the trends in these tissues match those of the % ions released. Ag NPs were distributed to breast milk to a greater extent early in the lactation period than later in the lactation period.

Morishita et al.[35] also found that the administered doses of Ag (NPs or ions) did not induce any apparent acute damage to the blood-milk barrier, thus their observed translocation seemed not to be caused by increased leakiness of this barrier. Substances in blood are thought to transfer to breast milk by means of transcytotic, membrane transport, and paracellular transport pathways. As the Ag NPs used by Morishita et al.[35] were not small enough to use the membrane transport or paracellular transport pathways, the Ag NPs might have transferred to breast milk by means of a transcytotic pathway. They found some experimental evidence that this transport was indeed through a nonparacellular and energy-dependent pathway. But, as indicated previously for Ag NPs, this might have been the ions instead of the NPs.

After inhalation of Au NPs by volunteers Au was demonstrated in blood and urine at 15 min after inhalation, although the calculated uptake from the lung was very low (0,02 % of the administered dose)[241][242].

Due to the low level of elimination, accumulation is possible and is indeed reported for various types of manufactured NMs and for various routes of exposure (e.g. for TiO_2 in rats[17], for TiO_2 in the environment[243], for Ag in rats[29], and for SiO_2 in rats[244]).

13 Conclusions

Based on the observations described above several summaries and conclusions can be made. The most important conclusion to be made is that the toxicokinetics and tissue distribution of NMs differs considerably from molecular/ionic substances. Table 1 gives an overview of important differences between toxicokinetic processes and aspects of dissolved (molecular/ionic) substances and NMs. These differences are due to the particulate nature of the NMs, which contrasts with the soluble nature of dissolved substances. There are indications for oxidation of organic NPs. It is unclear if metal oxides are metabolized but dissolution of some metals and metal oxides can occur. For those NMs that do show some dissolution, like for example nanosilver, zinc oxide and copper oxide NMs, the toxicokinetics and tissue distribution need also to consider their soluble fractions. For the dissolved molecules originating from the NMs the toxicokinetics will be similar to that of dissolved (molecular or ionic) substances.

Due to this aberrant toxicokinetic behaviour of NPs, the classical toxicokinetic parameters have typical values for NMs, or may not even be applicable. Table 2 summarises the limitations of applying some of the classical toxicokinetic parameters to NPs.

Table 1 — Kinetic aspects which distinguish nanoparticles from dissolved substances

Kinetic aspect	Dissolved (molecular/ionic) substances	Nanoparticles
Accumulation	Possible, both in plasma and tissues	Possible, merely in tissues, hardly in plasma
Absorption	0 % to 100 %	Mainly low (<<10 %)
Barrier transport	Gradient driven, or active transport by carriers	Often via endocytosis. Against gradient is possible
Conjugation	Yes, aids excretion	Probably not
Distribution	Flow and extraction ratio dependent	Active uptake by phagocytes. Distribution mainly to tissues with phagocytic capacity (MPS)
Excretion	Renal, hepatic, etc.	Clearance from tissues in general very low and probably related to dissolution
Interactions from mixture exposures	A number of mechanisms known, e.g. competition for metabolic enzymes	Unknown, none reported as of yet

Table 1 *(continued)*

Kinetic aspect	Dissolved (molecular/ionic) substances	Nanoparticles
Interspecies differences	Basic understanding	Unknown, some indications
Linearity	<, >, or = dose-proportional	< dose-proportional due to agglomeration/aggregation and/or saturation seen at higher doses
Lung deposition	Diffusion	Deposition related to aerodynamic diameter, impaction
Mechanism of accumulation	Hydrophobic or bound to cellular structures or proteins	Mostly in vesicles
Metabolism (enzymatic degradation)	0 % to 100 %	Not occurring for metal oxides; for organic NPs some indications of oxidation
PBPK models	Physiological parameterization is understood	Physiological parameterization is under development
Proteins	Protein binding decreases free fraction, free fraction determines activity	Corona formation which might affect kinetics
Route-to-route extrapolation	Basic understanding	Unknown, route-dependent kinetics seem plausible, related to changes in protein corona
Saturation	Transporters and metabolic enzymes can become saturated	Not reported, unknown
Substance form	Uniform	Pluriform (e.g. size distribution), also during internal exposure
Transporter molecules	Renal, hepatic transporters	Mechanism of clearance not fully understood
Uptake into tissue	Diffusion driven, carrier mediated	Active uptake by monocytes (macrophages), endocytosis, possibly in some cases passive

Table 2 — Limitation on applying some of the classical toxicokinetic parameters to nanomaterials (definitions are from https://sepia.unil.ch/pharmacology/) and OECD TG 417.

Toxicokinetic parameter	Description	Applicability for nanomaterials
Area under curve (AUC)	Area under the curve is a plot of concentration of a substance in plasma over time. It represents the total amount of substance absorbed by the body within a predetermined period of time. (might also be applied to tissues, AUCtissue)	Very low for plasma, since nanomaterials are quickly removed from the blood. Can be more relevant when based on tissue concentrations.
Bioavailability	Fraction of an administered dose that reaches the systemic circulation or is made available at the site of physiological activity.	Very low. Bioavailability of nanomaterials might be difficult to determine based on AUC_{plasma}. Bioavailability can be calculated on concentrations/amounts in tissues.
Cmax	Either maximal (peak) concentration in blood (plasma/serum) after administration or maximal (peak) excretion (in urine or faeces) after administration;	Might not be relevant for NM in view of rapid blood clearance.
Clearance	Quantitative measure of the rate at which a substance is removed from the blood, plasma or a certain tissue per unit time.	High clearance from blood, but this is not a representative value. Low rate of clearance from tissues, which is more representative of whole body clearance.

Table 2 *(continued)*

Toxicokinetic parameter	Description	Applicability for nanomaterials
Elimination kinetics	Zero order: Elimination of a constant quantity per time unit of the drug quantity present in the organism First order: Elimination of a constant fraction per time unit of the drug quantity present in the organism. The elimination is proportional to the drug concentration	Poorly soluble particles in the lung have been known to eliminated from the alveolar air space by 1st order via the bronchial mucociliary escalator. It is not clear for the lung interstitium and other tissues. See also Clause 9.4
Half-life ($T_{1/2}$)	The time taken for the concentration of the test substance to decrease by one-half in a compartment. It typically refers to plasma concentration or the amount of the test substance in the whole body.	Plasma kinetics hardly give information on particle kinetics in view of generally rapid disappearance from blood circulation. Can be relevant when based on tissue concentrations.
Hepatic clearance	Part of the total clearance due to liver enzyme activity and biliary excretion	Low rate of liver clearance, probably not by metabolism but due to dissolution or relocation
Renal clearance	Part of the total clearance due to renal excretion	Not known, might not even occur.
Tmax	Time to reach Cmax;	See Cmax.
Volume of distribution (Vd)	Fluid volume that would be required to contain the amount of drug present in the body at the same concentration as in the plasma	Parameter that provides an indication of distribution to tissues, but might give a skewed indication due to rapid blood clearance.

NOTE Most information on NM is based on metal, metal oxide and carbonaceous nanomaterials.

This overview shows that, instead of the kinetic parameters mentioned in Table 2, important kinetic parameters to determine for NPs are:

— % absorption;

— % of amount present in relevant organs (at least liver, spleen, lung, brain, kidney, lymph nodes at organ of entry, and bone marrow) at different time points. Additional tissues may be added when a toxicological concern for effects in these organs has risen, e.g. testes and ovaries in case of concern for effects on fertility;

— for organic NPs: identity of metabolites and level of these metabolites in each relevant organ at different time points;

— rate of elimination/accumulation, from the organ levels taken at different time points after the last dose.

Furthermore, the rate of dissolution in relevant media provides an indication for the dissolution in the body in time, and thus in the presence of potentially toxic ions or molecules and the likelihood for accumulation. Especially for slowly dissolving NPs also the presence of ionic substance needs to be considered in the toxicokinetic studies.

Table 3 finally summarizes the factors that can affect the outcome of a kinetics study with NPs, which need thus to be carefully considered when designing such a study. The physical-chemical properties mentioned in this table, are obviously determinant for the toxicokinetics in general, but it is still unclear to which extent specific physical-chemical properties affect which toxicokinetic process, and in what way. This needs more and systematic research.

Table 3 — Factors affecting the outcome of toxicokinetics study with nanoparticles

Factor	How the factor generally affects the toxicokinetics of NPs
Dissolution rate	The higher the dissolution rate, the lower the potential for accumulation. The transformation of molecules to potentially toxic ions needs to be considered.
NP size	Not entirely clear yet, but it seems smaller particles are absorbed more easily and cross internal barriers more easily.
Shape/morphology	Not entirely clear yet, but higher aspect ratios seem to lead to higher persistence in the lung. In contrast, the shape may also influence the dissolution rate as wirelike NiO particles dissolved almost completely whereas spherical NiO particles did not. Metabolism seems to occur with single-walled CNTs but not multi-walled, but this remains to be confirmed.
Surface charge	Not clear yet, but a positive charge might theoretically give easier attachment to the negatively charged cell membranes, perhaps facilitating endocytosis and thus cellular uptake and absorption.
Surface chemistry	Certain coatings and ligands can lead to recognition by membrane receptors and facilitate endocytosis and thus cellular uptake and absorption.
Protein corona	Certain proteins (opsonins) enhance phagocytosis, leading to greater distribution to monocyte rich tissues like liver and spleen. The protein composition also affects uptake in other cells types and thus e.g. absorption.
Sensitivity of analytical method	Low sensitivity can lead to underestimation, or even failure to detect, absorption and distribution to tissues.
Loss of radiolabel from NP	When using radiolabels that detach from the NP in the organism, both under- or overestimations of the absorption, organ levels, accumulation and elimination might be the result, depending on how the loose label behaves.
Dispersion state	Poorly dispersed mixtures contain larger and more agglomerates/aggregates, which are expected to decrease the dose reaching the alveoli, and for all exposure routes, lead to lower absorption.
Dose level	High dose levels have been reported to lead to gelation in the intestine, rendering the NPs unavailable for uptake. In the lung, high dose levels can lead to saturation of the macrophages (lung overload), changing the percentage that can be absorbed through the macrophages.
Single vs. repeated dosing	Accumulation can not be detected after single dosing, and effects from the absorbed particles on the uptake mechanisms might be missed, too.
Bolus dosing vs. continuous dosing	Bolus dose leads to temporary high dose, with potential consequences as described above for dose level. In lung, a bolus dose (i.e. intratracheal instillation) can not fully reach the deeper lung and has been reported to lead to uneven division of load over lung lobes. This can lead to under – or overestimation of lung load, depending on which lobe is analyzed. In intestine, a bolus dose (gavage) might lead to a different protein corona than a continuous dose, due to the diet in which a continuous dose in mixed and because a bolus dose evades the upper digestive tract environment.

Annex A
(informative)

Definitions as used in OECD Test Guideline 417:2010

Absorption: Process(es) of uptake of substances into or across tissues. Absorption refers to parent compound and all its metabolites. Not to be confused with "bioavailability".

Accumulation (Bioaccumulation): Increase of the amount of a substance over time within tissues (usually fatty tissues, following repeated exposure); if the input of a substance into the body is greater than the rate at which it is eliminated, the organism accumulates the substance and toxic concentrations of a substance might be achieved.

ADME: Acronym for "Absorption, Distribution, Metabolism, and Excretion".

AUC: (Area under the plasma concentration-time curve): Area under the curve in a plot of concentration of substance in plasma over time. It represents the total amount of substance absorbed by the body within a predetermined period of time. Under linear conditions, the AUC (from time zero to infinity) is proportional to the total amount of a substance absorbed by the body, irrespective of the rate of absorption.

Autoradiography: (Whole-body autoradiography): Used to determine qualitatively and/or quantitatively the tissue localization of a radioactive substance, this technique uses X-ray film or more recently digital phosphorimaging to visualize radioactively labelled molecules or fragments of molecules by recording the radiation emitted within the object under study. Quantitative whole-body autoradiography, compared to organ dissection, may have some advantages for the evaluation of test substance distribution and the assessment of overall recovery and resolution of radioactive material in tissues. One significant advantage, for example, is it can be used in a pigmented animal model to assess possible association of the test substance with melanin, which can bind certain molecules. However, while it may provide convenient whole body overviews of the high-capacity-low- affinity binding sites, this technique might be limited in recognizing specific target sites such as receptor- binding sites where relatively high-resolution and high-sensitivity are needed for detection. When autoradiography is used, experiments intended to determine mass balance of administered compound can be conducted as a separate group or in a separate study from the tissue distribution experiment, where all excreta (which may also include expired air) and whole carcasses are homogenized and assayed by liquid scintillation counting.

Biliary excretion: Excretion via the bile ducts.

Bioaccumulation: See "Accumulation".

Bioavailability: Fraction of an administered dose that reaches the systemic circulation or is made available at the site of physiological activity. Usually, bioavailability of a substance refers to the parent compound, but it could refer to its metabolite. It considers only one chemical form. Nota Bene: bioavailability and absorption are not the same. The difference between e.g. oral absorption (i.e. presence in gut wall and portal circulation) and bioavailability (i.e. presence in systemic blood and in tissues) can arise from chemical degradation due to gut wall metabolism or efflux transport back to the intestinal lumen or presystemic metabolism in the liver, among other factors (10). Bioavailability of the toxic component (parent compound or a metabolite) is a critical parameter in human risk assessment (high-to-low dose extrapolation, route-to-route extrapolation) for derivation of an internal value from the external NOAEL or BMD (applied dose). For liver effects upon oral administration, it is the oral absorption that suffices. However, for every effect other than at the portal of entry, it is the bioavailability that is in general a more reliable parameter for further use in risk assessment, not the absorption.

Biopersistence: See "Persistence".

Biotransformation: (Usually enzymatic) chemical conversion of a substance of interest into a different chemical within the body. Synonymous with “metabolism”.

C_{max}: Either maximal (peak) concentration in blood (plasma/serum) after administration or maximal (peak) excretion (in urine or faeces) after administration.

Clearance rate: Quantitative measure of the rate at which a substance is removed from the blood, plasma or a certain tissue per unit time.

Compartment: Structural or biochemical portion (or unit) of a body, tissue or cell, that is separate from the rest.

Detoxification pathways: Series of steps leading to the elimination of toxic substances from the body, either by metabolic change or excretion.

Distribution: Dispersal of a substance and its derivatives throughout an organism.

Enzymes/Isozymes: Proteins that catalyse chemical reactions. Isozymes are enzymes that catalyse similar chemical reactions but differ in their amino acid sequence.

Enzymatic Parameters: K_m : Michaelis constant and V_{max}: maximum velocity.

Excretion: Process(es) by which an administered substance and/or its metabolites are removed from the body.

Exogenously: Introduced from or produced outside the organism or system.

Extrapolation: Inference of one or more unknown values on the basis of that which is known or has been observed.

Half-life ($t_{1/2}$): The time taken for the concentration of the test substance to decrease by one-half in a compartment. It typically refers to plasma concentration or the amount of the test substance in the whole body.

Induction/Enzyme induction: Enzyme synthesis in response to an environmental stimulus or inducer molecule.

Linearity/linear kinetics: A process is linear in terms of kinetics when all transfer rates between compartments are proportional to the amounts or concentrations present, i.e. first order. Consequently, clearance and distribution volumes are constant, as well as half-lives. The concentrations achieved are proportional to the dosing rate (exposure), and accumulation is more easily predictable.

Linearity/Non-linearity can be assessed by comparing the relevant parameters, e.g. AUC, after different doses or after single and repeated exposure. Lack of dose dependency may be indicative of saturation of enzymes involved in the metabolism of the compound, an increase of AUC after repeated exposure as compared to single exposure may be an indication for inhibition of metabolism and a decrease in AUC may be an indication for induction of metabolism (see also Reference 11);

Mass balance: Accounting of test substance entering and leaving the system.

Material balance: See “mass balance”.

Mechanism (Mode) of toxicity/Mechanism (Mode) of action: Mechanism of action refers to specific biochemical interactions through which a substance produces its effect. Mode of action refers to more general pathways leading to the toxicity of a substance.

Metabolism: Synonymous with “biotransformation”.

Metabolites: Products of metabolism or metabolic processes.

Nanomaterials: Material with typical size range between 1 nm and 100 nm which is either confined in one, two, or three dimensions or has an internal or surface structure.

Oral Absorption: The percentage of the dose of test substance absorbed from the site of administration (ie: GI tract). This critical parameter can be used to understand the fraction of the administered test substance that reaches the portal vein, and subsequently the liver.

Partition coefficient: Also known as the distribution coefficient, it is a measure of the differential solubility of a substance in two solvents.

Peak blood (plasma/serum) levels: Maximal (peak) blood (plasma/serum) concentration after administration (see also "C_{max}").

Persistence (biopersistence): Long-term presence of a substance (in a biological system) due to resistance to degradation/elimination.

Read-across: The endpoint information for one or more chemicals is used to make a prediction of the endpoint for the target chemical.

Receptor Microscopic Autoradiography (or Receptor Microautoradiography): This technique may be used to probe xenobiotic interaction with specific tissue sites or cell populations as for instance in receptor binding or specific mode of action studies that may require high-resolution and high sensitivity which may not be feasible with other techniques such as whole-body autoradiography.

Route of administration (oral, IV, dermal, inhalation, etc.): Refers to the means by which substances are administered to the body (e.g., orally by gavage, orally by diet, dermal, by inhalation, intravenously, etc).

Saturation: State whereby one or more of the kinetic (e.g. absorption, metabolism or clearance) process(es) are at a maximum (read 'saturated').

Sensitivity: Capability of a method or instrument to discriminate between measurement responses representing different levels of a variable of interest.

Steady-state blood (plasma) levels: Non-equilibrium state of an open system in which all forces acting on the system are exactly counter-balanced by opposing forces, in such a manner that all its components are stationary in concentration although matter is flowing through the system.

Systems Modelling (Physiologically-based Toxicokinetic, Pharmacokinetic-based, Physiologically-based Pharmacokinetic, Biologically-based, etc.): Abstract model that uses mathematical language to describe the behaviour of a system.

Target tissue: Tissue in which a principal adverse effect of a toxicant is manifested.

Tissue distribution: Reversible movement of a substance from one location in the body to another. Tissue distribution can be studied by organ dissection, homogenization, combustion and liquid scintillation counting or by qualitative and/or quantitative whole body autoradiography. The former is useful to obtain concentration and percent of recovery from tissues and remaining carcass of the same animals, but may lack resolution for all tissues and may have less than ideal overall recovery (<90 %). See definition for the latter above.

T_{max}: Time to reach C_{max}.

Toxicokinetics (Pharmacokinetics): Study of the absorption, distribution, metabolism, and excretion of substances over time.

Validation of models: Process of assessing the adequacy of a model to consistently describe the available toxicokinetic data. Models may be evaluated via statistical and visual comparison of model predictions with experimental values against a common independent variable (e.g. time). The extent of evaluation should be justified in relation to the intended use of the model.

Annex B
(informative)

Quantitation methods for nanomaterials, advantages and challenges

Table B.1 — Techniques to measure composition or concentration of nano-objects and their aggregates and agglomerates (NOAA)

Technique	Acronym	Chemical Composition	Concentration	Advantages	Limitations
Acoustic spectroscopy			Yes	Does not require sample dilution; applicable for concentrated samples. Method is suitable for characterizing aggregated and structured systems. Measurement is not affected by stirring and/or pumping of the sample. Absolute method; does not require size calibration. Instrument verification can be conducted by measurement of water with known acoustic properties. Solvent-based samples (non-aqueous) can be analysed.	Applicable only to liquid-borne particles. Lower size limit ≈10 nm; upper size limit ≈3 mm. Lower volume fraction limit is ≈0,1 %; upper volume fraction limit is ≈50 %.
Auger electron spectroscopy (scanning)	AES	Yes	Yes	High spatial (<1 µm) and surface (≈0,1 nm) resolution [spatial in this context refers to horizontal plane of analysis (*x-y* direction) and surface refers to depth]. Elemental mapping possible when employing a scanning electron beam. Depth profiling capability when combined with ion sputtering. High detection sensitivity; capable of analysing a fraction of a surface monolayer.	Analysis of non-conducting samples can be problematic due to surface charging effect. Surface contamination can complicate data analysis.
Differential scanning calorimetry	DSC	Yes		Minimal sample preparation required. Fast scanning allows suppression of material decomposition at higher temperatures (scan at 400 °C/min and faster). Wide temperature ranges. Measurements can be conducted in different atmospheres. Can characterize qualitative bond strength between a nanomaterial and epoxy in nanocomposites (strong or weak) and examines rigid amorphous fraction (RAF) of nanocomposite system.	Many DSC instruments cannot scan at 400 °C/min or collect data at those speeds.

Table B.1 *(continued)*

Technique	Acronym	Chemical Composition	Concentration	Advantages	Limitations
Electron energy loss spectroscopy	EELS	Yes		Spatial resolution of the order of the electron beam size. Any solid can be analysed. Quantitative analysis is possible. Signal includes chemical information. Direct information can be obtained on the structure of solids and oxidative state of the elements.	Need very thin specimens, <30 nm. Intensity weak for energy losses >300 eV. Can only be done using transmission electron microscopy (TEM).
EM based X-ray spectroscopy	EDX/EDS/ WDS	Yes		EDS is capable of semiquantitative or with the appropriate controls and sample preparation quantitative analysis. EDS can be used to generate complex multi-dimensional elemental composition maps. WDS is more sensitive to low atomic number elements than EDS and has the capacity for quantitative analysis.	Samples need to be well-polished and representative of the bulk material. It might be necessary to coat non-conductive samples with a thin layer of carbon, gold, or platinum. Standards might be required for high-accuracy quantification.
Field flow fractionation	FFF	Yes	Yes	Can fractionate highly polydisperse samples into individual populations. Can employ a variety of detectors, e.g., differential refractometers, UV/Vis absorbance, fluorescence, dynamic light scattering, multi-angle static light scattering, or an inductively coupled plasma-mass spectrometer.	Lower size limit depends on particle density and the FFF method used but is typically 2 nm to 5 nm. Size can only be determined accurately by calibration, retention time, or by secondary detection. Distributions that range from the nm scale to greater than 1 µm will not be properly separated, as the elution order inverts above roughly 1 µm. Sample required to be dispersed in liquid.
Fluorescence spectroscopy	FL		Yes	Sensitive technique, down to single fluorophore level. Rapid technique for qualitative measurements. Compatible with various sample formats. Sensitive to changes in nano-object size and shape.	Interference from background fluorescence for some samples. Quantitative measurements are time-consuming and require calibration standards. Corrections for scattering are frequently required for quantitative measurements for nano-objects.
Fourier transform infrared spectroscopy/imaging	FTIR	Yes		Can determine chemical structure and compositional analysis of chemical mixtures in solid or gas phase. High throughput, high signal-to-noise ratio and high wavelength accuracy. Samples can be run under either ambient temperature (in purged air) or extreme environmental conditions such as ultra-high vacuum, cryo-temperature, high pressure, high temperature. Typically non-destructive and requires little sample preparation. Insensitive to stray light. Large penetration depth; can acquire spectra through low bandgap semiconductors.	Not suitable for aqueous-phased characterizations due to its sensitivity to water. Sensitive to CO_2 and requires the instrumentation to be purged to remove both H_2O and CO_2. Spectral complexity requires experienced users or access to spectral library for proper band assignment.

Table B.1 *(continued)*

Technique	Acronym	Chemical Composition	Concentration	Advantages	Limitations
Inductively coupled plasma-mas spectrometry and single particle ICP-MS	ICP-MS	Yes	Yes	High sensitivity analysis — detection limits of most elements are on the order of ng/l (ng/kg) or lower. Multi-element analysis possible. Can determine element identity and quantity, typically in less than one minute. Wide linear dynamic range with eight orders of magnitude (in conventional mode). Isotopic information possible. Isotope dilution calibration possible. Can differentiate between dissolved and particulate analytes.	Spectral interference occurs when the instrument cannot distinguish the spectrum of an analyte ion from the spectrum of a concomitant having the same nominal mass-to-charge ratio. Molecular ion interferences can often be mitigated using collision/reaction cells; elemental isobars need to be avoided. Contamination in blanks (acids, water, etc.) can sometimes limit detection and quantification. The use of high purity reagents and a clean laboratory environment is necessary, if ultra-trace levels are required. ICP-MS in single particle mode might not be an adequate technique to determine particle number and mass concentration in the event that the NM size distribution extends below 10 nm for monoisotopic metal nanoparticles. Coupling ICP-MS with field flow fractionation (FFF) or another separation technique will resolve this limitation. Sample required to be dispersed in liquid. Particles with sizes smaller than the particle size detection limit will be quantified as ionic. Multiple analyses are required if no preliminary information on the particle size and number concentration in a sample is available.
Liquid chromatography-mass spectroscopy	LC-MS	Yes	Yes	Commonly used in many laboratories for a wide variety of applications. There are multiple MS detectors available depending on application needs.	Will only detect organic materials. Liquids/dispersions only; no solid or gas analysis. Requires optimization for best separation.
Optical absorption spectroscopy	UV/Vis/NIR	Yes	Yes	UV/Vis analysis requires only a few seconds to provide a response. Wide concentration range of analysis by varying sample path length. Quantification is available with higher end instruments. Little to no sample preparation required. Little maintenance required. Wide range of measurement accessories available to measure characteristics in both liquid and solid form including angular dependent scattered measurements. Absorption properties for some nano-objects provide information on sizes.	Corrections for scattering might be required. Solid samples require reflectance accessory. There is a wide array of accessories available to allow multiple modes of reflectance measurements to be made. These include relative, absolute and diffuse reflectance. The actual mode of reflectance required is to be determined by the specific analysis.

Table B.1 *(continued)*

Technique	Acronym	Chemical Composition	Concentration	Advantages	Limitations
Raman spectroscopy/ imaging		Yes		Capable of chemical analysis of complex mixture. Insensitive to water, thus suitable for the characterization of aqueous-phased nano-objects. Suitable for in situ measurements in liquid, vapour and solid state and under both ambient or extreme environmental conditions such as ultra-high vacuum, cryo-temperature, high pressure, high temperature or under electrical bias, in a high magnetic field. Typically non-destructive and requires little sample preparation. Small foot-print fibre-optics-based field portable instrumentations are commercially available. Spectra can be collected from very small volume, e.g. in ≈1 μm^3 in confocal Raman microscope.	Raman scattering is very weak and can be swamped by fluorescence from trace impurities in the sample. Raman spectral complexity often requires even experienced users to access a spectral library for proper band assignment.

Table B.1 *(continued)*

Technique	Acronym	Chemical Composition	Concentration	Advantages	Limitations
Scanning electron microscopy	SEM	Yes		Wide range of magnification. Large depth of field with best resolution under 1 nm. Parfocal, i.e. the focus does not change with changing magnification. Two- and three-dimensional and topographical imaging with rich details. Fast, completing imaging and analysis often possible within a few minutes. Most samples require no or minimal preparation. Low landing energy operation or low pressure gas around a specimen allows for imaging and measurement of electrically non-conductive specimens. Low pressure gas around a specimen allows for nanometre-scale etching and deposition of materials. With specially equipped instruments hydrated, even live specimens can be examined in pressure greater than 600 Pa. The sample temperature can vary from 70 K to above 1 000 K and specimen manipulation at the nanoscale is possible. Different imaging modes giving different information, e.g. backscattered electron imaging sensitive to composition, secondary electron imaging sensitive to topography. Commercial calibration standards available for size in gold and silver. When using electron back-scatter diffraction, it is highly sensitive to orientation. When using electron back-scatter diffraction, phase identification is through crystal type.	It may be necessary to coat non-conductive samples with a thin layer of carbon before examination. The instrument may be large in size and required to be housed in an area free of significant electro-magnetic or mechanical interference and might require circulation of cooling water. Special training is required to operate the microscope and to learn the processes of sample preparation, and to recognize and minimize preparation-related artefacts. Works in vacuum, which requires solid samples small enough to fit inside the vacuum chamber. Electron irradiation can alter the sample. Measurements on small particles might have high measurement uncertainties. Distinguishing between agglomerated and aggregated particles might be difficult. When using electron back-scatter diffraction, only polished surfaces can be examined. When using electron back-scatter diffraction, crystallinity determination of phases with resolution is down to 10 nm size (when polished, cross-section of sample including embedded nano-objects is examined). For particle size distribution, a relatively large number of independent images need to be obtained in order to have a statistically relevant sample representation.

Table B.1 *(continued)*

Technique	Acronym	Chemical Composition	Concentration	Advantages	Limitations
Secondary ion mass spectroscopy	SIMS		Yes	Identification of trace elements up to 2 000 Da with transmission >10 %, with better than 1 parts per million sensitivity. 3D elemental distribution with 10 nm depth resolution in depth profiling mode. Detailed chemical isotope ratio information, used for exact quantification [spot to spot reproducibility (1s): 0,4 per mille; mean internal error (1s): 0,3 per mille]. Chemical imaging of surfaces with ≈500 nm spatial resolution. Depth profiling of inorganic with ≈10 nm depth resolution. NanoSIMS is a term used to denote the use of an ion beam with an extremely small spot size (down to 50 nm) to provide high spatial resolution analysis.	Samples analyzed under vacuum. Sample preparation (embedding in resins and polishing). Sample preparation is not necessarily required when running TOF-SIMS. Number of simultaneous monitored elements can be limited but a TOF analyser is capable of monitoring ions of m/e into the hundreds and thousands. Not considered a bulk analysis technique. Homogeneous material is needed to obtain nano-shape information. Extreme care needs to be taken during sample handling to avoid contamination.
Static light scattering	SLS/SMLS		Yes	Useful for aerosols (SLS only) as well as particles dispersed in liquid. Very wide dynamic range, from tens of nanometres to several millimetres. Real-time measurement. Capable of time-dependent measurement. Well-established quality assurance procedures. Time-dependent analysis is possible. For SMLS, no sample preparation is typically required (i.e. no dilution required for highly concentrated samples). For SMLS, sensitive to changes in dispersion state (e.g. agglomeration, aggregation, and sedimentation) when coupled to scanning technology.	Optical properties (refractive index) of the particles and suspending medium might be required for application of specific theoretical analyses (e.g. RGD). Strongly absorbing particles (e.g. metal nanoparticles associated with surface plasmon resonance effects) might not be amenable to analysis by SLS. Calculation of size assumes spherical particle shape. If shape factor is known, it can be incorporated into RGD analysis or used to convert *Rg* to a defined shape model (e.g. ellipsoid or cylinder). For SLS, size range is limited by the source wavelength and the angular range of detectors. Using RGD or Guinier analysis with the wavelength of a He-Ne laser (633 nm), *Rg* smaller than about 60 nm can be extracted. Upper number concentration limit subject to the onset of coincidence. For SMLS, volume fraction and refractive indexes are required for mean particle size calculation.
Single particle light interaction methods			Yes	High size resolution possible. No size distribution assumptions required. Real-time measurement. Capable of time-dependent measurement. Well-established quality assurance procedures	Applicable either to airborne or liquid-borne NPs. Lower size limit ≈60 nm; upper size limit ≈100 µm. Upper number concentration limit subject to the onset of coincidence.

Table B.1 *(continued)*

Technique	Acronym	Chemical Composition	Concentration	Advantages	Limitations
Thermogravimetric analysis	TGA	Yes		Various atmospheres available. Scanning: subambient to 1 000 °C. Isothermal studies at a constant temperature for times of seconds to hours.	Samples over 1,5 mg required. Samples that are wet required to be let dry before running.
Transmission electron microscopy	TEM	Yes		70 pm imaging resolution with best instruments. Chemical information with EDS and EELS. Tomography for full 3D shape analysis. Imaging at the sub-nanometre scale. Automated image analysis for improvement of statistical relevance and work-flow. Commercial calibration standards available for size in gold and silver.	Long-time consumption is required to get good statistics for size measurements. Time and expense of equipment and maintenance. Limited primarily to electron-dense materials; soft materials lack sufficient contrast unless dyed. Electron beam damage or modification of sample. Magnification calibration required for accurate size measurements. Generally unable to differentiate between agglomerates, aggregates and artefacts. Sample preparation is essential to good results. Calibration might change as the lenses system is changed.
X-ray photoelectron spectroscopy	XPS	Yes	Yes	Analysis depth of order 1 nm to 10 nm (sample dependent). Analysis area can be as small as 10 nm. Chemical state/bonding information. Simultaneous detection of Auger electrons can assist in elemental identification.	Contamination of the surface can complicate qualitative and quantitative analysis. Surface damage due to prolonged X-ray exposure is possible for certain types of samples. Incapable of detecting hydrogen and helium. Ultra-high vacuum conditions can limit the type of samples that can be analysed.

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