A discussion on existing nanomedicine regulation

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ABSTRACT:

Due to the large diversity of nanomaterials and to their partly unknown behavior in human, nanomedicine regulation is taking shape at a slow pace. Here, I discuss the following aspects linked with the emergence of such regulation: i), nanomaterial definition/classification, which may be extended beyond the sole notion of size or dimension and overcome the confusion arising from the distinction between their intentional and non-intentional use or exposure, ii) nanomaterial interacting behaviors that lead to the formation of aggregates or agglomerates depending on their interacting strength, opsonization following capture of biological material at their surface, and the apparition of assemblies of dispersed or soluble nanomaterials depending on their level of degradation in a given medium, iii) nanomaterial dose whose value depends on the method used for measurement and on the type of dose considered (injected versus effective), iv) the different steps of nanomaterial preparation whose most critical one is often sterilization that should remove bacterial contamination without affecting nanomaterial properties, v) the different characterization methods and properties that they measure, vi) the toxicological assessment of these materials that necessitates accurately selecting the toxicity tests and associated operating conditions, vii) nanomaterial biodistribution properties that notably depend on the frequency with which the organism is monitored, the types of studied tissues/organs, and the nature of the nanomaterial used for the study (transformed or not), viii) the risk analysis that should determine the severity of potential damages caused by a nanomaterial as a function of the probability of this damage.

KEYWORDS:

Nanomedicine, regulation, dose, preparation, biodistribution, toxicity, risk analysis, standard, ISO, OECD, nanoparticle, nano.

INTRODUCTION

Regulating the nanomedicine field is not an easy task because of the large diversity of nanomaterials, comprising among others polymers, liposomes, dendrimers, nanocrystals, nanocomplexes, nanoemulsions, nanoparticles, metal colloids, micelles, quantum dos, fullerene, carbon nanotube, liposome, and virosome. Indeed, all these materials have different properties such as various compositions, sizes, shapes, interactions, charges, resulting in a very broad range of activities and behaviors. A series of non-scientific communications have generalized certain behaviors, notably of toxicity, to a whole set of nanomaterials, whereas in fact they only apply to one type of nanomaterial examined in a specific situation. This has created some fears among the general population, often without a sound scientific basis. In addition, clinical data are often missing, making it difficult to evaluate the benefit/risk ratio of nanomaterials. Despite these difficulties, regulation governing the use of these materials in the medical field is slowly taking shape as discussed elsewhere, by describing the different available standards and guidelines (1, 2), by presenting the principles of a harmonized approach of standardization (3), by analyzing the nanomaterials already in the market to define the bases for new nano-regulation (4), by highlighting the controversies in nanomaterial regulation (5), by debating about the link between regulation and innovation in nanomedicine (6), by pointing out differences in nanomaterial regulation between various regions of the world (7), and by listing different types of nano-drugs and regulatory issues associated with their entry to market (2).

Here, the different aspects of nanomedicine regulation are discussed starting from the analysis of the standards presented in Figure 1. They concern the definition and the classification of the nanomaterials, which essentially rely on an analysis of size or dimension (1). Other important properties of these materials should probably also be taken into account. They discuss the different types of interactions between nanomaterials, as well as methods for measuring dose, preparation and characterization of nanomaterials, which can determine their behavior and benefit/risk ratio. The studies of toxicity and biodistribution of these materials as well as a risk analysis are necessary steps described in these

standard that need to be undertaken to be able to start clinical trials and later obtain marketing authorization. Furthermore, while developing new standards for nanomaterials, the pitfalls encountered during nanomaterial development and characterization (8, 9) should also be avoided. Nanomaterial standards directly impact the translation of these drugs into clinical trials, making their design important for the scientific community, the society as a whole, and more importantly the patients who could benefit from new and more efficient treatments provided by specific nano-therapies (10).

NANOPARTICLE DEFINITION

According to existing standards, a material is considered nano-metric when at least one of its dimensions (internal or external), or surface structure, is at the nano-scale. This definition raises a number of concerns. Firstly, the size of a nanomaterial can't always be measured, for example when nanomaterials form aggregates from which individual nanomaterials can't be isolated or when they are comprised in an environment such as the interior of the organism, where nanomaterial characterization tools can't easily be used. Thus, this definition of a nanomaterial may exclude nanomaterials whose size can't be measured. In addition, the size of a nanomaterial depends both on the method employed to measure it and on nanomaterial environment or configuration. For example, nanomaterial size deduced from EM (electron microscopy) is usually smaller than that estimated by DLS (dynamic light scattering). It may be suggested to identify a nanomaterial not only from its size but from some of its other properties such as catalytic ones. Secondly, it was suggested to make a distinction between the internal and external dimension of a nanomaterial, without however clarifying what this distinction implies. One might argue that the external dimension may correspond to the dimension of the surface of a nanomaterial that is exposed to an external environment while the internal dimension may be that of the inner core of the nanomaterial. However, this view has some limitations. Indeed, it does not describe the transition between the internal and external dimensions of the nanomaterials, *i.e.* one does not know when and where the internal and/or external dimension(s) start(s). In addition, it is not clear what the terms *''nano-material surface structure''* refer to. They seem to describe an assembly of different materials rather than a single nanomaterial. Furthermore, this definition seems insufficient to fully

define a nanomaterial. To be more precise, the definition could be complemented by specifying: i) the situation of a nanomaterial relatively to injection, *i.e.* before, during, or after its administration to a living organism, ii) nanomaterial environment, for example an inorganic, organic, solid, liquid, or gaseous medium, iii) nanomaterial methods used to measure its properties, and iv) other nanomaterial properties than only their sizes.

Another concern in nanomaterial definition is the distinction between intentional and unintentional nanomaterial production and use. Nanomaterials synthesized by animals such as birds or fishes or by bacteria may be considered as intentionally or unintentionally produced depending on whether one considers that an animal or a bacterium can have an intention (or not). When nanomaterials are produced by the organism of a human, for example by having free iron react with specific enzymes such as ferritin, it is not clear whether this would be considered as an intentional or unintentional process. Another example of a confusing situation can be provided when one is willing to fabricate another type of material than a nanomaterial, but ends up by making a nanomaterial. In this case, it is difficult to know whether the nanomaterial is intentionally or unintentionally produced or used. The distinction between these two situations (intentional and unintentional) was probably introduced to determine how much control can be reached over these materials, since an intentional process should in principle result in a better control than an unintentional one. However, to address this concern, it may be better to directly attribute a certain level of control over a given nanomaterial, which would depend on how much knowledge one has gained on this material.

NANOMATERIAL CLASSIFICATION

The most simple classification of nanomaterials distinguishes between materials with three dimensions at nanometer scale (nanoparticles, quantum dots), two dimensions at this scale (nano-fiber, nano-rod, nanotube), or one dimension at this scale (Nano-leaf). Here again, the question arises about whether this classification exclusively by size or dimension is not too restrictive. To be broader, the classification could define the nano-power of a nanomaterial that would be measured not only according to the

number of nano-metric dimensions, but also to other properties that arise due to nanomaterial specific sizes, such as their heating property or more generally medical activity.

COMMON PITFALLS TO BE OVERCOME

Nanomaterial studies are prone to a number of pitfalls. Regulation could indicate how to overcome them, especially by discussing the following aspects (8):

• Do nanomaterial studies need to focus on nanomaterials themselves, their environment such as their supernate, or both? A number of compounds such as endotoxins may be in equilibrium or diffuse between the surface of nanomaterials and their environment, in which case such environment may be studied, *e.g.* by measuring its endotoxin concentration.

• When nanomaterials degrade, for example by transforming into ions, should degradation products be studied? Specific standards indicate how to study products of degradation, but they don't describe the transition between nanomaterials and products of degradation. On the one hand, the nature of this transition could be specified by explaining where it comes from (a change in nanomaterial size, composition, charge etc…). On the other hand, kinetic of degradation could be undertaken under detailed conditions.

• Nanomaterial properties depend on the environment in which they are located. They are different in water, a culture medium, inside a cell, in tissue, in blood etc... Regulation could list a series of environments that are representative to those encountered by nanomaterials during their use, and in which nanomaterial properties could be studied.

• Nanomaterials can interfere with methods used for their characterization, due to their size or interaction (aggregation/agglomeration). Regulation could explain how to avoid or remove these interferences.

NANOPARTICLE INTERACTIONS

Nanomaterials form assemblies, in which they interact with each other and their environment. Hence, its interacting properties should be taken into account. Regulation seems to distinguish between three types

of interacting properties, associated with aggregation/agglomeration, opsonization, or dispersion/sedimentation. They are discussed below.

- **Aggregates versus Agglomerates.** Nanoparticles used in nanomedicine are assemblies of nanoparticles in weak or strong interactions with each other. In order to take into consideration these different interactions, assemblies of nanoparticles were divided between those of strongly bound or fused nanomaterials (aggregates) and those of weakly bound nanomaterials (agglomerates). To provide a physical meaning to the terms *''aggregates''* and *''agglomerates''*, these assemblies have been described as having their external surface that is either similar to the sum of the surface areas of each individual nanomaterial (agglomerates) or smaller than the sum of the calculated surface areas of each individual nanomaterial (aggregates). These definitions raise some concerns. On the one hand, they are only valid if nanomaterials are sufficiently distant from each other for agglomerates or close to each other for aggregates. It would therefore be beneficial to consider a separating distance between nanomaterials, which could be lower than an average distance in the case of aggregates and larger than this distance in the case of agglomerates, as illustrated in Figure 2. This distance could be estimated for each nanomaterial according to its size, composition, charge, and surrounding environment. On the other hand, when nanomaterials are interacting with each other they are in a dynamic system that evolves over time and can often easily switch from an aggregated to an agglomerated state or vice versa from an agglomerated to an aggregated state. Thus, it would also be valuable to specify the condition in time at which the aggregate/agglomerate is measured or observed, *i.e.* at one or several different time(s), or in a situation of equilibrium or saturation reached over time. In the literature, to distinguish between an aggregated and agglomerated condition, a difference between aggregated and well-dispersed nanomaterials is often highlighted. The author believes that the words agglomerated and well-dispersed describe a relatively similar situation.

- **Opsonization.** One of the main interactions of nanoparticles with the organism is the so-called opsonization (11-13). In this mechanism, nanoparticles are covered with opsonins that bind at nanoparticle surface following their administration. Opsonins are then recognized by defense cells,

which possess receptors for them, *e.g.* scavenger receptors, resulting in nanoparticle phagocytosis and clearance. Opsonization, whose efficacy depends on several parameters such as nanoparticle shape or type of coating, is an important factor that determines nanoparticle biodistribution properties. A standard that would explain how to deal with opsonization, *i.e.* measure its presence and/or efficacy as well as determine if/how/when such mechanism should be avoided or sought for, is currently lacking in current regulation

- **Dispersibility versus solubility.** Interaction between nanomaterials is also governed by their soluble or dispersed state. Current regulation makes a distinction between these two conditions, since nanomaterial toxicity profiles can dependent upon this state. However, this approach seems to oversimplify the real situation of nanomaterials, which is often in-between a soluble and insoluble state. Indeed, nanomaterials considered as insoluble can disintegrate or lose some of their constituents which end up as ions in solution or in the body and are solubilized. On the other hand, a nanomaterial may consist of both soluble and insoluble parts, for example when it is made of a vesicle with an aqueous core.

DOSE MEASUREMENTS

The injected dose is an essential parameter in the determination of the benefit/risk ratio of a nanomaterial. The current standards suggest considering the following parameters for its estimation. First, the administered volume can determine the quantity of nanomaterials in a targeted organ. Although an increase in such volume can result in a larger quantity of nanomaterials in such organ, the opposite situation can also occur, *e.g.* when nanomaterials induce an immune response that removes nanomaterials from this organ. Second, the method used for dispersing a nanomaterial, *e.g.* a proper solvent, pH or ionic strength of the dispersing medium, or the correct dispersing method often chosen among sonication, stirring, or shaking, can be essential in determining the quantity or dose of nanomaterial that is effectively administered. The dispersing method should disperse nanomaterials without damaging or modifying them and enable a control over certain dispersion parameters such as the volume/temperature of dispersion, or the type or quality of dispersing medium (14). Third, the

length of time between the step of homogenization of the nanomaterial suspension and that of administration should be optimized. Often when this lengths of time is increased, sedimentation and/or aggregation become(s) more important, especially for nanomaterials in liquid suspension. Fourth, the types of forces involved between nanomaterials, such as diffusion, convection and/or sedimentation forces, which depend on nanomaterial properties such as nanomaterial size, charge, composition, or environment, determine the way in which nanomaterials organize in a given medium, directly impacting their distribution before and after administration and therefore also the dose that is effectively injected or effective after injection. Fifth, the size distribution and by extension the distribution in other nanomaterial properties than sizes can also determine the quantity of injected nanomaterials that is efficient, *e.g.* a large size distribution may hamper the heating power of a nanomaterial, resulting in a percentage of nanomaterial that is efficient, which is lower for a wide than narrow size distribution. Sixth, the effective dose can also depend on interactions between nanomaterial and the organism, especially cells, *e.g.* nanomaterials could be more cytotoxic when they are internalized than when they remain outside of cells.

Furthermore, the most widely used parameters to estimate the dose of nanomaterial are the mass and number of nanomaterials per unit volume. Standards also recommend measuring the surface of nanomaterials, which depends on nanomaterial size and size distribution. The author guesses (since it is not explicitly mentioned in standards) that such measurement is recommended to gather some indication about the catalytic power of a nanomaterial, since the latter is believed to essentially occur at nanomaterials surface. Such assumption is however only partly true. To provide an example when a metallic nanoparticle is heated by the application of an alternating magnetic field, the external source of energy couples with the magnetic moment of the nanoparticle core without necessarily directly involving nanoparticle surface.

A final consideration concerns the method used for dose measurement, which often inaccurately estimates nanomaterial dose at least for two reasons, the presence of aggregates/agglomerates that

distorts the measurements and the conditions of nanomaterials used for the measurement (for example in powder) that can be far from their real situation in the organism.

PREPARATION OF NANOMATERIALS

Figure 3 summarizes the recommended rules for nanomaterial preparation, which are summarized below (15). First, to approach the situations that would occur in the individual, a formulation, which is close from its ready-for-injection stage, should preferentially be used for toxicity/efficacy assessments. Second, in order to correctly disperse nanomaterials, it is advisable to use an appropriate coating agent, dispersion medium, and/or mixing method. Third, to prevent nanomaterial contamination by impurities, the presence of impurities or toxic contaminants in the media used for making the preparation should be minimized. Fourth, the preparation should be stable, *i.e.* the dissolution and/or degradation of nanomaterials over time should be minimized. This may be achieved by preventing the change of one of its properties, such as the loss of its coating, or of part of its composition, and by monitoring such changes. Fifth, the dispersing medium should be adapted by adjusting its ionic strength, calcium, magnesium, or anion concentration, pH, presence of organic additives, and identity or concentration of dispersing agents. Sixth, the properties of the preparation should be described before and after its administration to the organism. Seventh, a proper method of sterilization should be chosen, mainly among filtration, autoclaving, irradiation, and chemical treatments, which enables to get rid of endotoxins and contaminants without destroying the nanomaterials, (16-18). Seventh, the stability of the nanomaterial over time should be ensured.

CHARACTERISATION OF NANOMATERIALS

Characterization of nanomaterials is an essential step in their evaluation (19). A framework has been set in place by current regulation to ease such task, which includes a descriptor (description to identify a nanomaterial parameter), clarification (additional information about such parameter), pertinence (toxicological relevance of a parameter), measureande (nanomaterial variable that is measured). It shall not only determine the properties of a nanomaterial as a whole, but also lead to a description of the different constitutive parts of such material, such as its central part, coating, corona, surrounding

medium, or various types of molecules or entities associated or in interaction with such material, such as antibody, protein, lipid, drug, targeting agent, as shown in Figure 4. It needs to distinguish between properties of interacting and non-interacting nanomaterials. It also has to define the variability in nanomaterial properties between one batch and another, the method and conditions used for characterization, *e.g.* nanomaterial inside or outside its packaging, in powder, liquid, or gas form, homogenized or not, before or after its administration, at various concentrations, pH, temperature, redox state, or exposed to various types of stress. The different nanomaterial properties, which can influence their toxicity/efficacy or behavior *in vivo*, are listed below:

- **Size/size distribution,** evaluated by an estimate of the spherical diameter, length, width, surface, volume of a nanomaterial, and distribution of these parameters.
- **Aggregate/Agglomerate,** which are nanomaterial assemblies in strong (aggregates) or weak (agglomerates) interaction, characterized by their size and frequency relatively to those of non-interacting nanomaterials.
- **Shape,** which corresponds to the contour of nanomaterial surface, can be various for the same nanomaterial, and is defined by specific words designing the type of shape, for example spherical, rectangular, or cubic.
- **Isotropy/anisotropy**, which is measured by estimating either the ratio between the smallest and largest length of a nanomaterial for geometry anisotropy or by measuring the anisotropy constant for magnetic anisotropy.
- **Surface area,** which is defined as the surface of a nanomaterial accessible to a gas, liquid, or adsorbent surrounding the nanomaterial.
- **Composition,** which includes a chemical compositions with information about the level of impurities and stoichiometry of a nanomaterial and a crystalline composition defining the parameters of the crystalline and spatial group of a nanomaterial.

- **Surface charge or zeta potential,** which is the electric charge at nanomaterial surface, where large values of zeta potential can stabilize nanomaterial dispersion by promoting repulsions between them and low zeta potential values can lead to stronger nanomaterial attraction than repulsion resulting in nanomaterial aggregation.
- **Solubility/dispersibility,** which is the degree or concentration up to which a nanomaterial can be homogenously or uniformly dissolved or mixed in a solvent or material without forming a precipitate, aggregating, dissolving, or changing one of its property (size, shape, charge for example).

When feasible, an uncertainty should be associated with the values of the above parameters, which comes on the one hand from the different values of these parameters estimated when the measurements are repeated and on the other hand from the uncertainty associated with the measuring device.

In order to measure the above parameters, various methods of nanomaterial characterization have been recommended by current nanomaterial regulation, as summarized in table 1. They rely on:

- A balance between different forces applied on nanomaterials in: i), analytical centrifugation (AC), where nanoparticles are inserted in an ultracentrifuge and exposed to centrifuge and diffusion forces while the formation of different fractions is monitored by an optical detection system, enabling the measurement of nanoparticle molecular weight, density, and diameter (20), ii), aerosol particle mass analyzer (APMA), in which nanoparticles are placed inside a rotating cylinder in the presence of an applied voltage, and exposed to centrifugal and electrostatic forces, allowing the measurement of nanoparticle mass/charge ratio.
- The temperature increase of a nanomaterial followed by: i), the measurement of the amount of energy released or absorbed by a nanomaterial as a function of temperature, nanomaterial specific heat capacity, the temperature or energy of melting, fusion, crystalline phase transition, precipitation, and denaturation of a nanomaterial (21), using differential scanning calorimetry (DSC), ii), the production of ions detected by a mass spectrometer, which enables the determination of nanomaterial concentration, (22), using Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

- The application of a uniform electric field on nanomaterials, inducing the separation of nanomaterials of different sizes, charges, or masses, followed by the measurement of nanomaterial electrophoretic mobility, often converted to a 'zeta potential', using: i), for nanoparticle in suspension, electrophoretic light scattering (ELS), which was further refined in capillary electrophoresis (CE) (23), ii), for nanoparticle in a gas, differential mobility analyzer (DMA), where these techniques enable the determination of nanoparticle size and size distribution (24, 25).
- The application of a vertical separating force onto a horizontal laminar flux of nanomaterials using field flow fractionation (FFF), which enables the separation of nanomaterials with different sizes, masses or compositions in individual fractions, where each fraction can be analyzed using a light scattering technique (26).
- Interactions between an electron beam and nanomaterials in: i), Auger electron spectroscopy (AES), where a beam of electrons with relatively low energy (< ~ 2 keV) and penetration depth focused on nanomaterials provides information about nanomaterial surface, (27), ii), Electron energy loss spectroscopy (EELS), where a beam of electrons is inelastically scattered by a nanomaterial, leading to a series of interactions such as inner shell ionization, from which the composition of the nanomaterial can de deduced, iii), Scanning electron microscopy (SEM), where the surface of a nanomaterial is exposed to a beam of electron, providing information about surface topography and composition, iv) Secondary ion mass spectrometry (SIMS), in which a beam of electrons is focused on nanomaterial surface producing secondary ions whose mass/charge ratio provides the composition of the nanomaterial, iv), Transmission electron microscopy (TEM), in which a beam of electrons crosses a thin layer of nanomaterials, given access to nanoparticle size, size distribution, shape, geometry.
- Interactions between X-rays and nanomaterials using: i), Energy-dispersive X-ray spectroscopy (EDX), where a nanomaterial is exposed to a beam of protons, electrons, or X-rays, leading to the emission of an X-ray, whose measured energy is characteristic of the emitting element and therefore enables nanomaterial composition to be determined, ii), Small-angle X-ray scattering (SAXS), which

measured the difference in density between nanomaterials and leads to an estimate of nanomaterial size and mass, iii), X-ray photoelectron spectroscopy (XPS), where a nanomaterial is irradiated with a X-ray beam and the nanomaterial surface chemistry is determined from the properties of the emitted electrons, iv), X-ray diffraction (XRD), where the intensity of X-rays scattered by a nanomaterial enables the determination of nanomaterial composition and structure.

- Interactions of light with nanomaterials, including: i), UV/Vis absorption spectroscopy from which the composition and concentration of a nanomaterial can be deduced, ii), fluorescence spectroscopy (FS), where the fluorescent properties of a nanomaterial can be correlated to its structure ii), Raman scattering imaging (RSI) in which light is scattered by nanomaterial inducing a shift in its frequency that can be related to nanomaterial composition, ii), infra-red absorption spectra of nanomaterials exposed to light with a broad spectrum of wavelengths, using FT-IR spectroscopy, giving access to the different types of bonds between a nanomaterial and the molecules at its surface iii), the creation of a nanomaterial grating under the application of an electric field that diffracts light using induced grating (IG), and enables the determination of nanomaterial sizes, iv), laser diffraction by nanomaterials, which can provide information about nanomaterial size and shape (28), v), analysis of light scattered by a single nanomaterial to estimate nanoparticle size and composition, using single particle light interaction (SPLI), vi), static light scattering (SLS) in which the molecular weight of a nanomaterial is deduced from the relation between the intensity of light scattered by a nanomaterial and its molecular weight and size, as determined by the Rayleigh theory, vii), dynamic light scattering (DLS), where nanomaterials suspended in liquid that are prone to Brownian motion are illuminated leading to a light scattering signal from which nanomaterial hydrodynamic size, size distribution, and zeta potential can be deduced (29, 30), viii) nanoparticle tracking analysis (NTA), which works similarly to DLS except that it enables to determine the size of individual nanomaterials whereas DLS monitors the behavior of nanomaterials collectively.

- The use of acoustic systems in: i), acoustic detection in acoustic spectroscopy (AS), where nanomaterials are exposed to light producing acoustic waves that are detected, hence revealing the presence and quantity of nanomaterials, (31), or ii) electroacoustic velocity (EV) (32).
- The adsorption of a gas at the surface of nanomaterials using the Brunauer-Emmett-Teller (BET) method, yielding an estimate of the specific surface area of a nanomaterial (33)
- The formation in a mixture of nanomaterials and supersaturated gas of droplets larger than nanomaterials, which can be detected laser nephelometry (34), using a technique called condensed particle counter (CPC).
- The separation of nanomatetrials having different surface properties, charges, sizes, hydrophobicity/hydrophilicity, geometries using various types of liquid chromatography, such as size exclusion chromatography or high performance liquid chromatography, which is combined with a method such as mass spectroscopy to enable the identification of the nanomaterial composition, using liquid chromatography mass spectroscopy (LCMS).
- The deposition of nanomaterial on top of quartz crystal, leading to a change in the oscillation frequency of the crystal and enabling the nanomaterial mass measurement with a resolution of up to ~ 2 ng per cm^2, using a quartz crystal microbalance (QCM)
- The flow of nanomaterials through a fluidic channel, which modifies the frequency of a contilver, providing an estimate of the buyant and dry mass as well as size of a nanomaterial, using a technique called resonant mass measurement (RMM).
- The use of probe positioned above the surface of a nanomaterial to detect various nanomaterial properties using scanning probe microscopy (SPM). As an example of property, the force applied by the nanomaterial on the probe can be measured using atomic force microscopy (AFM).

Table 2 summarizes the different nanomaterial parameters that can be measured by these various methods of characterization.

The results of the characterization shall be included in a report that mentions the description of the nanomaterials using the measured parameters, nanomaterial preparation, the conditions under which nanomaterial measurements are made, the methods, equipment, and laboratory used to determine nanomaterial properties, the values of the measured parameters and their associated uncertainty, and the experimental protocols relying on identified standards.

TOXICOLOGIC EVALUATION OF NANOMATERIALS

The types of toxicity test that need to be carried out on a nanomaterial depend on the duration of interaction between the nanomaterial and the organism, the type and number of organs, cells, biological entity with which a nanomaterial is in contact, are listed below :

- **Cytotoxicity**, evaluated according to ISO 10993-5, is a toxicity induced at cellular level by nanomaterials (35). During its evaluation, as summarized in Figure 5, a number of parameters that can have an impact on it can be taken into consideration, such as: i), the type of test used to measure it, e.g. nanoparticle cytotoxicity was shown to be slightly more pronounced with the MTT test than with neutral red assay, or alarm blue assay (36), ii), the composition of nanomaterials, e.g. iron oxide nanomaterials appear to be relatively non-cytotoxic compared with other compositions iii), the type of coating surrounding the nanomaterials, *e.g.* oleic acid is reported to be less cytotoxic than silica (36), iv), the type of tested cells, by distinguishing between those inducing an absorption, such as phagocytizing cells (murine macrophages RAW264,7, human THP-1 cells as differentiated macrophages), and those not responsible for such mechanism (murine fibroblasts 3T3, murine fibroblasts L929, human keratinocytes HaCaT), v) the incubation time, vi), the presence (or not) of a serum surrounding cells, vi), cellular internalization, which was usually shown to increase with increasing quantity and duration of nanomaterial incubated, (37), vi), the quantity of nanomaterial incubated with cells, vii), the rate of nanomaterial sedimentation / aggregation, e.g. nanomaterials were reported to be more cytotoxic when they sedimented and/or aggregated, (38), viii) diffusion versus sedimentation, xi) the production of radical oxygen species that can induce the production of cytokines and chemokines involved in pro-inflammatory responses , xii) degradation of nanomaterials leading to

401 the release of certain atoms / ions from nanomaterials. During cytotoxicity assessment, it is necessary to
402 take into consideration the interferences between nanomaterials and both calorimetric/fluorescent agents
403 whose luminescence can be decreased by light absorption from nanomaterials and the compounds of the
404 cellular growth medium.

405 • **Genotoxicity**, whose method of evaluation are described 10993-3, include : i) the in vitro
406 micronucleus test in which the number of cells with two nucleus in the presence of CytoB is counted
407 (OECD LD 487), ii), the search for DNA alterations when the thymidine kinase gene expresses
408 hypoxanthine-guanine phosphoriboxyl transferase (HPRT) or xanthine-guanine phosphoriboryl
409 transferase (XPRT) using mammalian cells (OECD 476), iii) the monitoring of DNA or chromosome
410 damages when the genes L5178Y and TK6 are used to express mouse lymphoma (OECD 490), iv) the
411 in vivo alkaline comet assay in which DNA strand breaks are looked for in cells or nuclei isolated from
412 animal tissues (OECD489), v) the in vivo micronucleus test where micronucleus are searched in
413 erythrocytes of mammals (OECD 474), vi) mammalian in vivo chromosome aberration test in which
414 chromosome aberrations are monitored in bone marrow cells of animals (OECD 475), vii) TGR gene
415 mutation assays in which mutations are measured in rodent tissues (OECD 488).

416 • **Cancerogenicity** can be evaluated according to OCDE451, OCDE 453, and ISO 10993-3, using
417 rodents receiving nanomaterials frequently during their life while monitoring the apparition of tumors as
418 well as other side effects.

419 • **Toxicity for reproduction**, which may be assessed according to ISO 10993-3, ODCE 421,
420 ODCE 414, ODCE 415, ODCE 416, or ODCE 422, using female rodents or rabbits receiving
421 nanomaterials a few days after coupling followed by the monitoring of toxicity effects on fetus
422 developments.

423 • **Immuno-toxicity**, assessed following ISO/TS 10993-20, EHC 180, EHC 236, through: i) the
424 detection of the first signs of immunosuppression/immunostimlation following repeated administration
425 of nanomaterials *in vivo*, ii), the monitoring of nuclear factor kappa B produced by immune cells, or iii)

the evaluation of nanomaterial phagocytosis, chimotaxis, or of the production of nitric acid by macrophages.

- **Sensibilisation**, evaluated according to ISO 10993-10, using: i), the application of a patch containing nanomaterials at the surface of animal skin that releases nanomaterials followed by the monitoring of erythema and edema (tests BT, GPMT), ii) the local application of nanomaterials at the back of animal ears followed by the measurement of the proliferation of lymphocytes in lymph nodes (LLNA test).
- **Irritation**, assessed following ISO 10993-10, tested on rabbits in which 0.5g/0.5mL of nanomaterials is applied on their skin, the nanomaterial region is covered by a bandage for a few hours, and the presence of erythema and edema is monitored following removal of the bandage.
- **Hemocompatibility,** assessed according to ISO 10993-4, measures thrombosis, coagulation, plaquette agregation, leucocyte activation, hemolysis, activation of complement system, using the co-culture model of endothelial and monocyte cells.
- **Systemic toxicity**, evaluated following ISO 10993-11, measures the potential toxicity of nanomaterials towards organs, using rodents receiving a unique (acute toxicity), or repeated (chronic toxicity) nanomaterial administration. The frequency and dosage of the administration are adapted depending on the foreseen clinical protocol. To assess toxicity, the variations in corporal mass or water/food consumption of the animals are measured. The macroscopic or clinical pathologies are monitored. Toxicity is also examined through histological analysis, variations in masses, or analysis of organs, tissues, or blood. Certain specific nanomaterial properties can have an impact on systemic toxicity such as: i), their faculty (or not) to cross physiological barriers, ii), their potential dissolution in contact with organs or tissues, iii), their possible accumulation in organs/tissues. Specific care should be given to reticulum endoplasmic system (liver and spleen), kidney, brain, bone marrow, where some nanomaterials have been shown accumulate.
- **Pyrogenicity**, assessed according to ISO 29701, ISO 10993-11, USP 85, USP 151, ANSI/AAMI ST72, could be due to the presence of endotoxins such as lipopolysaccharides at nanomaterial surface.

Such presence can come from the large exposed surface that can capture these entities (39). The American pharmacopeia recommends treating nanomaterials at a sufficiently high temperature for a long enough time to reduce the concentration of endotoxins to an acceptable level (generally a minimum temperature of 250 °C for more than 30 min, USP 1995). Pyrogenicity can be measured using: i), the Limulus amebocyte lysate (LAL) test following ISO 29701 after removing the interferences between this test and nanomaterial (40), ii), the monocyte activation test (MAT), or iii), the rabbit test.

NANOMATERIAL BIODISTRIBUTION

Nanomaterial biodistribution properties depend on their intrinsic properties such as their size, their chemical composition, their charge, their hydrophobicity/hydrophilicity, their shape, their geometry, their tendency to form aggregates/agglomerates. They are also function of the conditions under which the biodistribution tests are carried out, *i.e.* their mode of administration, the choice of animal species, nanomaterial environment such as injection medium, the injected dose, the types of examined samples/tissues, and the frequency of the examinations. Finally they are influenced by the characterization method, which is employed. A first remark concerns the large number of these parameters, which can lead to different biodistribution profiles for the same nanomaterial tested under different conditions. To the author knowledge, a standard specifying a set of parameters that should be used for biodistribution studies is lacking. Biodistribution behaviors are difficult to generalize to all nanomaterials because of the differences between these materials. However, some studies have attempted such generalization by examining the size effect. They suggested that nanomaterials smaller than 15 nm distribute over the entire body, nanoparticles of intermediate size (between 15 nm and 200 nm) accumulate in mononuclear phagocytic system (SPM) organs such as liver and spleen, while nanoparticles larger than 200 nm are preferentially found in spleen. However, their analysis mainly relies on a presumed size-dependent diffusion of nanoparticles through the orifices, which give access to various organs. They don’t seem to fully take into consideration other mechanisms such as immune reactions that can play an important role in distribution properties and are influenced by other factors than size such as nanomaterial surface properties. Furthermore, the method used to evaluate the

biodistribution properties is also subject to unresolved discussions in current standards. First, if the material used for such studies is not identical to that injected in humans, for example if it is associated with a fluorescent or radioactive substance to facilitate its detection, is it still considered that its behavior is representative to that expected in a human? More generally, what level of modification of a nanomaterial can be acceptable for carrying out a valid biodistribution study? The same type of consideration surrounds the choice of the animal species that must have a metabolism comparable to that of a human. In this respect, the choice of the rodent model is still questionable. Second, should one focus primarily on the modification of the nanomaterial in the body, or rather on the effect induced by the presence of nanomaterials on the organism? Both aspects require different methods of characterization. Third, how frequently should the study be performed? Nanomaterials can furtively diffuse through certain organs, making their presence undetected if organs are not often enough monitored. Fourth, to which types of nanomaterials do the parameters describing nanomaterial kinetic of elimination and biodistribution, such as clearance and half-life, apply? Most of the time, nanomaterials gradually degrade over time in the body. Thus, when measuring one of these parameters, it characterizes a nanomaterial during its transformation, for example changing size between before and after administration or even, in a more extreme case, being completely dissolved. What is the level of transformation after which one considers that the nanomaterial studied is no longer the same? A transformation threshold value could be introduced such as an acceptable decrease in size or percentage of variation of certain properties, beyond which the nanomaterial would be considered different from the original one. Above this threshold, a value of a kinetic parameter could no longer be attributed to a nanomaterial.

RISK ANALYSIS

The aim of the risk analysis (41, 42), carried out according to ISO15499, ISO14971, ISO10993-22, is to estimate the probability and the severity of possible damages caused by nanomaterials depending on the level of exposure and the foreseen clinical application of these materials. The different aspects of a risk analysis are summarized in Figure 6. They should enable a comparison between the damages that they

might cause with well-established harms induced by similar materials. Damages could come from the misuse of nanomaterials. Their severity essentially depends on how long nanomaterials remain in the organism, the way in which they are administered, the quantity of nanomaterials that is absorbed by the organism, and the duration of nanomaterial interaction with biological material. They can result from the use of an incorrect method of characterization, which fails to provide relevant characteristics, lacks sensitivity, or evaluates the properties of nanomaterials in an unsuitable configuration, which is not representative of its condition during its use. In addition to being enriched by the analysis of the state of the art on a given material, the risk analysis should consider factors that yield damages such as wrongly determined properties of nanomaterials, erroneous sample preparation, false measurement of nanomaterials, or inappropriate methods used for evaluating the bio-distribution and toxicity of these materials. The risk analysis relies in large part on the evaluation of the toxicity and biodistribution properties of nanomaterials as a function of nanomaterial doses. To achieve a proper risk analysis, the type of tests carried out on nanomaterials should be function of the mode of action of these materials in the foreseen medical indication. The risk management process is summarized in Figure 1 of ISO10993-22.

CONCLUSION

In this article, I have presented different aspects of current nanomaterial regulation, which concerns the definition, fabrication, characterization, interactions of these materials as well as their toxicity, biodistribution, and risk assessment. It appears important that such regulation covers the various aspects of nanomaterial development. In additions to the previously mentioned unresolved questions, it seems that the following aspects have not been fully addressed by current regulation:

- The definition of some criteria to help choosing the most relevant characterization and toxicity assessment methods among the large number of different ones presented in standards,
- The determination of specific conditions in which these methods should be employed since they can have strongly impact the study outcomes,

- The setting of quality control tests that could ensure reproducibility and accuracy of all protocols (43), especially during fabrication, characterization, toxicity, and efficacy assessments of nanomaterials,
- The listing of reference materials (44) used to compare the properties of a given nanomaterial with those of a similar material with well-established behaviors,
- The description of specific conditions for the up-scaled production of nanomaterials (45, 46), which could result in significant changes of nanomaterial properties, where standards could clarify the extend up to which such modification would be acceptable.

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FIGURES AND TABLES:

Figure 1: List of standards that apply to nanomedicine, which were taken into consideration to write this manuscript.

Figure 2: A schematic picture showing the different types of interactions between nanomaterials, forming: i) aggregates when nanomaterials interact witch each other with a force (f) larger than a critical force (f_c), $f>>f_c$, and the distance separating the centers of two nanomaterials (d_{av}) is similar to the nanomaterial diameter, or ii) agglomerates when nanomaterials interact with each other with a force f that is smaller than a critical force (f_c), $f<<f_c$, and $d_{av}>>d$. The picture also shows the transition between dispersed and dissolved states of nanomaterials.

Figure 3: A schematic picture summarizing the recommendations for nanomaterial preparation.

Figure 4: A schematic picture showing the different parts of a nanomaterial that need to be characterized, *i.e.* inner part, coating, corona, surrounding medium, as well as bound entity such as antibody, protein, lipid, drug, or targeting agent.

Figure 5: A summary of the different aspects to be considered to carry out cytotoxicity assessment of a nanomaterial.

Figure 6: A declination of the different steps to lead a risk analysis, based on an analysis of the severity of damage caused by a nanomaterial as a function of its probability.

Table 1: The mechanisms of action, drawbacks and advantages and parameters measured for different nanomaterial characterization methods.

Table 2: The characterization methods used to measure the various nanomaterial parameters.

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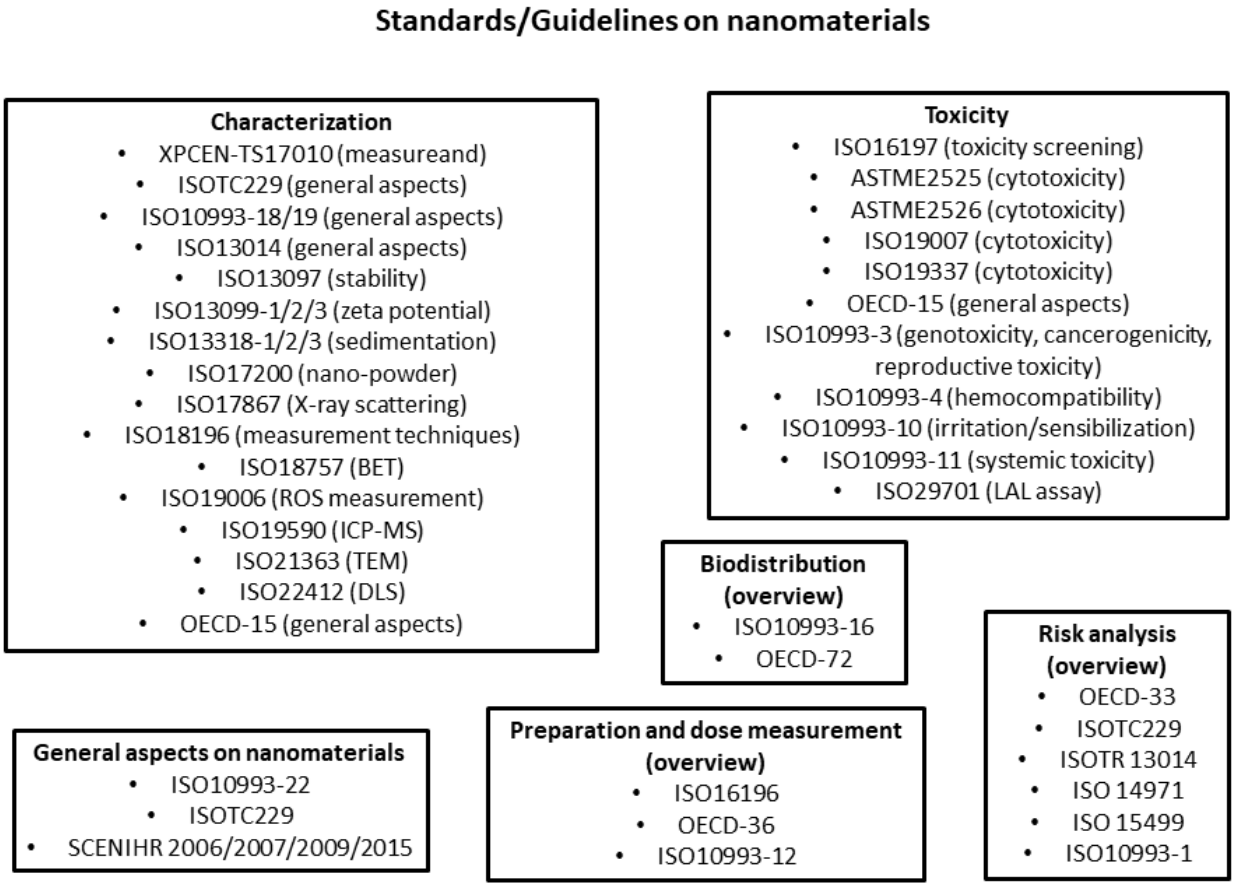
Standards/Guidelines on nanomaterials
Characterization
XPCEN-TS17010 (measureand)
ISOTC229 (general aspects)
ISO10993-18/19 (general aspects)
ISO13014 (general aspects)
ISO13097 (stability)
ISO13099-1/2/3 (zeta potential)
ISO13318-1/2/3 (sedimentation)
ISO17200 (nano-powder)
ISO17867 (X-ray scattering)
ISO18196 (measurement techniques)
ISO18757 (BET)
ISO19006 (ROS measurement)
ISO19590 (ICP-MS)
ISO21363 (TEM)
ISO22412 (DLS)
OECD-15 (general aspects)
Toxicity
ISO16197 (toxicity screening)
ASTME2525 (cytotoxicity)
ASTME2526 (cytotoxicity)
ISO19007 (cytotoxicity)
ISO19337 (cytotoxicity)
OECD-15 (general aspects)
ISO10993-3 (genotoxicity, cancerogenicity, reproductive toxicity)
ISO10993-4 (hemocompatibility)
ISO10993-10 (irritation/sensibilization)
ISO10993-11 (systemic toxicity)
ISO29701 (LAL assay)
Biodistribution (overview)
ISO10993-16
OECD-72
Risk analysis (overview)
OECD-33
ISOTC229
ISOTR 13014
ISO 14971
ISO 15499
ISO10993-1
General aspects on nanomaterials
ISO10993-22
ISOTC229
SCENIHR 2006/2007/2009/2015
Preparation and dose measurement (overview)
ISO16196
OECD-36
ISO10993-12

Figure 1

676

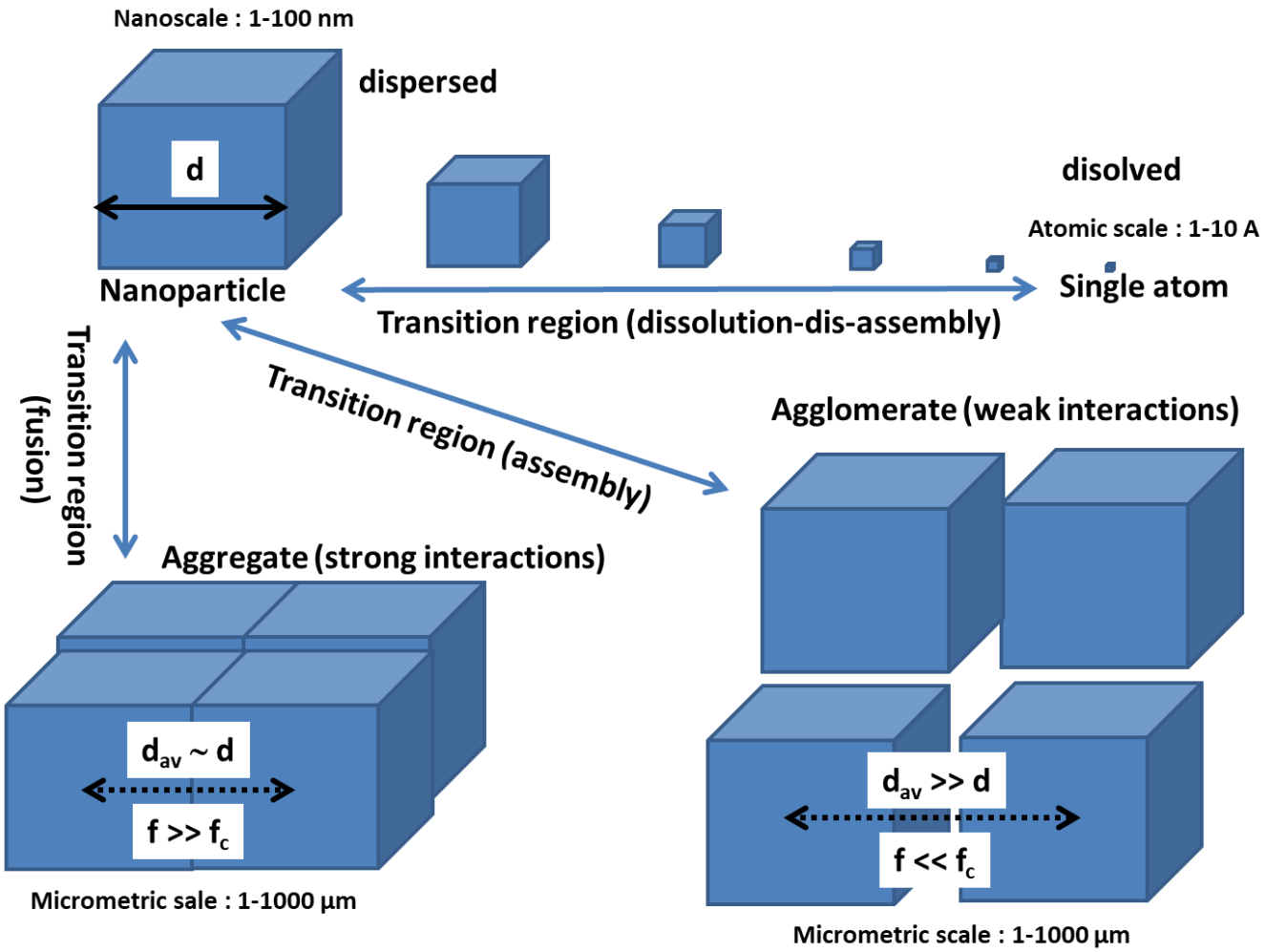
Nanoscale : 1-100 nm
dispersed
d
disolved
Atomic scale : 1-10 A
Nanoparticle
Single atom
Transition region (dissolution-dis-assembly)
Transition region (fusion)
Transition region (assembly)
Agglomerate (weak interactions)
Aggregate (strong interactions)
$d_{av} \sim d$
$f >> f_c$
$d_{av} >> d$
$f << f_c$
Micrometric sale : 1-1000 µm
Micrometric scale : 1-1000 µm

Figure 2

677

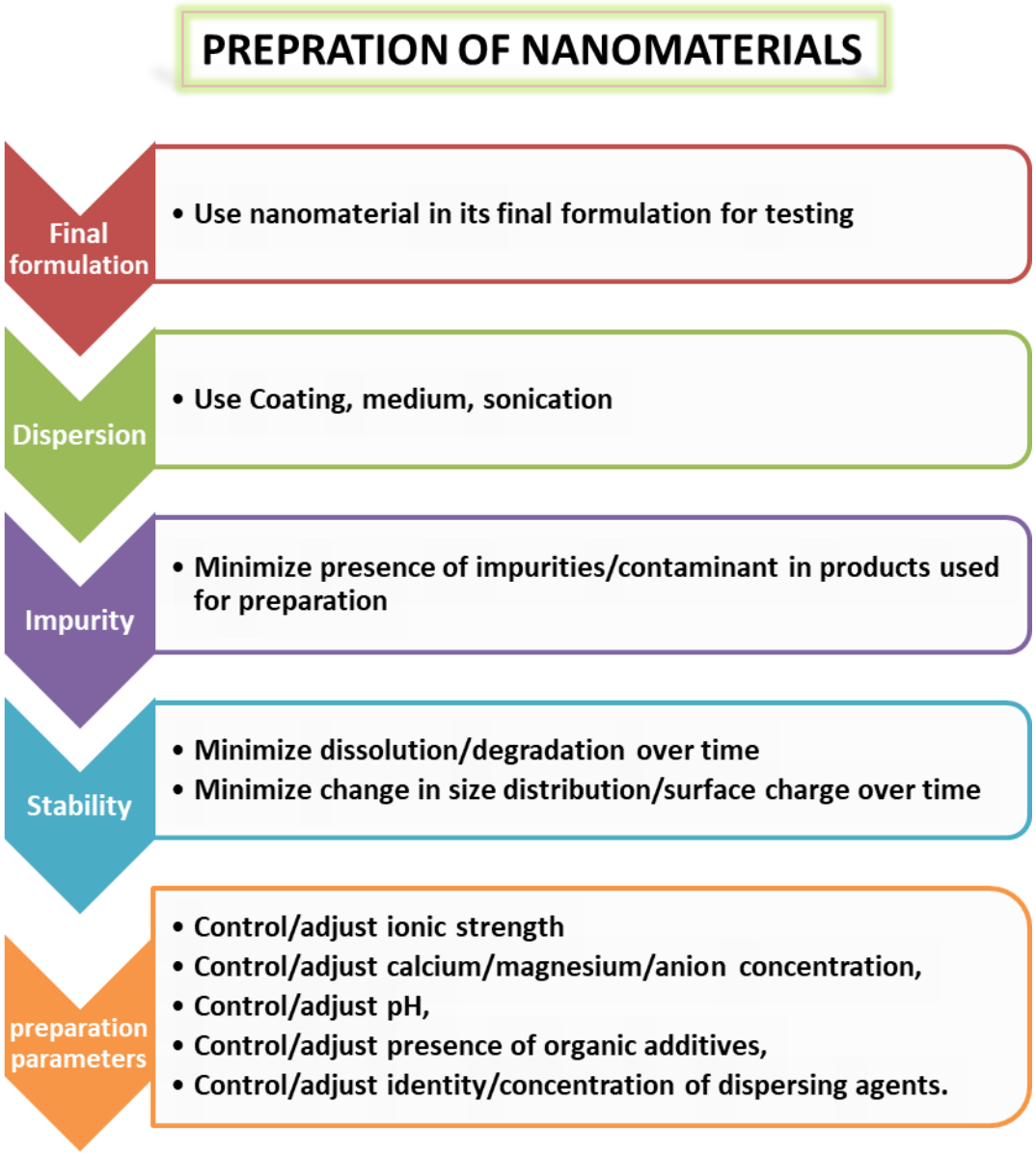
PREPRATION OF NANOMATERIALS
Final formulation
• Use nanomaterial in its final formulation for testing
Dispersion
• Use Coating, medium, sonication
Impurity
• Minimize presence of impurities/contaminant in products used for preparation
Stability
• Minimize dissolution/degradation over time
• Minimize change in size distribution/surface charge over time
preparation parameters
• Control/adjust ionic strength
• Control/adjust calcium/magnesium/anion concentration,
• Control/adjust pH,
• Control/adjust presence of organic additives,
• Control/adjust identity/concentration of dispersing agents.

Figure 3

678

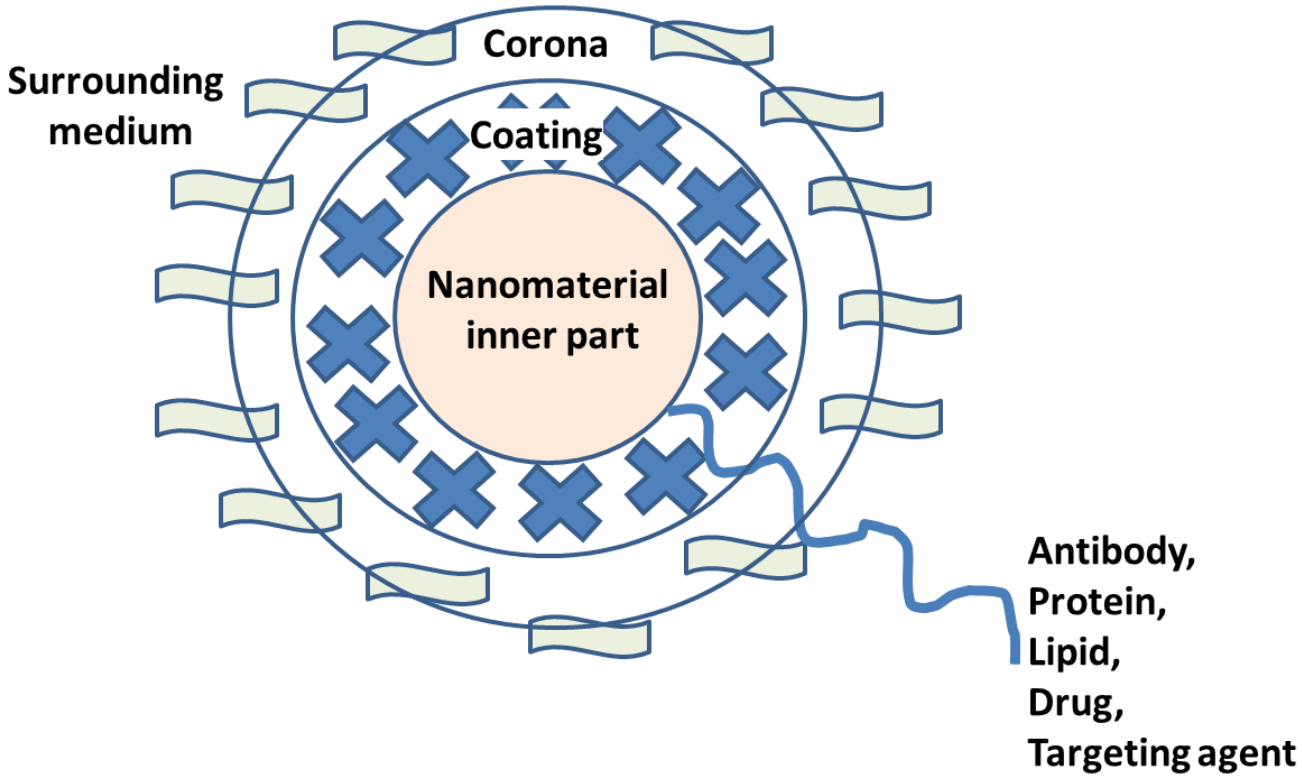
Surrounding medium
Corona
Coating
Nanomaterial inner part
Antibody,
Protein,
Lipid,
Drug,
Targeting agent

Figure 4

679

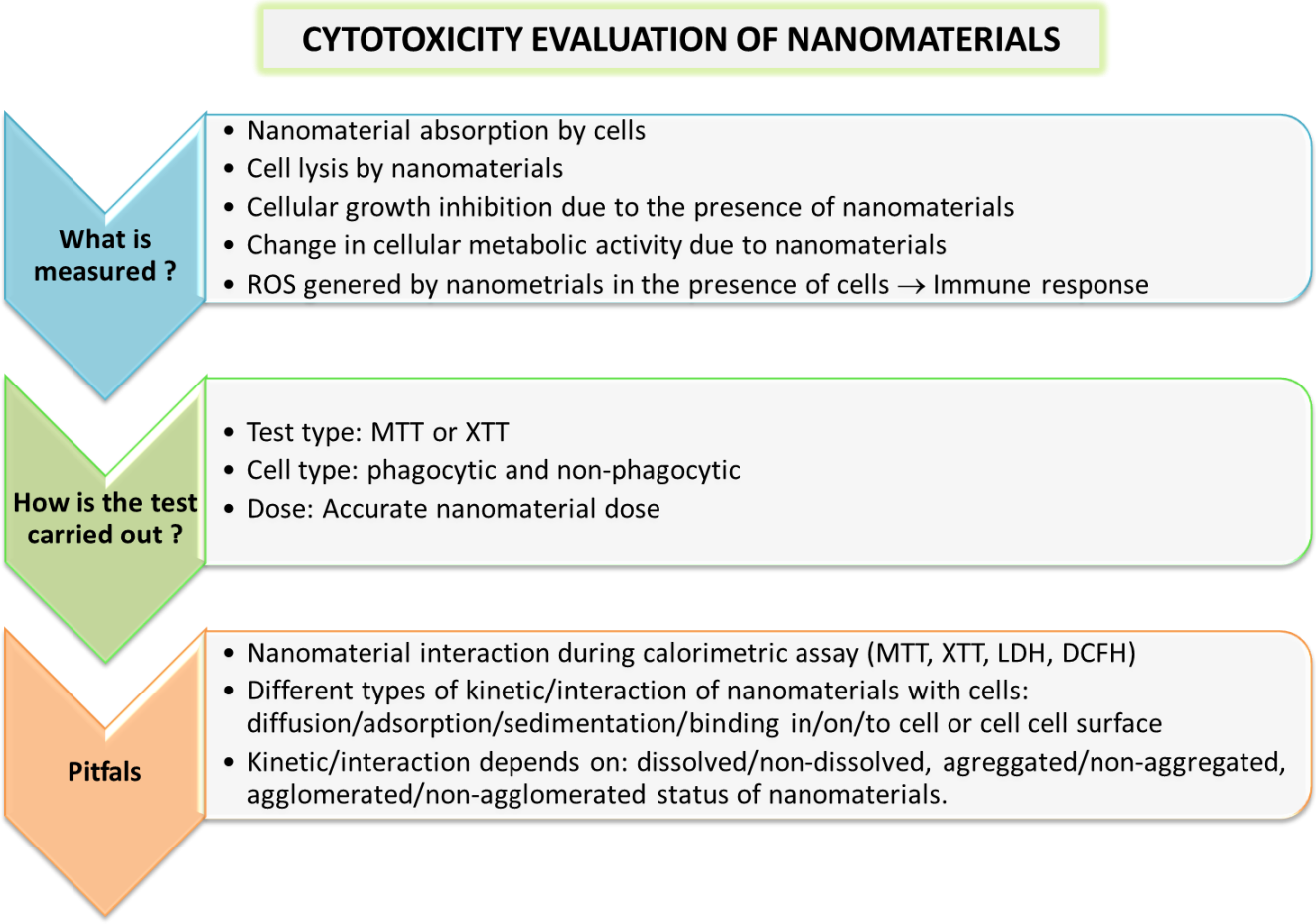
CYTOTOXICITY EVALUATION OF NANOMATERIALS
What is measured ?
• Nanomaterial absorption by cells
• Cell lysis by nanomaterials
• Cellular growth inhibition due to the presence of nanomaterials
• Change in cellular metabolic activity due to nanomaterials
• ROS genered by nanometrials in the presence of cells → Immune response
How is the test carried out ?
• Test type: MTT or XTT
• Cell type: phagocytic and non-phagocytic
• Dose: Accurate nanomaterial dose
Pitfals
• Nanomaterial interaction during calorimetric assay (MTT, XTT, LDH, DCFH)
• Different types of kinetic/interaction of nanomaterials with cells: diffusion/adsorption/sedimentation/binding in/on/to cell or cell cell surface
• Kinetic/interaction depends on: dissolved/non-dissolved, agreggated/non-aggregated, agglomerated/non-agglomerated status of nanomaterials.

Figure 5

680

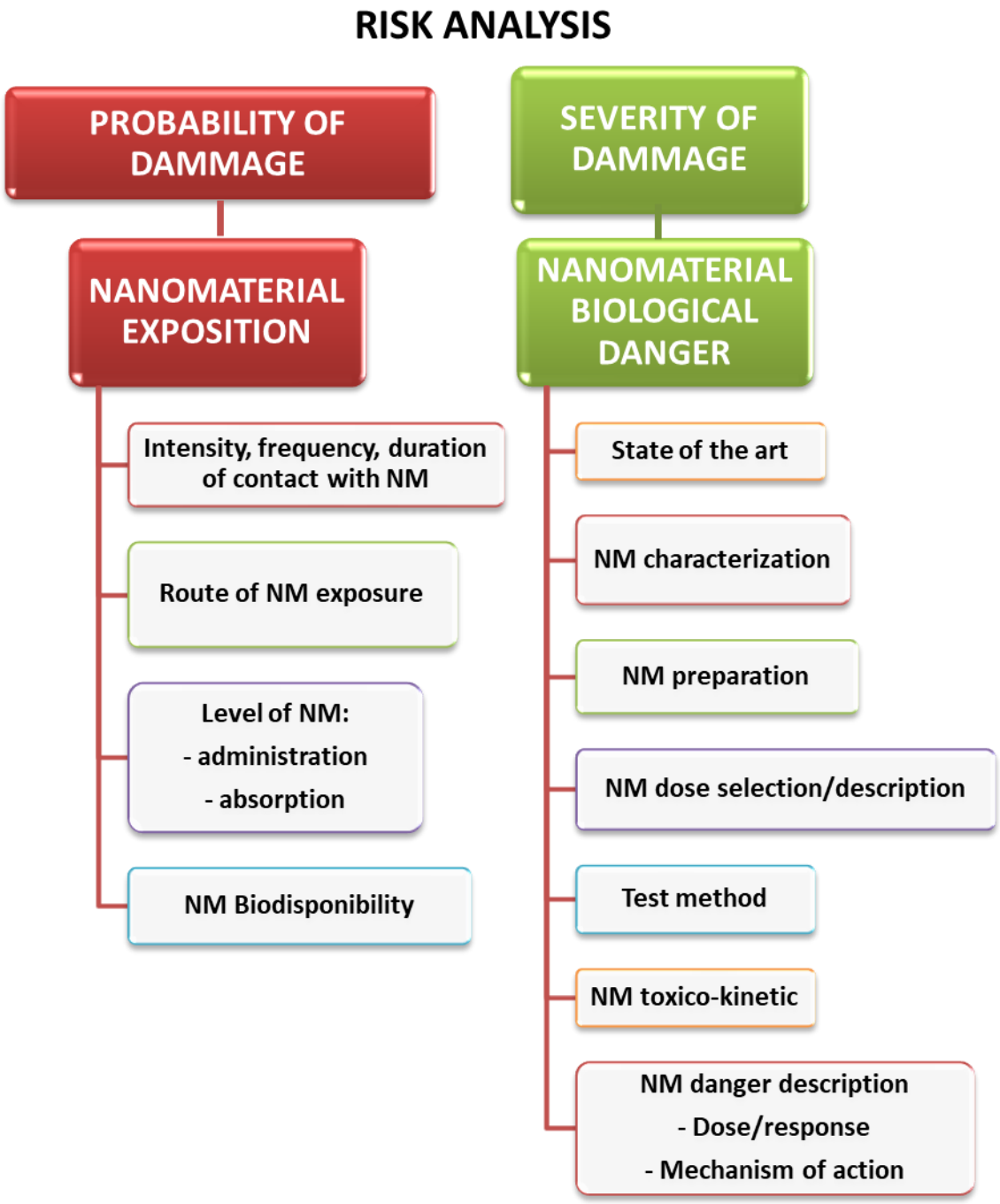
RISK ANALYSIS
PROBABILITY OF DAMMAGE
SEVERITY OF DAMMAGE
NANOMATERIAL EXPOSITION
NANOMATERIAL BIOLOGICAL DANGER
Intensity, frequency, duration of contact with NM
Route of NM exposure
Level of NM:
- administration
- absorption
NM Biodisponibility
State of the art
NM characterization
NM preparation
NM dose selection/description
Test method
NM toxico-kinetic
NM danger description
- Dose/response
- Mechanism of action

Figure 6

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CHARACTERIZATIONS OF NANOMATERIALS (NM)				
Characterization method	**Principle**	**Advantage**	**Drawback**	**Quantity measured**
Analytical centrifugation (AC)	NM exposed to centrifugal force → sedimentation depends on NM size	High resolution Large range of measured sizes (1-5000 nm)	Non uniform chemical composition → uncertainty in measurement	Velocity of sedimentation under centrifugal force
Auger electron spectroscopy (AES)	Excitation of NM with electron beam → Determines NM surface properties	High spatial and surface resolution	Not possible with non-conductive and/or contaminated surface.	NM surface composition
Aerosol particle mass analyser (APMA)	Aerosol NM exposed to centrifugal force in the the presence of an applied DC voltage	Determination of concentration as a function of mass	Applicable only to NM in air	Number/concentration of NM as a function of mass/charge.
Acoustic spectroscopy (AS)	Ultrasound (velocity/attenuation) measured as a function of frequency	Measures aggregated/non aggregated NM	Limited measured size range (3-10 mm)	Particle size distribution
Brunauer-Emmet-Teller (BET) method	Measurement of adsorbed gaz at NM surface	High resolution	Applicable to NM in powder NM heated → aggregation	NM Surface area
Condensed particle counter (CPC)	Growth of NM to droplets by condensation in supersaturated vapour → measured optically	time-dependent measurement possible	Applicable only to NM in air	NM number/concentration
Dynamic light scattering (DLS)	Light scattering of NM prone to random motion in liquid.	Reproducible, rapid, non-destructive, non-invasive	NM (Non-spherical or with broad size distribution) can't be measured	NM hydrodynamic size
Differential mobility analysis system (DMA)	NM flowing in a chanel exposed to DC voltage (NM velocity depends on NM size/concentration)	time-dependent measurement possible High resolution	Applicable only to NM in air	NM concentration, size, size distribution
Differential scanning calirometry (DSC)	Energy absorebed by a NM when the NM is heated	Easy operating conditions	The rate/speed of temperature increase can be limited depending on the equipment.	Heat capacity, specific heat of a NM.

Table 1-1

682

CHARACTERIZATIONS OF NANOMATERIALS				
Characterization method	**Principle**	**Advantage**	**Drawback**	**Quantity measured**
Energy dispersive X-ray spectrometry (EDS/EDX and WDS)	NM exposed to electron beam → individual photons	Precise composition determination	Requires treated surface (conductive/polished) and standards.	NM composition
Electron energy loss spectroscopy (EELS)	NM exposed to electron beam → inelastic interactions → measurement of resulting energy spectrum.	High spatial resolution (electron beam size)	Requires very thin samples and combination with TEM	NM Structure/oxidative state/composition
Electrophoresis Electrophoretic light scattering Capillary electrophoreis	Different velocities of charged NM in an electric field.	Does not necessarily require calibration	Only for NM in liquid.	Electrokinetic potential/electrophoretic mobility
Electroacoustic velocity (EV)	Measures electric field generated by ultrasound field Measures ultrasound field generated by electric field.	Measure aggregated/non-aggregated NM Rapid measurement time/ small sample volume	Only for NM in liquid.	Electrokinetic potential
Field flow fractionation (FFF)	Electric field applied to NM in liquid suspension	Can separate NM with broad size distribution	Only for NM larger than 2-5 nm. Only for NM in liquid	NM size, size distribution, concentration
Fluorescence spectroscopy (FS)	Emission of light by NM exposed to UV/visible light	Can go down to single NM measurement	Interference due to background or light scattering	Intensity versus wavelength (quantum yield)
Fourier transform infrared (FT-IR) spectroscopy/imaging	Excitation of NM molecular bonds by infrared spectrum → absorption spectrum	High resolution (well-defined peak at given wavelength, large signal to noise ratio)	Difficult to attribute a peak to a chemical function (several functions can have the same peak position).	nanomaterial surface composition
Inductively coupled plasma-mass spectrometry (ICP-MS)	Ions produced at high temperature/pressure → detected using mass spectrometer	High sensitivity (ng/l)	Requires specific preparation	NM concentration, size, size distribution Trace element concentration

Table 1-2

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CHARACTERIZATIONS OF NANOMATERIALS				
Characterization method	**Principle**	**Advantage**	**Drawback**	**Quantity measured**
Induced grating method (IG)	Measurement of NM size using dielectrophoresis and light diffraction	Sensitive to size NM	Only for NM in liquid Limited size range	Hydrodynamic diameter
Liquid chromatography-mass spectroscopy (LC-MS)	NM in liquids are separated by LC and analyzed by MS	Commonly used	Only detects organic material	Organic ligands attached to NM
Laser diffraction (LD)	Light scattering by NM depends on nanomaterial size	Can be used for NM in liquids or powder Characterizes individual NM/aggregates	Does not work on very small or very large NM	Size, size distribution
Particle tracking analysis (PTA)	NM in solution exposed to laser light to monitor NM movement and deduce NM size	Requires small sample volume	Not adapted to non-spherical NP. More tedious than DLS	Size, size distribution, number, concentration, electrokinetic potential
Quartz crystal microbalance (QCM)	NM on quartz crystal → Change in frequency of quartz resonatior → change in NM mass	Small sample required	Sensitive to environmental conditions	NM mass with high resolution
Resonant mass measurement (RMM)	Change in frequency of a cantilever connected to NM flowing through a microfluidic channel.	Characterizes protein aggregation Distinguishes between proteinaceous and non-proteinaceous material	Can't measure particles with the same density as the liquid	NM buoyant/dry mass, NM size.
Raman spectroscopy/Imaging (RSI)	NM exposed to radiation → energy loss/gain from rotational, vibrational, phonon excitation	Non destructive	Difficult to detect signal → requires specific skills for detection	NM crystal property/composition
Small angle X-ray scattering (SAXS)	NM exposed to X-rays → measurement of elastically scattered signal	Non destructive, Wide concentration range.	Not suitable for polydisperse NM	Size, size distribution, shape, aggregation/agglomeration state
Scannng electron microscopy (SEM)	NM exposed to primary electrons → emission of secondary electrons	High resolution, 2-3 dimensional imaging.	Consudtive NM surface required, Works in vacuum, Requires special skills.	Structure, composition, topography, size, size distribution, composition of NM

Table 1-3

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Characterization method	Principle	Advantage	Drawback	Quantity measured
Secondary ion mass spectroscopy (SIMS)	NM exposed to ions → production of secondary ions	Identification of trace elements, 3D elemental distribution.	Vacuum necessary for measurement. Frequent sample contamination	Unified atomic mass unit
Static light scattering (SLS)	NM exposed to laser light → Time-average intensity of elastically scattered light measured.	Real time measurements, braoad range of sizes (nm to mm), no sample preparation required	Difficult to use with strongly absorbing NM (e.g. metalic ones)	Size, concentration
Single particle light interaction methods (SPLI)	Analysis of scatterd light by a single NM to deduce concentration, size, size distribution of NM	High size resolution; Real time measurement	Applicable to NM in gaz or liquid	NM concentration, size.
Scanning probe microscopy (SPM)	Imaging of conductive surface of NM	Topologycal information of NM surface, no complicated sample preparation.	NM immobilization may be required, Difficult to distinguish between aggregates/agglomerates	Size, size distribution, shape of NM.
Transmission electron microscopy (TEM)	NM exposed to electron beam → electron diffraction pattern	Imaging at sub-nm scale	Results depend on sample preparation (NM concentration)	NM size, shape, size distribution, electron diffraction spectra
Thermogravimetric analysis (TGA)	Change in mass of NM measured as a function of NM temperature	Wide range of temperature available (up to 1000 °C)	More than 1.5 mg of NM required	Chemical composition
Optical absorption spectroscopy (UV/Vis/NIR)	Absorbance of electromagnetic radiation by NM as a function of absorption wavelength	Rapid/easy measurements Very useful to assess the stability of NM in suspensions	Often does not give a direct access to NM properties (requires a pre-calibration)	Absorption spectrum → NM concentration, size, chemical composition
X ray photoelectron spectroscopy (XPS)	NM exposed to X-ray → Energy distribution of photon/Auger electrons emitted by NM	Limited analysis depth Chemical state/bonding information	Surface contamination can complicate measurement	NM Concentration
X-ray diffraction (XRD)	Diffraction pattern when NM exposed to X-ray beam	Non destructive, gives crystallite size information	Time consuming, requires large amount of sample	NM crystal property, size, atomic structure

Table 1-4

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EXAMPLES OF METHODS RECOMMENDED FOR CHARACTERIZING NANOMETARIALS (NM)	
Measured NM parameter	**Method used to characterize nanomaterial parameter**
Chemical composition and purity	UV/Vis/NIR, AES, RSI, TGA, MEB, RMN, ICP-MS, EDS/EDX.
Size/Size distribution	DLS, MEB, TEM.
Aggregation/agglomeration	AES, SEM, SPM/AFM, SAXS, TEM
Shape	AES, SEM, SPM/AFM, SAXS, TEM
Surface area	BET
Concentration	AES, CPC, DMA, FFF, ICP-MS, LC-MS, UV/Vis/NIR, SEM, TGA, TEM, XPS
Crystal properties	TEM, XRD, SEM.

Table 2

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