Basis for the toxicological evaluation of engineered nanomaterials

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Abstract: The increasing annual production of nanomaterials enhances the human and environmental exposure, as well as the possible impact on public concern. In order to regulate the production, international organizations such as the Organization for Economic Cooperation and Development (OECD) and the European Commission through the REACH regulation have established some measures to assess its health and environmental safety. This review tries to analyze these proposed measures according to the standard test used in toxicology, the different classification of nanomaterials and the main mechanisms of toxicity known for nanomaterials. Resulting from this analysis, it is considered convenient to continue the development of specific tests for nanomaterial evaluation, as the measures established by the organizations of reference are not enough to establish standard basis for testing nanomaterials. This is mainly due to the huge diversity of manufactured nanomaterials and the importance of its handling, techniques and experimental systems chosen in the toxicity results.

Keywords: Nanotoxicology, toxicology, nanoparticle, risk

Resumen: Bases para la evaluación toxicológica de los nanomateriales manufacturados El aumento de producción anual de nanomateriales eleva la exposición humana y ambiental, y tiene un posible impacto en la opinión pública. Con el fin de reglamentar esta producción, organizaciones internacionales como la Organización para la Cooperación y Desarrollo Económico (OECD) y la Comisión Europea a través de la reglamentación REACH han establecido algunas medidas para evaluar la seguridad para la salud y el medio ambiente de los nanomateriales. Esta revisión intenta analizar las medidas propuestas por estas instituciones de acuerdo con las pruebas estándar usadas en toxicología, las distintas clasificaciones de los nanomateriales y los principales mecanismos de toxicidad conocidos de los nanomateriales. Como resultado de este análisis se cree conveniente continuar desarrollando tests específicos para la evaluación de nanomateriales, ya que las medidas establecidas por las organizaciones de referencia no son suficientes para conseguir unas bases estándares para testar nanomateriales. En gran parte esto es debido a la gran diversidad de nanomateriales existentes y la importancia de la manipulación, técnicas y sistemas experimentales escogidos en los resultados de toxicidad.

Palabras clave: Nanotoxicología, toxicología, nanopartícula, riesgo

Introduction

This review arises from the increasing production and subsequent human and environmental exposure to nanomaterials, and its possible impact on public concern. In order to regulate this production, international organizations such as OECD and REACH have established some measures to assess its environmental and health safety.

Engineered nanomaterials (ENM) production is increasing each year. The use of nanotechnology in different fields such as electronic components, cosmetics (sunscreen…), filters, sprays, cleaning products and biomedicine (drug delivery, medical imaging, diagnostics or medical device) is increasing [1-5]. ENMs have an estimated annual turnover in the range of 1.1-2.5 trillion US dollars by 2015 [5]. Today, ENMs can be found in more than 800 consumer products (Woodrow Wilson International Centre for Scholars, http://nanotechproject.org) and it is expected that nanotechnology will be utilized in more commercial products in the near future[4,6]

However, should consumers be worried about this increase in production? Society is already asking to the scientific community which is the real risk-benefit balance for the use of ENMs. It is well-known that anything can be toxic at a high enough dose, but as scientific our main question should be: how toxic are ENMs at the potential concentrations at which they might be used?

Unfortunately, the assessment of toxicity of ENMs (nanotoxicology) continues under development. Recently, the Organization for Economic Cooperation and Development (OECD) recognized that the Test Guidelines were not specifically designed for the testing of ENMs and the guidance provided on these guidelines regarding preparation, delivery of test substances to test system, exposure quantifications, dose metrics, measurement, and metrology in all of these test guidelines is considered to be insufficient for testing ENMs. In addition, there are currently no final conclusions on the best metric to use although often mass or particle number are been used. However, the existing guidance on toxicological data information requirements is considered applicable for the assessment of ENMs. Just a new section is recommended to address sample preparation issues applicable to the determination of all properties and endpoints and further recommendations include the evaluation for the suitability of existing methods [7]. Nevertheless modifications of such protocols are in some cases required for ENMs [8]. Di Guglielmo reported the need to modify “classic EST” (ECVAM-European Centre for the Validation of Alternative Methods –validated protocol INVITTOX no.113) because it was noticed that the nature of NP suspension prevented the normal development and attachment of the embryonic bodies to the plate surface following day 5, alternatively a new method was proposed and validated [9]. Furthermore the great variability between ENMs makes difficult to establish a consensus about which methods are really useful to test their toxicity. Any toxic effect caused by an ENM will be specific to
the type of material, size, shape and/or coating among other parameters [10].

In order to define the best strategy to test nanoparticles (NPs), the OECD is currently spearheading a coordinated strategy focused on an initial selection of ENMs and characterization properties [11]. In fact, the use of reference materials to validate and evaluate potential tests have been discussed by several organizations such as the European Commission within different projects such as NANOReg (http://nanoreg.eu/), the Institute of Occupational Medicine within REFNANO or the Engineer Research and Development Center and National Institute of Standards and Technology (ERDC-NIST) [8]. Nowadays several initiatives such as National Institute of Standards and Technology (NIST, USA) and Joint Research Centre—Institute for Reference Materials and Measurements (JRC-IRMM, European Commission) are developing such materials [11].

The main objective of this review tries to complement these measures according to the standard test used in toxicology, the different classification of ENMs and the main mechanisms of toxicity found for ENMs.

**Classification of engineered nanomaterials**

ENMs are defined by the U.S. National Nanotechnology Initiative as materials that have at least one dimension in the 1- to 100-nm range. However, there is no clear size cut-off and the 100 nm boundary appears to have no solid scientific basis [12]. Indeed, research carried out with particles above 100nm also uses the term nano.

Nano-sized materials can be originated from natural sources such as forest, fires and volcanoes, in viral particles and even protein molecules such as ferritin; and from unintentionally and intentionally anthropogenic sources such as combustion by-products, and ENMs, respectively [13].

Several authors have provided a classification for ENMs [8,10,14]. But there is no consensus and legislation has neither provided a clear suggestion.

In order to study the mechanisms of toxicity derived from exposure to ENMs with different properties, NPs have been classified according to its size, morphology, composition, agglomeration state and uniformity [15]. Morphology have been approached depending on its aspect ratio: ENMs with a high aspect ratio are nanotubes and nanowires, while spherical NPs have a low aspect ratio. The state of agglomeration and uniformity are strongly dependent on its chemistry composition and electromagnetic properties (e.g. magnetic NPs tend to cluster and behave as bigger particles) [15].

Due to the great heterogeneity, ENMs come be classified in general groups in accordance of its chemical composition, shape, size and surface (Table 1).

**Characterization of engineered nanomaterials**

ENMs are quite complex. For instance, evaluation, generalization, and prediction of important toxicological aspects derived from exposure to nanomaterials is extremely difficult [16]. In order to

<table>
<thead>
<tr>
<th>Table 1. Classification for engineered nanomaterials</th>
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![Classification Diagram](image)

SWCNT: Single-Walled Carbon NanoTube; MWCNT: Multi-Walled Carbon Nanotube
assess the potential (eco)toxicity of ENMs, there is consensus that representative sample preparation and accurate physico-chemical characterization are essential. Thus, NP sizing standards, as well as standardized methods for sampling and measurement, are urgently required to overcome the problem of inconsistent data. In general, the guidance on physic-chemical properties is considered to be applicable to ENMs. But in fact, current standard guidelines need to be adjusted for the testing of ENMs and/or additional tests have to be carried out to avoid misinterpretation of results. Some of these guidelines have limited relevance and applicability, such as the property and methods for surface tension, flash point and viscosity. On the other hand, further evaluation on the suitability of existing methods for other properties such as water solubility, partition coefficient, and adsorption/desorption has been recommended (see Table 2) [7].

Table 2. Physico-chemical properties relevant to characterize ENMs according to RIP-oN 2.

<table>
<thead>
<tr>
<th>Differences between nano and non-nanomaterials</th>
<th>RELEVANT TO NANOMATERIALS</th>
<th>NOT RELEVANT TO NANOMATERIALS</th>
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<tbody>
<tr>
<td>Guideline status</td>
<td>Suspected important differences</td>
<td>Guideline updates recommended</td>
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<tr>
<td>Water solubility</td>
<td>Granulometry</td>
<td>Purity</td>
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<tr>
<td>Flammability</td>
<td>Adsorption/desorption</td>
<td>Elemental composition</td>
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<tr>
<td>Explosive properties</td>
<td>Partition coefficient</td>
<td>Surface chemistry</td>
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<td>Porosity</td>
<td>N-octanol/water</td>
<td>Cell-free ROS/RNS production capacity</td>
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<tr>
<td>Surface energy</td>
<td>Dissociation constant</td>
<td>Surface tension</td>
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<tr>
<td>Surface acidity</td>
<td>Particle shape/Aspect ratio</td>
<td>Flash point</td>
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<td>Surface charge</td>
<td>Surface area</td>
<td>Viscosity</td>
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<td>Redox potential</td>
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<td>Melting point/Tefloning point</td>
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<td>State of dispersion</td>
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<td>Boiling point</td>
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<td>State of aggregation/aggleromeration</td>
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<td>Relative density</td>
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<td>Dustiness</td>
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<td>Vapour pressure</td>
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<tr>
<td>Crystallinity/crystal phase</td>
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<td>Stability in organic solvent</td>
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<td>Primary particle size /</td>
<td></td>
<td>and degradation products</td>
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<tr>
<td>Particle distribution</td>
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<td>Self-ignition temperature</td>
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<tr>
<td>ROS (Reactive Oxygen Species); RNS (Reactive Nitrogen Species)</td>
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When comparing the intrinsic properties relevant to ENMs in accordance with specific advice on requirements for nanomaterials under REACH (RIP-oN 2) and preliminary guidelines by OECD, with one of the first initiatives in the field of characterization of ENMs [17], it is possible to appreciate that the list of intrinsic properties that can have a role in the (eco)toxicology response of ENMs has not decreased. Bucher et al suggested that it was essential to focus on specific physicochemical properties including: size, shape, surface area, surface porosity, roughness, crystallinity, solubility, chemical composition, surface chemistry and reactivity; together with images, dispersibility and dosage [17]. Table 3 compares the priority properties to characterize ENMs in media proposed by different authors then, and the recommended in the current guideline. 

Whilst characterization of ENMs as-produced or as-supplied is the most direct and currently realistic approach to obtain physico-chemical information about the material being studied, this data may not appropriately represent the properties of the material when in contact with the environment in which is being observed (air or physiological environments). It has been suggested that adequate particle characterization should be performed in 3 distinct phases: 1. On particles as-synthesized or as-received in its dry native state; 2. On particles in the wet phase as a solution or suspension in aqueous media; 3. On particles following interactions with cells under in vivo or in vitro conditions [7].

The huge list of recommended physico-chemical properties for ENMs described in RIP-oN 2 together with the fact that no individual technique can satisfy a meaningful characterization of ENMs, and for instance multiple techniques should be used, did not allow a complete standardized characterization of the ENMs being tested. In addition, the required techniques should be selected based on the specific ENM type and form under investigation.

RIP-oN 2 evaluates the current REACH regulation in order to reflect if it can be used for ENMs. The conclusion is that an ENM cannot be properly characterized with the data normally required under REACH regulation. As above mentioned, some properties are not relevant for ENMs, and others commonly not considered may have a role in assessing its toxicity. Table 4 reflects the herein suggestion of properties relevant to be characterized in any toxicological study for ENMs, taking into account RIP-oN 2 guideline and other scientific advice.

Water solubility is considered a significant relevant property for ENMs compatible with determining state of agglomeration (as at strong agglomeration, the material would be considered not dispersible, analogue of “not soluble”) in accordance with RIP-oN 2 advices. Currently available standard methods for measuring dissolution may not be applicable. OECD concluded that the test guideline relevant to characterize water solubility might be applicable under some circumstance or to some classes of manufactured ENMs, whereas ISO guidance should be reviewed.

Table 3. Comparison of physicochemical properties relevant to be characterized for ENMs in accordance with guideline 2011 (RIP-on 2) and the first scientific initiatives in 2004

<table>
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<tbody>
<tr>
<td>Size distribution</td>
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<tr>
<td>Agglomeration state/dispersion</td>
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<tr>
<td>Crystal structure</td>
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<td>Chemical composition</td>
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<td>Surface area and Porosity</td>
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<tr>
<td>Surface chemistry</td>
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<tr>
<td>Surface charge</td>
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<tr>
<td>Shape and morphology</td>
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<tr>
<td>Dissolution/Solubility</td>
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<td>*</td>
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<tr>
<td>Physical/chemical properties (purity)</td>
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<td>*</td>
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<tr>
<td>Methods of synthesis</td>
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<td>Flammability</td>
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<td>Explosive properties</td>
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<td>Gransometry</td>
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<tr>
<td>Adsorption/desorption</td>
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<td>Partition coeff (n-Octanol/water)</td>
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<td>Dissociation constant</td>
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<td>Melting/Freezing/Boiling point</td>
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<td>Relative density</td>
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<td>Vapour pressure</td>
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<td>Self-ignition temperature</td>
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<tr>
<td>Oxidizing properties</td>
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<tr>
<td>Stability in organic solvent and degradation products</td>
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<tr>
<td>Surface energy</td>
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<td>Surface acidity</td>
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<td>Redox potential</td>
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<td>Dispersity</td>
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<tr>
<td>Cell-free ROS/RNS production capacity</td>
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ROS (Reactive Oxygen Species); RNS (Reactive Nitrogen Species)

Table 4. Suggested parameters to be characterized

<table>
<thead>
<tr>
<th>Parameter</th>
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<tbody>
<tr>
<td>Particle size and Size distribution</td>
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<tr>
<td>Agglomeration state</td>
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<tr>
<td>Chemical composition</td>
</tr>
<tr>
<td>Surface area</td>
</tr>
<tr>
<td>Surface chemistry</td>
</tr>
<tr>
<td>Surface charge</td>
</tr>
<tr>
<td>Shape</td>
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<tr>
<td>Water Solubility</td>
</tr>
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</table>

However, it should be noted that measuring solubility is different from dissolution, since dissolution is the process by which a solid or liquid forms a homogeneous mixture with a solvent (when characterizing NPs this can be related to size distribution and/or state of agglomeration) whereas solubility is the maximum equilibrium amount of solute that can be dissolved per amount of solvent [17].

Particle size is a key factor of the potential toxicity of ENMs. Decreasing size leads to an exponential increase in surface area relative to volume, thus the ENM surface becomes more reactive on itself (aggregation) and to its surrounding environment (biological components). There is a known relationship between primary particle size and deposition and translocation potential. Particles below 10 nm present high deposition (>80% retained in the human respiratory tract if inhaled), particles between 10 and 30 nm present a moderate deposition (60-80% deposited in the human respiratory tract), while above 30 nm particles show low deposition (less than 60% are retained). Regarding translocation potential, it is high for particles below 2.5 nm and low above 5 nm. Translocation to other organs was also strongly dependent on both the size and surface area [7]. For instance, particle size is important for the response, distribution and elimination of the materials in organisms [16, 19]. It can affect the mode of endocytosis, cellular uptake, and the efficiency of particle processing in the endocytic pathway [3, 23]. In vitro studies of non-phagocytic cellular uptake of latex spheres have demonstrated slower uptake and processing of large spheres (200 nm) relative to small ones (50 and 100 nm) [23]. Increased uptake into certain tissues may lead to accumulation, where they may interfere with critical biological functions [3, 24]. Measured particle size values should be regarded as “method-dependent”. Because different methods based on different measurement principles, may yield to different results when measuring the same nano-object or structural feature. It is recommended that multi-analytical techniques and/or multiple preparation techniques should be used when characterizing NPs (because no single technique can be considered to be without artifacts or can be employed in all cases when determining particle sizes). The appropriate measurands for this property are both average size of individual particles and size distribution of the sample of particles.

The great effort to achieve non aggregated/agglomerated ENM in the test media, makes us wonder the relevance to study monodispersed ENMs in human and environmental exposure. It has been suggested that the state of agglomeration is important to assess the potential toxicity of ENMs. Aggregation behavior in the media might follow a non-linear concentration-aggregation relationship. NP-NP interactions can be affected by relevant parameters such as surface energy, surface charge and salvation.

Increased surface area relates to increased potential for biological interaction. The reduction in size to the nanoscale is accompanied by an inherent increase in the surface-to-volume ratio, and therefore a greater proportion of entities at the surface compared to the bulk (non-nanoscale) material. Effects correlate with surface area to a greater extent than mass as a dose metric.

It is essential to measure zeta potential (surface charge) as a function of pH because this allows the determination of the point of zero charge, where a dispersion of ENMs exhibits the highest propensity to aggregate. Thus, the value of zeta potential can be related to the stability of NP dispersions. The surface charge of NPs has been reported to influence particle uptake and interaction with exposed cells. In addition, size and charge can influence the adsorption of ions, contaminants and biomolecules.

It is suggested that shape/aspect ratio is an important property to characterize in relation to NP toxicity and fate, because it may play a role in effective clearance. Longer fibres promote the development of frustrated phagocytosis, reduced clearance and the potential to persist and to increase their propensity for damage. Champion and Smitragotri have described macrophage interaction with different shaped ENMs [25].

The nature of the surface functionalization and coatings, commonly called surface chemistry, is another relevant property to characterize as when modified: agglomeration, dustiness, zeta potential, surface area and water solubility can be altered. This property can define their chemical interactions since the surface is in direct contact with the cell or organism whereas the limited bulk volume is hidden [16]. The presence of hydrophilic surface groups on the surface of NPs may be used as an indicator of their reactivity (in relation to their potential uptake and bioaccumulation), being particles with hydrophilic...
surface groups highly reactive, and particles without reactive surface
groups low reactive. Many ENMs are functionalized on the surface
for target therapy or to increase blood circulation time or,
biocompatibility. While functionalization has shown promise in
many applications, functional groups added to the surface can
potentially interact with biological components, alter biological
function, and allow passage of ENMs that would not normally be
taken up by certain cells [26].

Degradability is also important when studying acute and long-term
toxicity of nanomaterials. Non-degradable ENMs can accumulate in
organs and also inside cells where they can cause detrimental cell
effects, similar to lysosomal storage diseases [2]. In contrast,
biodegradable ENMs can lead to unpredictable toxicity due to
unexpected toxic degradation products [27]. ENMs may contain
transition metals (e.g. quantum dots) or other compounds with known
toxicity, which are “masked” by functionalization. Degradation of
this material may release toxins to the biological media, leading to
free radical formation and resulting in cellular damage [3,27].
Dissociation constant is a particularly relevant aspect according to
OECD because ionizable sites may influence surface charge playing
a major role in colloidal particle stability and it may inhibit migration
into hydrophobic phases. This property is applicable to solutions but
it is not known in colloidal suspension.

Other properties mentioned in REACH guidelines (RIP-oN 2) can be of
interest and should be provided when possible such as crystallite
size, dustiness, boiling/melting point, relative density or explosivity
and flammability among several others characteristics. But the main
objective of this review is to highlight which standard properties
should be characterized in order to compare results between studies.

Despite ENMs have been completely and properly characterized for
its intrinsic properties, it is not enough. Sample preparation and
dosimetry are also of key importance when comparing obtained
results in different studies. It is important to take into account the
storage and stability of the test material, the dispersion protocol,
the chemical composition of the test media, the exposure method,
the light conditions, the dose selected, the species used or the cell type
under investigation, and the characterization of samples prior to
administration/testing. All these factors can potentially impact on the
toxicity of ENMs.

Different processes for dispersion of NPs can be used: solvents,
surfactants, proteins and mechanical process (centrifugation and
sonication). However, the use of sonication to disperse NPs in
solution has the potential to change the size distribution of NPs and
introduce defects, while the use of surfactants can reduce cell
viability directly at all concentrations. Stability can be controlled by
determining the state of agglomeration and zeta-potential. And the
chemical composition of the test media can be studied regarding its
ionic strength, calcium concentration, hardness, pH, dissolved
organic matter, alkalinity and dispersing agents [7]. Similarly, and
despite it is not specifically mentioned in RIP-oN 2 guideline, the
form of supply of NPs, mainly powder or dispersion forms, can also
have an effect on sample preparation. Generally dispersions are
considered to be relatively more stable, but powder forms would be
most appropriate as they are usually freshly prepared just before
exposure, however in some cases they can lead to problems of
dispersion and reproducibility. This difference in ENM preservation
is particularly important in the case of ENMs that may dissolve, and
thus may be unsuitable for storage as stock solutions. In addition,
dispersions/suspensions can be used at a limited concentration range,
and usually needs additional substances to stabilize the dispersions
[8].

Prioritization of engineered nanomaterials

The lack of consistent reference materials and standards further
exacerbates the inconsistent data results. Some progress has been
made recently regarding reference materials for characterization.
Furthermore, it is essential to prioritize the order in which ENMs
should be approached, and to use reference materials to validate and
compare test results [8].

Several criteria may be applicable for prioritization. Some
researchers demonstrated a strong interest in ENMs that have been
deeply studied in human toxicology models, in order to provide a
comparison between species; whilst others argued against this point
because some materials may have a lower relevance in the
environment. Another criteria emphasized that such materials may
provide ease of detection against large background of natural
particles, organic matter, or trace metals in many environmental
samples [8]. The OECD has provided a list of representative
manufactured ENMs for testing in a communication, taking into
account those materials which are already in commerce (or close to
commercial use), and other criteria such as production volume, the
likely availability of such materials for testing and the existing
information that is likely to be available on such materials. The
materials comprised in this list will become a set of reference
materials to support measurement, toxicology and risk assessment
of ENMs. This OECD list published on November 2012 is considered as
a “snapshot in time”, at the moment it comprises fullerenes (C60),
single- and multi-walled carbon nanotubes (SWCNTs and
MWCNTs, respectively), silver NPs (Ag), iron NPs (Fe), titanium
dioxide (TiO2), aluminium oxide (Al2O3), cerium oxide (CeO2), zinc
oxide (ZnO), silicon dioxide (SiO2), dendrimers, nanoclays, and gold
NPs (Au) [28]. In February 2011 the Institute for Health and
Consumer Protection (IHCP) inaugurated a repository of
representative ENMs, which can serve as international benchmarks.
Currently, the repository brings together the principal ENMs from the
OECD sponsorship program including titanium dioxide, silicon
dioxide, zinc oxide, cerium oxide, nano-silver, nanoclays and multi-
walled carbon nanotubes. It is then possible to use those reference
materials worldwide and allow comparison between results obtained
in different laboratories and with different conditions
(http://ihcp.jrc.ec.europa.eu/our_activities/nanotechnology
/nanomaterials-repository).

Fullerenes are being produced by tons each year. It is likely that they
will eventually be found in the environment at measurable
concentrations. Fullerenes are lipophilic and localize into lipid-rich
regions such as cell membranes in vitro, and they are redox active.
Initially uncoated fullerenes can be modified with biocompatible
coatings, but it is unknown how long those coatings will stay on the
fullerenes during weathering in an environmental setting [29].

SWCNTs and MWCNTs are considered to be useful test materials in
the future. It would be difficult to obtain reproducible samples due to
small differences in production conditions affecting the quality of the
nanotubes and additional differences being introduced during
purification. Until large volume methods are available that yield high
quality, homogeneous batches of nanotubes over time, it would be
unwise to include nanotubes among the test materials to be
prioritized.

Ag NPs could be used as positive control, because these particles are
associated with relatively high toxicity in a great number of studies.
This toxicity might be driven by release of soluble material, with a number of researchers agreeing that the particle form might alter exposure, bioavailability, potential for uptake and fate within organisms, therefore influencing toxicity [30]. Furthermore silver NPs have high prevalence in consumer products for health remedies, wound dressings, clothing, food processing surfaces and computer keyboards, increasing the potential for exposure to humans and the environment [8].

Zero-valent iron NPs are candidates due to the field-scale application of this product for groundwater remediation. It is important to note, however, that the redox chemistry of certain substances such as those containing iron, titanium or copper, may act as a complicating factor in the various media used for ecotoxicological testing.

TiO₂ NP, one of the most widely manufactured ENMs [31] has received much attention in materials sciences and engineering due to its optoelectronic properties [32]. It is a common additive in many food, personal care, and other consumer products used by people which after use can enter the sewage system and, subsequently, enter the environment as treated effluent discharged to surface waters or biosolids applied to agricultural land, incinerated wastes, or landfill solids. For several high-consumption pharmaceuticals, the titanium content ranged from below the instrument detection limit (0.0001 μg Ti/mg) to 0.014 μg Ti/mg. Although several of these product classes contained low amounts of titanium, their widespread use and disposal down the drain and eventually to wastewater treatment plants (WWTPs) deserves attention [33].

In comparison to TiO₂, SiO₂ NP has been studied more widely due to an occupational lung disease called silicosis, which is linked to crystalline phase silica. However, amorphous SiO₂ NPs are considered generally highly biocompatible and non-cytotoxic unless engineered to be otherwise [32].

The Al₂O₃ NP is one among the most abundantly produced NPs, estimated to account for approximately 20% of the 2005 world market of NPs. Al₂O₃ NP have been widely used in diverse fields ranging from medical, military and industrial purposes as abrasives, wear resistant coatings and as drug delivery systems to increase solubility. However, recent studies have shown that nano-sized aluminum (10 nm in diameter) can generate adverse effects, such as pulmonary response [34, 35].

CeO₂ NPs are used in multiple applications. It is a very potent oxidation catalyst which promotes chemical reactions such as CO oxidation in automobile catalytic converters, it can be used for instance as an additive to diesel fuel to act as a fuel borne catalyst or as an electrolyte material of solid oxide fuel cells and many others. There exist discrepancies about the mode of toxic action of CeO₂ NPs. Some studies report a cytotoxic effect, others show no toxic effect and the availability to protect cells from radiation or oxidative stress [36]. In the last few years, several works involving ceria NPs on their surfaces can be functionalized (therefore they can be used in in vivo applications), and therefore toxicology is dose and generation dependent with higher dose and higher generation dendrimers leading to more toxicity in vivo. Dendrimer toxicity profiles are also closely related to the chemical structure of dendrimer and size [43].

Gold nanocompounds are applied in great amount of fields due to their unique properties and inert chemistry [45]. Due to a straightforward synthesis, stability and ease of incorporation of functional groups for targeting capabilities, gold ENMs have great application in gene and protein delivery, biological imaging, cancer treatments and implants [46-48]. Gold NPs have slow dissolution rate, they are available as reference materials in a variety of sizes, their surfaces can be functionalized (therefore they can be used in labelling of ENMs), and finally, gold NPs are easy to detect within biological specimens and environmental matrices [8].

Other possible ENMs which can be of interest as candidates for comparing the results obtained with well-known ENMs, although not mentioned in the OECD communication for the list of representative ENM for testing; are Cupric oxide (CuO) NP and combustion derived particles. CuO NP can be useful due to its relatively low dissolution rate but its potentially high toxicity towards organisms [49], though this remains to be shown in experimental studies at the moment. Regarding combustion derived particles there is no clear consensus. It was suggested the use of existing samples (NIST diesel exhaust particles), especially since they would allow direct comparisons between ecotoxicology models and human toxicology studies. However, it was commented that such samples consist of a mixture of components and are therefore not sufficiently well characterized and defined to be suitable as a reference material for ecotoxicological studies. On the other hand it was also acknowledged that ENMs can act as carriers of impurities or already existing environmental contaminants, and therefore materials with well-defined impurities could be interesting [8].
Mechanisms of engineered nanomaterial toxicity

The mechanisms of toxicity are not completely elucidated for most NPs at the moment. Membrane disruption, protein oxidation, genotoxicity, interruption in the energy transmission, ROS formation, inflammatory reactions and delivery of toxic compounds have been signaled as possible mechanisms implicated in its pathogenicity [50]. Although it is believed that the main molecular mechanism of in vivo nanotoxicity is the induction of oxidative stress by free radical formation [3]. In excess, free radicals cause damage to biological components through oxidation of lipids, proteins, and DNA. Oxidative stress may have a role in the induction or the enhancement of inflammation through up-regulation of redox sensitive transcription factors (NF-κB..., activator protein-1, and kinases involved in inflammation [3,51,52].

Free radicals can originate from several sources including phagocytic cell response to foreign material, insufficient amounts of antioxidants, presence of transition metals, environmental factors, and physicochemical properties of some ENMs [3]. Slow clearance and tissue accumulation of potential free radical producing ENMs, as well as prevalence of numerous phagocytic cells in the organs of the reticuloendothelial system (RES), makes organs such as the liver and spleen main targets. Additionally, organs of high blood flow (such as kidneys and lungs) can also be affected.

Inside cells, ENMs may interact with cellular components, disrupt cell function or originate reactive oxygen species (ROS). Although still under debate, ENMs may be involved in the up-regulation of NADPH oxidase and xanthine oxidase, which are free radical sources in macrophages and neutrophils [3]. The interaction between ENMs and mitochondria or cell nucleus are being considered as main sources of toxicity. ENMs such as silver-coated gold NPs, fullerene, block copolymer micelles, and carbon nanotubes may be located in mitochondria and induce apoptosis and ROS formation; or in the cell nucleus and cause DNA damage, cell-cycle arrest, mutagenesis, and apoptosis [53].

Additionally, other mechanisms of toxicity should be considered since ENMs immediately interact with their surrounding environment. Once in the bloodstream, NP can interact with blood components and cause hemolysis or thrombosis. Contact with the immune system can lead to immunotoxicity [6]. And further metabolic modification of ENMs in the liver (by cytochrome P450) may result in hepatotoxicity by reactive intermediates [3].

Toxic effects of some engineered nanomaterials

As there are no established protocols to study the toxicity of ENMs and its safety is of current concern, toxicity studies of ENMs are increasingly being published in a wide range of test methods, tested concentrations, cell lines and culture conditions, among other variables. This heterogenic information difficulties the comparison of toxic effects observed for a same kind of NP in different studies in the bibliography. The lack of understanding of its mechanism of toxicity, did not allow to discern which are the parameters that are masking the final toxicity results [10,26].

Available data clearly shows that pristine fullerenes (C60) has no acute or sub-acute toxicity in a large variety of living organisms, from bacteria and fungi to human leukocytes, and also in drosophila, mice, rats and guinea pigs. In contrast to chemically—either covalently or noncovalently—modified fullerenes, some C60 derivatives can be highly toxic. Furthermore, under light exposure, C60 is an efficient singlet oxygen sensitizer. Therefore, if pristine C60 is absolutely nontoxic under dark conditions, this is not the case under UV-Visible irradiation and in the presence of O2, where fullerene solutions can be highly toxic through 1O2 formation [54]. In vivo, C60s may induce inflammatory responses in the lung of mice [55].

Several research groups have examined the uptake and potential hazards of CNTs, particularly MWCNTs, to humans and other biological systems. Studies have implicated size (aggregation), CNT length, and manufacturing impurities as sources for potential toxicity in vivo. There is evidence of cytotoxicity for carbon-based ENMs, which follows this sequence on a mass basis: SWCNTs > MWCNTs > quartz>C60 [56]. Several studies indicated that CNTs exhibit substantial cytotoxicity in vitro, including induction of oxidative stress, inhibition of cellular proliferation, and induction of apoptosis/necrosis [57-64]. MWCNTs activate genes involved in cellular transport, metabolism, cell cycle regulation, and stress response in human skin fibroblasts [65]. In addition, MWCNTs produced DNA damage, among others it can activate the tumor suppressor protein p53 within 2 h of exposure [66].

CNTs have the potential to induce adverse pulmonary effects, alveolar macrophage activation, various chronic inflammatory responses (alveolitis and fibrosis), and severe pulmonary granuloma formation [67,68]. Ultrafine carbon particles show greater lung penetration than larger particles and are able to cross the blood-brain barrier and impact on the central nervous system. Its toxic effects appear quickly after exposure and it is suggested that carbon NPs travel from the lungs to the bloodstream rather than release clotting agents from the lungs. Since inhalation of asbestos fiber is known to induce asbestosis, lung cancer, and malignant mesothelioma of the pleura, there would seem to be a high probability that CNTs are likely to have significant toxic effects on human health due to their structural resemblance to asbestos [57,58]. Improper phagocytosis and macrophage translocation may be due to the large diameter of the MWCNT. Reduction of CNT accumulation and aggregation is achieved by functionalization [69-71]. Smaller size and length of CNT result in less aggregation and better uptake by macrophages. It was suggested that MWCNTs are recognized and interact with macrophage receptors on the plasma membrane, MWCNTs can rupture the membrane causing cytotoxicity and damage to the macrophage [72].

Primarily, accumulation of CNT was determined to be in the liver, but also in the spleen and lungs. No acute toxicity was observed in any tissues up to 24 h [73-75]. But its accumulation in the liver may result in hepatic injury and oxidative stress, ultimately resulting in inflammation. No acute toxicity was determined histologically up to 90 days post dose [74].

The cytotoxicity of silver NPs of 15 nm in diameter is likely to be mediated through oxidative stress although Ag NPs have been observed inside the mitochondria and nucleus, implicating their direct involvement in the mitochondrial toxicity and DNA damage [76,77]. The viability metrics significantly decreases with increasing dose (10-75 μg/mL) of 15 and 30 nm Silver NPs [76]. Ag NPs reduced ATP content of cell, caused damage to mitochondria and increased production of ROS in a dose-dependent manner. DNA damage was also dose-dependent. The NP treatment caused cell cycle arrest in G2/M phase possibly due to repair of damaged DNA. No massive apoptosis or necrosis was observed [77]. Regarding their distribution, silver NPs were found in brain, heart, yolk, and blood of zebrafish embryos, were they also induce a dose-dependent toxicity.
Generally, concerns regarding the toxicity of Zero-valent iron NPs have been minimal. This is largely due to the formation of iron oxides, which are already present in the ground as rust. However, this topic is gaining interest within the research and regulatory communities. Although iron is a required nutrient for oxygen transport, electron transfer, and catalysis in the body, it can accumulate to a level that causes DNA damage, oxidative stress, lipid peroxidation, and severe diseases such as hemochromatosis and carcinogenesis. The toxicity of iron is based on its ability to catalyze the formation of hydroxyl radicals (OH•) from superoxide (O2•−) and hydrogen peroxide (H2O2). Free radicals are highly reactive, unstable molecules that are in need of an additional electron for stabilization. Because of this, they can affect antioxidant enzymatic activities, peroxidation of membrane lipids, modification of nucleic acids, and eventually cause cell death and tissue injury. Iron toxicity studies have primarily focused on Fe2+ and its oxides and little is known about the toxicity specific to Zero-valent iron NPs. However, zero-valent iron can produce free radicals through this transformation process [79].

There is a significant increase in oxidative stress at higher TiO2 NP concentrations (> 60 µg/mL). As the concentration of TiO2 NPs increased in the culture medium, the levels of reactive oxygen species and lactate dehydrogenase increased in mouse fibroblast (L929)[80] and BEAS-2B cells [81]. Inhalation exposure of 2–5 nm TiO2 NPs, showed an inflammatory response with recovery at week 3 post-exposure [82]. NPs penetrated the plasma membrane and were located in the peri-region of nuclear membranes, indicating that NPs may have direct interactions with cellular molecules to cause adverse biological responses. With the induction of ROS, the expressions of oxidative stress-related genes, including heme oxygenase-1 or inflammation-related genes like interleukine-8, were increased [81]. It has been also reported that exposure of cultured cells to TiO2 NPs may lead to cell apoptosis, Interleukine 8 (IL-8) level increase, reduced glutathione (GSH) decrease and induction of micromuclei. Exposure to TiO2 NPs in mice caused acute toxicity and accumulate particles in the liver, spleen, kidneys, and lung [83].

Limited phytotoxicity studies are reported, but the results obtained were both positive and negative depending on the study. For example, TiO2 NPs were reported to promote photosynthesis and nitrogen metabolism and to improve growth of spinach at an optimal concentration [84,85].

There is growing evidence that amorphous SiO2 NP can cause an inflammatory response in the lung. Again a size-dependent toxicity is assessed: while SiO2 NP of 150 and 500 nm exerted no toxic effects, the nanotoxicity of amorphous 10 nm SiO2 NP was associated with inflammation, the release of ROS leading to apoptosis, and decreased cell survival [86]. DNA damage has also been reported for nano-sized SiO2 particles [83]. This increased cytotoxicity and cell death was time and concentration dependent, with a lethal concentration (LC50) of 9.7 µg/mL after 24h. As the nanotoxic effects of 10 SiO2 NP can be decreased by fisetin and catalase treatment, this injury implies oxidative stress [86].

The AlO2 NPs are able to rapidly enter into cells, and get distributed in the cytoplasm and intracellular vesicles [83]. Aluminium oxide NPs induces cytotoxicity [86] in a low but significant level, and in a dose-dependent manner. This dose-related cytotoxic effect can be due to changes in lysosomal and mitochondrial dehydrogenase activity, although the mechanism of cytotoxicity caused by aluminium metal oxide NPs is unclear, and need to be further explored. However, AlO2 NPs seem to be less cytotoxic than other NPs (such as ZnO, TiO2 or SiO2 NP) at the same concentration [83]. CeO2 NPs were taken up into caveolin-1 and LAMP-1 positive endosomal compartments, respectively, in BEAS-2B and RAW 264.7 cells, without inflammation or cytotoxicity. CeO2 also suppressed ROS production and induced cellular resistance to exogenous source of oxidative stress [87]. ZnO NP showed effects on cell viability as well as DNA damage, at lower doses than TiO2 particles. ZnO is the most toxic NP with the lowest LD10 value in comparison with TiO2, SiO2, and AlO2 NPs [83]. ZnO induced toxicity leading to the generation of ROS, oxidant injury, excitation of inflammation, and cell death [87].

Once introduced into the systemic circulation, positively charged dendrimers and cationic macromolecules have been found to interact with blood components, destabilize cell membranes, and cause cell lysis [40–42]. Overall, these studies indicated that dendrimer toxicity is dose and generation dependent with higher dose and higher generation dendrimers leading to more toxicity in vivo. The lethal dose for a third generation dendrimer was 160 mg/kg, giving rise to 100% mortality 6–12 h post injection. However, lower doses (40 mg/Kg) for both acute and subchronic exposures lead to significant increase in ALT activity and extensive liver necrosis in the histopathological evaluation [88]. Dendrimer modification with chemically inert polyethylene glycol (PEG) or fatty acids reduced dendrimer toxicities in vitro [42,43,89,90]. The toxicity profiles of dendrimers also depend greatly on the surface charge of the dendrimers: cationic dendrimers such as -NH2 PAMAM have shown concentration and generation dependent hemolysis and induced morphology changes in red blood cells after 1 h incubation at a low concentration of 10 µg/mL; whereas, anionic dendrimers were not hemolytic at concentrations up to 2 mg/mL [43]. No evidence of immunogenicity [90] or humoral immune response due to dendrimers was found. Finally, biodistribution studies revealed high kidney and liver accumulation for different generations of dendrimers [43,90–92]. The renal elimination rate decreased with increasing dendrimer size. Minimal renal excretion and high RES uptake were observed when PAMAM-Gd size exceeded 10 nm [93–96].

Little information exists on the toxicological hazards associated to organo-modified clays. The cytotoxicity of a series of pristine and organo-modified nanoclays has been evaluated in different cell lines. The calculated IC50 values for cell viability range from 1.4 to 47 µg/mL for the nanoclays tested and are above 100 µg/mL for the pristine nanoclays. The IC50 values of the organoclays are driven by the proportion and structure of the quaternary ammonium compound used as surface organic modifier. No differences in cell toxicity can be observed between the large and small-sized nanoclay batches, although their size differences related mostly to upper range of the size distribution. Despite their lower toxicity, pristine nanoclays induce apoptosis and can be found in cytoplasmic vesicles of exposed cells. Organoclays can be also found in cytoplasmic vesicles, although the size of the agglomerates is larger and the efficiency of uptake, considerably lower [97].

Gold NPs are considered biocompatible because they do not produce acute cytotoxicity. However, the response can vary depending on their coating, surface charge, size, shape, and in the biological system applied. In addition, these ENMs can cause non-lethal effects such as morphological changes, inflammation or cell damage [45]. In this sense, Di Guglielmo reported that despite AuNP were not cytotoxic at concentrations up to 1000 µg/mL, they resulted to be genotoxic due to
indirect mechanisms [98]. Nanoclusters of 1.4 nm exhibited increased cytotoxicity (IC_{50} is 30 and 46 μM), whereas nanoclusters of 0.8, 1.2, and 1.8 nm where four to six-fold less toxic. Larger sizes (15 nm) exhibited no cytotoxicity even at high concentrations (6.3 mM)[99]. Immune system cells also showed not cytotoxicity for gold NPs and decrease of potentially harmful reactive oxygen species [100].

Regarding shape, nanorods were described as more cytotoxic than spherical gold ENMs in human Hacat keratinocytes [101]. Cationic particles are generally toxic at much lower concentrations than anionic particles, this was explained as a consequence of the electrostatic interaction between the cationic NPs and the negatively charged cell membranes [100].

Studies for cell uptake and exocytosis revealed the role of size. 50 nm spheres were more quickly taken up by endocytosis than both smaller and larger sizes [102], and the rate of exocytosis reported a greater accumulation for larger gold NPs [103]. In addition, it has been reported that gold ENMs can penetrate easily through HaCaT cells and accumulate in the cell nucleus. Gold nanoclusters of 1.4 nm can selectively and irreversibly bind to the major grooves of DNA and cause increased cytotoxicity compared to larger particles (18 nm). The lack of interaction of larger particles with DNA is suggested to be due to steric hindrance. Regarding shape, nanorod uptake was slower relative to spherical particles in HeLa cells [102].

When studying biodistribution in vivo, the smaller particles (10-50 nm) were found to disperse quicker to almost all tissues, mainly accumulating in liver, lungs, spleen, and kidneys at 24 h post injection. Larger particles (100-200 nm) were also found in liver, lungs, spleen, and kidneys but they were not as widely dispersed into other tissues [46,101].

Relevance to studies of environmental fate and ecotoxicity

For the purpose of environmental hazard identification of ENMs, the PBT-profile (persistence, bioaccumulation, toxicity) is of major importance as defined by REACH. With the main goal degradability [104], characteristics that affect mobility and bioavailability in the environment and ultimately the processes leading to bioconcentration, biomagnification and ecotoxicity must be also studied [8].

Particle size can certainly be identified as a characteristic that will affect mobility and uptake in organisms, though the smallest particles are not necessarily the most mobile [105]. On the other hand, coating with organic polymers seems to have a major impact on environmental mobility [106] due to the charge at the surface.

Persistence is a key issue for organic environmental contaminants like polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs), as this will not only affect the toxic profile of a chemical (the risk of long-term exposure is low for ready biodegradable compounds) but a low degradability is a prerequisite for biomagnification to occur. Both abiotic and biotic degradation should be included in these considerations. Even though the persistence of metals is never discussed (metals cannot be degraded) the dissolution and speciation of metals may be affected by abiotic and biological interactions yielding changes in metal bioavailability [107]. Metals and metal oxide ENMs that in their bulk form traditionally may be regarded as more or less stable against dissolution, can release ions into solutions and thus exhibit persistence that is similar to conventional contaminants. In this way by dissolving ENMs, which are accumulated by organisms as ENMs, the ADME of the compound may be governed by characteristics related to the particles form, while the effects may be related to the dissolved element. For instance a ENM like ZnO with fast dissolution rates in aqueous media may be regarded less of a nano-specific hazard and therefore could be considered a less interesting candidate test material in an environmental context.

To study bioaccumulation, it is essential to be able to trace and quantify ENMs within organisms, tissues and cells. Unless special labelling techniques are used, it is an advantage to work with ENMs that may easily be detected through unique chemical signatures or inherent properties that permit detection. Furthermore, it is important to consider the background levels in the studied organisms in order to be able to discriminate compounds taken up from those naturally occurring in the media or in the organisms. For this purpose ENMs like Au, Ag, TiO, and polystyrene are suitable as test materials. As for bioaccumulation studies, the study of mobility of ENMs in the environment is feasible only if the materials studied have unique chemical signatures or carry some label (chemical, radioactive, fluorescent or immunologic). In environmental matrices like soils, sediments, sludges and water, the background levels of many elements are far higher than in organisms. This is one of the reasons that make rare earth elements like Au good for studying environmental fate and behaviour of ENMs [8].

One of the largest gaps that affect the investigation of the environmental behavior of NPs is the lack of analytical methods for their analysis at environmentally relevant concentrations [89]. Although work is being carried out in this sense. The first paper in this issue by Sobek and Bucheli reports a method by which carbon nanotubes can be isolated from natural samples and thus provides an important step forward in the analysis of these compounds in natural systems [108] and another report about the detection of an ENM in the environment is already available [109].

Discussion

This review arises from the increasing production and subsequent human and environmental exposure to ENMs, and its possible impact on public concern. In order to regulate this production, international organizations such as the Organization for Economic Co-operation and Development (OECD) and the European Comission through the REACH regulation had established some measures to assess its environmental and health safety. In this review we had focused in the information provided by guidelines, trying to complement it in order to have a more scientific evidence of its relevance.

From this work it can be revealed that the definition for ENMs is poor and without scientific relevance. It can be interesting to take into account definitions provided by Kreyling W G et al. who suggested a classification based on volume specific surface area (VSSA) [12]. No international organization has classified ENMs in categories, despite some preliminary attempts based on chemical classification system had been suggested [8]. Although it was agreed that current chemical classification systems used for normal substance are not adequate because they do not account for particle shape, size or other non-chemical physical properties. However, an established classification is needed in order to facilitate legislation and scientific comparison among studies and results. Herein, a general table has been suggested in order to classify ENMs in regard of its chemical composition, size, shape and surface coating characteristics. In the future, it may be possible to reclassify ENMs as more information emerges (toxicity...
mechanism, environmental behavior, surface reactivity or other physical properties).

The intrinsic properties relevant to ENMs in accordance with REACH have been compared with the properties suggested by several authors. The conclusion is that a ENM cannot be properly characterized with the data normally required under REACH, as some properties are not relevant for ENMs while others not considered can have a relevant role. Size distribution and particle size, agglomeration state, chemical composition, surface area, surface chemistry, surface charge, shape and water solubility had been selected as necessary physicochemical properties to be characterized in any study assessing risk of ENMs. However, despite a correct characterization is carried out in all studies, it is well known that it is not enough. Sample preparation and dosimetry are also of key importance. REACH and OECD are developing this point.

Some progress has been made regarding reference materials. These materials are promising, as they can be used worldwide to compare and validate test results. The OECD has provided a list of representative manufactured ENMs for testing in a communication, comprising: fullerenes, single-and multi-walled carbon nanotubes, silver, iron, titanium dioxide, aluminium oxide, cerium oxide, zinc oxide, silicon dioxide, dendrimers, nanoclays and gold NPs[28]. All of them have been reviewed in order to corroborate its scientific relevance. Cupric oxide NPs and combustion derived particles have also been suggested as possible additional reference materials.

The mechanisms of toxicity for ENMs have been summarized as well. Despite differences among different ENMs, the toxicity of ENMs is attributed frequently to oxidative stress induction by free radical formation, which in turn can lead to DNA damage and inflammation. In vivo, liver and spleen are considered main target organs, although other relevant organs can be also affected. Toxicity for the listed reference materials is also detailed in regard to its chemical composition.

Finally, some considerations for ecotoxicity studies have been mentioned in order to avoid confusions when assessing risk of ENMs in the environment. As above mentioned, degradation, persistence and dissolution rates of ENMs are key factors to assess bioaccumulation, which can be related with the toxicity of ENMs in the environment. In order to assess bioaccumulation, it is also necessary to find an analytical method to quantify ENMs within the environment and the tested organisms at relevant concentrations. Due to technical limitations, usually rare earth elements like gold are commonly used.

**Conclusion**

A classification for ENMs has been suggested in regard of its chemical composition, size, shape and surface coating characteristics.

It has been suggested that ENM cannot be properly characterized with the data normally required under REACH. A list of physico-chemical properties that should be provided in any study in the field of nanotoxicology has been detailed, including: particle size and size distribution, state of agglomeration, chemical composition, surface area, surface chemistry, surface charge, shape and water solubility. Sample preparation and dosimetry are also of key importance, and should also be described in the test methods. In ecotoxicity studies: degradation, persistence and dissolution rates of ENMs are key factors to assess bioaccumulation, which can be related with the toxicity of ENMs in the environment.

The use of reference materials can help in the process of homogenization of results. As these materials can be used worldwide to compare and validate test results.

The toxicity of ENMs is attributed frequently to oxidative stress induction by free radical formation. Liver and spleen are considered main target organs.

As a summary, much work is in development regarding safety assessment of NPs. And despite scientific groups use different techniques to evaluate different ENMs, it is important to take into account guidelines and recommendations of international organizations in order to allow comparison and correct understanding of the obtained results. However, guidelines and recommendations are not enough at the moment, due to the huge diversity of manufactured nanomaterials and the importance of its handling, techniques and experimental systems chosen in the toxicity results. Further work is required. It is considered convenient to continue developing specific test for nanomaterial evaluation.

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