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Research paper

Challenges on the toxicological predictions of engineered nanoparticles

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ABSTRACT

The perceived enormous potential of nanotechnology in contributing to sustainable innovation has led to the growth of investments into new industrial applications and consumer products. However, the lack of tools that are needed to generate early knowledge about the potential adverse effects, combined with the uncertainties regarding the health and safety risks of engineered nanoparticles (ENPs), are a potential threat to the acceptability by society of the nanotechnology innovations, due to the rising societal concerns that are based on generic worries. In order to tackle these issues, it has been necessary to adopt a more proactive approach into nanotechnology safety assessments. Multiple projects have been initiated around the world in order to understand how ENPs interact with living organisms, but the validation of most of the emerging knowledge may take years. This is while robust risk assessment results are urgently needed, in order to support timely regulatory decisions and risk management actions. The goal of this paper has been to review the present knowledge on the physicochemical characteristics of ENPs, focusing on titanium dioxide (TiO_2), gold (Au), copper oxide (CuO), and zinc oxide (ZnO), as well as on their biological interactions. In addition, the paper has been aimed at the identification of the main challenges on the current toxicological characterisation of these ENPs. Focus will also be given in this article to those ENPs that have been described by the Consumer Product Inventory as having prevalent nanomaterials present in consumer products, but also, with those having therapeutic and diagnostic applications, due to their physical (ex: confined plasmon resonances) and biological (biocompatibility and antimicrobial) properties.

Abbreviations: ENPs, Engineered Nanoparticles; TiO_2, titanium dioxide; Au, gold; CuO, copper oxide; ZnO, zinc oxide; MNM, manufactured nanomaterials; GHS, globally harmonized system; 3Rs, three Rs principles; QSAR, quantitative structure–activity relationship; DLS, dynamic light scattering; NTA, Nanoparticle Tracking Analysis; FFF, Flow Field-Flow Fractionation Analysis; GLC-TEM, Graphene Liquid Cells - Transmission Electron Microscopy; EELS, electron energy-loss spectroscopy; Cu^{2+}, copper ions; Zn^{2+}, zinc ions; ICP-MS, Inductively Coupled Plasma Mass Spectrometry; IARC, International Agency for Research on Cancer; FDA, Food and Drug Administration; FP, fine particles; NIOSH, National Institute for Occupational Safety and Health; NEDO, New Energy and Industrial Technology Development Organization; ROS, reactive oxygen species; OECD, Organisation for Economic Co-operation and Development; SCCS, Scientific Committee on Consumer Safety; A549, Lung Carcinoma Cell Line; THP-1, Human Monocytic Cell Line; MAPKs, activated protein kinases; ERK, Extracellular Signal–Regulated Kinases; JNK, c-Jun N-terminal Protein Kinase; hESCs, Human Embryonic Stem Cells; SOPs, standard operating procedures; GLP, good laboratory practice; PLGA, poly (lactic-*co*-glycolic acid; MTT, water-soluble tetrazolium salts (WST-1), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; XTT, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H–tetrazolium-5-carboxanilide; ELISA, Enzyme-linked Immunosorbent Assay; AgNPs, silver nanoparticles; TNFα, tumour necrosis factor alpha; HTS, high throughput screening; HCA, High Content Analysis; RMs, proper reference materials

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1. Introduction

Humans and other living organisms are continuously exposed to nanometer-sized materials (Buzea et al., 2007; Oberdörster, 2010; Aschberger et al., 2011). Modern science has learned how to synthesise tailored nanomaterials by manipulating matter at the atomic scale, in order to have well-defined properties for specific purposes. These so called engineered nanoparticles (ENPs) are commonly used in therapeutics, cosmetics, sporting goods, tyres, stain-resistant clothing, sunscreens, toothpaste, and food additives, among many others (Buzea et al., 2007; Oberdörster, 2010; Becker et al., 2011). In fact, intentionally produced nanometer-sized particles are inhaled every day. They are absorbed through the skin (when using consumer care products) and/or they are consumed in processed food and beverages (Buzea et al., 2007; Aschberger et al., 2011; Chen et al., 2016; Monteiro-Riviere et al., 2011; Smijs and Pavel, 2011; Jeon et al., 2016).

Most of these nanoparticles are expected to cause little or no effects on human health and be unnoticed. But in some cases, they might cause appreciable harm to organisms (Buzea et al., 2007; Becker et al., 2011; Martirosyan and Schneider, 2014). The amount of man-made nanomaterials ranges from several million tons/year (*e.g.* carbon black for car tyres) to microgram quantities for fluorescent quantum dot markers for biological imaging (Schulte et al., 2013; Bogart et al., 2014). As a consequence, workers and consumers are exposed to potentially hazardous substances when they are involved in activities such as research, development, synthesis, and the usage of ENPs or ENP-containing products (Buzea et al., 2007; Bogart et al., 2014; Bitounis et al., 2015). The lungs, the gastrointestinal tract, as well as human skin, are the most likely points of entry for ENPs into the human body. Injections (*e.g.* ENPs for drug delivery) and biomedical implants (ENPs generated by surface degradation) are other feasible routes of exposure to these engineered materials (Margarethe et al., 2015).

The lack of communication by stakeholders, as well as issues in the regulatory robustness of data (exposure and toxicological studies), together with that is generated by unsuitable methods, are factors that are potentially increasing the risk perceptions by consumers, and at the same time, decreasing perceptions of the benefits (Grobe et al., 2012). This is while a lack of robust knowledge contributes to regulators' insecurity, such that this too, has the potentiality to nurture public fear, in the light of nano-related media-driven accidents, hence, restraining the economic development of manufactured nanomaterials (MNM).

As referred to above, there are already a reasonable number of products in the marketplace, as reported in Woodrow Wilson's Database/Consumer Product Inventory (http://www.nanotechproject.org/cpi/), as well as in the periodic reporting of the French Registry of Nanomaterials (https://www.r-nano.fr/?locale=en). Among the 1814 products that are listed in the Consumer Product Inventory, 47% of them advertise the composition of at least one nanomaterial component (Vance et al., 2015). Titanium dioxide, zinc oxide, gold, and copper oxide are considered by the Consumer Product Inventory to be the most prevalent nanomaterials present in consumer products (Vance et al., 2015). Nevertheless, currently, the available industry-derived data regarding ENPs is limited (Becker et al., 2011; Vance et al., 2015). Essential information is not being incorporated on Safety Data Sheets. However, it is also not clear how nanomaterials should be classified and labelled, in order to follow the globally harmonized system (GHS) (Schulte et al., 2013; Hodson et al., 2009).

Some precautionary guidelines and recommendations for the safe handling of ENPs have been produced by organisations and agencies around the world, in order to protect their workers (Schulte et al., 2013; Hodson et al., 2009). The current regulatory frameworks for risk assessment (RA) are in principle applicable to ENPs, but adjustments are considered necessary, at least in terms of the testing guidelines (Schulte et al., 2013; Hristozov and Malsch, 2009; Hristozov et al., 2012, 2014; Seaton et al., 2010; Landsiedel et al., 2010; Steiling et al., 2014; Petersen et al., 2015; Kühnel et al., 2016; Schwirn et al., 2014). The principles of chemical risk assessments do not reflect some important properties of ENPs (size, specific surface area, reactivity) that are considered to be determinants of their toxicity (Schwirn et al., 2014). The risk assessments of ENPs are a massive task, because the regulatory frameworks require a case-by-case approach (Hodson et al., 2009; Hristozov and Malsch, 2009). Due to the huge number of existing and emerging ENPs, RAs are time and money consuming, conflicting with the three R principles (3Rs) of to replace, reduce and refine animal testing (Oomen et al., 2000). Significant developments overcoming these limitations (*e.g.* intelligent testing and grouping strategies), in favour of effective regulatory control, are under evaluation (Stone et al., 2014; Arts et al., 2015).

The scientific community is working hard in order to develop methods and tools that regulators can apply to a wide array of nanomaterials (overcoming the need of case-by-case assessments). The development of standardised methods and new risk assessment tools, such as foresight approaches, tiered schemes, grouping schemes, quantitative structure–activity relationship models (QSAR models), safe-by-design approaches, high throughput and high content methods, are some of the present strategies. These methods are now being followed-up by technical and scientific communities (Schwirn et al., 2014; Stone et al., 2014). In addition, there is a clear trend for the development of decision supporting frameworks that are based upon iterative dialogues, the engagement of all stakeholders, as well as considerations for the socio-economic, cultural and political contexts (Oomen et al., 2000). These complementary approaches can also serve as research prioritisation tools, which can help industry in identifying the relevant sources of risk in ENP life-cycles and pinpoint the areas of knowledge deficits (Stone et al., 2014; Arts et al., 2015).

This review has aimed to: (1) highlight the important aspects of the physicochemical characteristics of ENPs, focusing on titanium dioxide (TiO_2), gold (Au), copper oxide (CuO), and zinc oxide (ZnO) and their biological interactions; and (2) identify the main challenges on the current toxicological characterisation of these ENPs. Two of the NPs that have been reviewed in this work are inert (TiO_2 and Au), while the other two (CuO and ZnO) are known to release metal ions, resulting in a Trojan-horse mechanism of toxicity.

2. Nanoparticle physicochemical characteristics and their biological relationships

The field of nanotoxicology aims to establish the relationships between nanoparticle physicochemical properties and their toxic potentials. In fact, nanoparticle toxicity depends upon various physicochemical characteristics, such as size, number, mass, aggregation, composition, crystallinity, surface functionalisation, among many others (Pettitt and Lead, 2013). Some of the physicochemical properties that are relevant for toxicological studies are reported in Table 1. However, it is still a challenge to identify the physicochemical parameters which are most relevant for eventual adverse health effects. In the last few years, different publications have come out regarding the nanoparticle characterisation required, in order to evaluate human health hazards from ENPs. In some of them, there is some overlap on the proposed parameters that are being considered as essential or desirable (Oberdörster et al., 2005; Emond et al., 2013). The biological effects of ENPs are affected by their physicochemical properties, such as size, surface area, solubility, shape, crystalline structure, surface charge, catalytic activity, and chemistry, as well as by their number. Most probably, it will not be single parameters, but various combinations that need to be considered, in order to be decisive on their ENP toxicities.

Systematic studies concerning which physicochemical properties are the most relevant for hazard assessments have revealed the following rankings (Orts-Gil et al., 2013): surface area (100%), elemental composition (96%), surface chemistry (89%), particle size (86%), particle size distribution (86%), surface charge (86%), agglomeration state

Table 1
Physicochemical properties of ENPs relevant for toxicological studies.

Physical & chemical properties	Biological effects
Chemistry and coatings	ENPs with different properties and surface modifications use different routes of uptake and elicit different cellular responses (Pettitt and Lead, 2013).
Size and surface area	Smaller particles of the same material tend to be more toxic than larger nanoparticles *e.g.* smaller ENPs cause adverse respiratory health effects (Pan et al., 2007; Karlsson et al., 2009).
Surface charge	Surface charge will inform on the ENP interactions with cells and organisms and will determine the corona formation (Hristozov et al., 2012; Pettitt and Lead, 2013; Oberdörster et al., 2005).
Corona formation (proteins, lipids)	Corona will determine the ENP uptake and the distribution in cells and organisms (Hristozov et al., 2012; Pettitt and Lead, 2013; Oberdörster et al., 2005; Verma and Stellacci, 2010).
Shape and aspect ratio	ENPs with a high aspect ratio (fibres) tend to be more toxic (Hristozov et al., 2012).
Agglomeration/aggregation	Size is a key factor with respect to translocation across the cell barriers (Meißner et al., 2014; Allouni et al., 2009).
Crystalline structure	Crystalline structure may impact on other properties of the material (*e.g.* reactivity, zeta potential) in a manner that affects human toxicity (Shi et al., 2013; Braydich-Stolle et al., 2008; Zhang et al., 2003).
Solubility (dissolution rate)	Solubility gives information on how many ions/molecules are released from the ENPs over time, dictating the toxicity in biological systems (*e.g.* Trojan-horse type toxicity) (Oberdörster et al., 2005; Dekkers et al., 2016).
(Photo) reactivity	Photo reactivity is an important parameter that informs of the potential of ENPs to elicit toxicity *via* oxidative stress pathways (Oberdörster et al., 2005).
Band gap	The conduction band energy levels can be used in order to predict toxicological potentials at cellular and whole animal levels (Zhang et al., 2012).

(71%) and crystalline structure (61%). The chemical composition of ENPs is fundamental for understanding the human health effects of ENPs. However, the surface characteristics of the nanoform can exhibit a different behaviour when compared to non-nanoforms of the same chemical composition. For some of these properties, internationally standardised test procedures are already available, in order to characterise the nanomaterials, such as ISO 92761-6, ISO 13317-1, ISO 9277 and ISO 15901-1.

In fact, several analytical techniques are available for characterising ENP properties. Fig. 1 shows some characterisation techniques that are commonly used in toxicity studies. Transmission Electron Microscopy is the gold standard technique for size distribution, shape and morphology, however, it is expensive, time consuming and unsuitable for systematic monitoring. Dynamic Light Scattering (DLS) is quite a fast technique that allows for obtaining a size distribution profile of the NPs in solution, but it has some limitations, *e.g.* larger aggregates may cause some interference. Regarding the characterisation of ENPs in a liquid media, DLS (as was referred to above), Nanoparticle Tracking Analysis (NTA), as well as Flow Field-Flow Fractionation Analysis (FFF), are all suitable techniques for NP size distribution measurements (Pettitt and Lead, 2013; Roebben et al., 2011; Hassellöv et al., 2008). However, the understanding of ENP surface reactivity and the measurement of *in situ* ENP reactivity are also required. Together with this, a quantitative characterisation of surface composition and surface chemistry is also essential (Yuk et al., 2012a; Wang et al., 2014a). *In situ* Transmission Electron Microscopy using Graphene Liquid Cells (GLC-TEM) has recently been applied to nanomaterials (Yuk et al., 2012a; Wang et al., 2014a, 2014b; Chen et al., 2013; Park et al., 2015). The GLC-TEM technique has offered the opportunity to reveal, at atomic resolutions, the structure of the nanocrystals, together with their chemical information, through the use of Electron Energy-Loss Spectroscopy (EELS) (Yuk et al., 2012b; Evans et al., 2011; Liao et al., 2013).

Nowadays, it is still not possible to really determine the toxicity of ENPs based upon their physicochemical properties, even though they are strongly interconnected (Rivera-Gil et al., 2013; Suttiponparnit et al., 2010; Chusueia et al., 2013). A recent article studying the cytotoxicity of fourth period metal oxide (TiO_2, Cr_2O_3, Mn_2O_3, Fe_2O_3, CuO, and ZnO) ENP cytotoxicity, has suggested that an increment in the atomic number of the transition metal oxide results in a higher toxicity (Chusueia et al., 2013). The number and the surface area also seem to pose an increment of hazard in the pulmonary field. Studies with rodents, when exposed to several ENPs (*e.g.* titanium dioxide, carbon black, barium sulphate) have revealed that for a fixed mass of particles, ENPs in the form of agglomerated and aggregated nano-objects produced a superior effect, rather than larger particles of similar chemical compositions and surface properties. Nevertheless, cytotoxicity seems to also be dependent on the particle surface charge, the number of available particle surface sites, as well as the metal ion dissolutions of the ENPs (Ortega et al., 2014). The dissolutions of ENPs give information about the release of ions from the ENPs over time. This release depends upon the chemical compositions, the particle sizes, the coatings, the surface treatments, the stability, the synthesis process, and the biological environment. In fact, the toxicity of some ENPs is often related to the number of ions that are released from them. For instance, the release of Cu^{2+} from CuO nanoparticles has been associated with lung cell toxicity (Ortega et al., 2014). In addition, cell death that was induced by zinc oxide (ZnO) nanoparticles has been found to be related to the extracellular liberation of high amounts of Zn^{2+} cations, their fast uptake by the cells, and the induction of apoptosis pathways (Chibber et al., 2013). For analysing particle stability and ion dissolution, in general, the particles are separated from suspension. So far, ultracentrifugation and ultrafiltration are mainly used, coupled with plasma mass spectrometry. However, depending upon the method, the protocol, and the medium, huge differences in the determinations of the dissolution rates were observed (Jemec et al., 2016). It has been suggested that ENP dissolutions should be tested in a relevant biological media, since this fundamentally affects the bioavailability of substances in a biological environment. In this regard, the metal speciation in different media also needs consideration (Lapresta-Fernández et al., 2014; Jemec et al., 2016). The quantification of nanoparticle uptakes by the cells and tissues after *in vitro* or *in vivo* (Fig. 1) tests is mandatory, in order to understand ENP toxicity, since aggregation, dissolution, in addition to surface modification, are dynamic processes that are correlated with toxicity (Oberdörster et al., 2005). The literature has revealed that Inductively Coupled Plasma Mass Spectrometry (ICP-MS-based) techniques and flow cytometry can be used in order to estimate and quantify the ENP internalisation by cells (Oberdörster et al., 2005; Allouni et al., 2009; Lapresta-Fernández et al., 2014; Jemec et al., 2016). Surface charge is also known to influence systemic distribution and the cellular uptake of ENP toxicities. There are already some studies linking zeta potential to the inflammogenicity of nanoscale particles of metals (Cho et al., 2012).

The understanding of nano-bio interactions, meaning by this, the understanding of the interfacial phenomena occurring between ENPs and the biological environment (cells, tissues, macromolecules, organs) is essential for the safety evaluations of nanomaterials (Cho et al., 2012; Pelaz et al., 2012; Xu et al., 2012; Ribeiro et al., 2016). When ENPs enter the blood stream, a variety of serum proteins build a protein corona on their surface (forming a protein corona), leading to their recognition and their internalisation by different cells (Foroozandeh

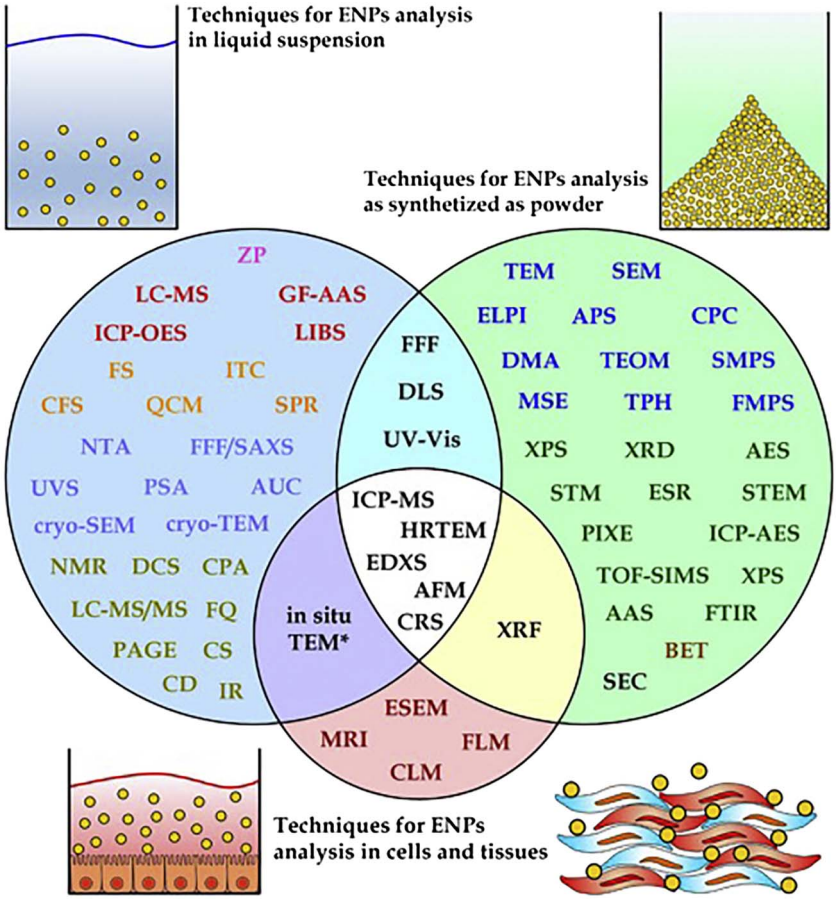

Fig. 1. Analytical techniques available for characterising ENP properties: particle size, size distribution, shape as synthesised in powder (dark blue) abbreviations (abbrev): Transmission Electron Microscopy (TEM), High-Resolution Transmission Electron Microscopy (HRTEM), Scanning Electron Microscopy (SEM), Scanning Transmission Electron Microscopy (STEM), Scanning Probe Microscopy (SPM), Atomic Force Microscopy (AFM), X-Ray Diffraction (XRD), Differential Mobility Analysis (DMA), Size Exclusion Chromatography (SEC), Field Flow Fractionation (FFF), Magnetic Sedimentation (MSE), Thermophoresis (TPH), Electrical Low Pressure Impactor (ELPI), Scanning Mobility Particle Sizer (SMPS), Fast Mobility Particle Sizer (FMPS), Tapered Element Oscillating Micro Balance (TEOM), Condensation Particle Counter (CPC), Aerodynamic Particle Sizer (APS). Particle size, size distribution and morphology in liquid suspension (light blue) abbrev: Dynamic Light Scattering (DLS), Particle Size Analyser (PSA), Analytical Ultracentrifugation (AUC), Field Flow Fractionation (FFF) combined with FFF/SAXS, Particle Tracking Analyser (PTA), UV–Visible Spectroscopy (UVS), Cryo-Transmission Electron Microscopy (Cryo-TEM), Cryo-Scanning Electron Microscopy (Cryo- SEM), liquid TEM (*in situ* TEM), Environmental Scanning Electron Microscopy (ESEM). Number density and size distribution of ENPs internalised in cells and tissues (dark red) abbrev: Magnetic Resonance Imaging (MRI), Fluorescence Microscopy (FLM), Confocal Light Microscopy (CLM), X-ray Fluorescence Microscopy (XRF). Chemical composition, crystalline structure and purity of ENPs as synthesised in powder (dark green) abbrev: X-ray Photoemission Spectroscopy (XPS), Electron Spin Resonance (ESR), Auger Electron Spectroscopy (AES), X-ray Diffraction (XRD), Inductively-Coupled Mass Spectroscopy (ICP-MS), Inductively-Coupled Plasma Atomic Emission Spectroscopy (ICP-AES), Atom-Absorption Spectroscopy (AAS), Time-Of-Flight Secondary Ion Mass Spectroscopy (TOF-SIMS), Scanning Tunnelling Microscopy (STM), Fourier-Transform Infrared Spectroscopy (FTIR), Particle Induced X-ray Emission (PIXE). Chemical composition and purity of ENPs in cells and tissues (black) abbrev: Confocal Raman Spectroscopy (CRS), Energy-Dispersive Dispersive X- Ray Spectroscopy in an Electron Microscope (EDXS) and ICP-MS. Surface charge in liquid suspension (pink) abbrev: Zeta Potential (ZP). Surface area as synthesised in powder (light brown) abbrev: Isothermal Gas Adsorption (BET); quantification, dose/concentration in liquid (light red) abbrev: Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES), Liquid Chromatography–Mass Spectrometry (LC-MS), Laser-Induced Breakdown Spectroscopy (LIBS), Graphite Furnace Atomic Absorption Spectroscopy (GF-AAS). Protein corona binding affinity (orange) abbrev: Isothermal Titration Calorimetry (ITC), Fluorescence Spectroscopy (FS), Quartz Crystal Balance (QCM), Surface Plasmon Resonance (SPR), Atomic Force Microscopy Fluorescence Correlation Spectroscopy (CFS). Protein structural changes after binding (light green) abbrev. Circular Dichroism Spectroscopy Fourier Transformed Infrared Spectroscopy (IR), Nuclear Magnetic Resonance (NMR), Differential Centrifugal Sedimentation (DCS), Size Exclusion Chromatography (SEC), Colorimetric Protein Assays (CPA), Poly (Acrylamide) Gel Electrophoresis (PAGE), Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS), Circular Dichroism (CD), Fluorescence Quenching (FQ), Isothermal Titration Calorimetry (ITC). Abbreviations naming the techniques were used to improve comprehension. Black abbreviations are those techniques with more than one application.

and Aziz, 2015; Saptarshi et al., 2013). In fact, an opsonisation of ENPs can occur when they are brought into contact with biological systems, meaning that they can induce an immune response (Hassellöv et al., 2008; Cho et al., 2012). The understanding of the content and the structure of the protein corona that is formed around ENPs may help to explain the differences in the biological responses that have been observed after an *in vitro* exposure to the cells or after *in vivo* experiments (Oberdörster et al., 2005; Walkey and Chan, 2012; Caracciolo et al., 2016; DeLoid et al., 2017; Monopoli et al., 2012).

In summary, the correlation of the physicochemical properties of ENPs, with their hazard potential explained in a reproducible and meaningful way, needs an accurate physicochemical characterisation. Nevertheless, the dynamic nature of body fluids that offer a repeated source of new biomolecules onto the ENP's surface should be taken into account (Caracciolo et al., 2016). The use of microfluidic cell culture devices that are aimed at simulating the physiological response of organs and that are under development may possibly contribute to a better understanding of the mechanisms of protein corona formation under more realistic conditions (Caracciolo et al., 2016; Dell'Orco et al., 2010). Clearly, the combination of different techniques (Fig. 1) should be used in order to characterise the physicochemical properties of ENPs in an adequate media. However, in order to adopt this approach, it would be necessary for a vast array of equipment that is sometimes not available. Some recommendations with regard to the minimal requirements on nanomaterial characterisation have already been proposed (Kühnel et al., 2016; Pettitt and Lead, 2013).

3. Physicochemical properties of titanium dioxide, gold, copper oxide and zinc oxide nanoparticles and their toxicological events

Regarding titanium dioxide (TiO_2), gold (Au), copper oxide (CuO), and zinc oxide (ZnO), a short description on their physicochemical characteristics and their biological effects is as follows:

3.1. Titanium dioxide

At the moment, TiO_2 is classified by the International Agency for Research on Cancer (IARC) as a "possible carcinogen to humans" (Shi et al., 2013; National Institute for Occupational Safety and Health, 2011; Boffetta et al., 2004). The U.S. Food and Drug Administration (FDA) approved TiO_2 as a food colour additive (not exceeding 1% w/w) and as a "food contact substance" in food packaging (Weir et al., 2012). In the nano form, titanium is one of the nanomaterials manufactured at a high volume. It is being widely used in a broad number of consumer products (*e.g.* toothpaste, sunscreens, cosmetics, food products, paints, plastics), as well as a component for surface coatings, in addition to medicine (*e.g.* implantable metallic materials, drug delivery systems) (Shi et al., 2013; Magdolenova et al., 2012; Hext et al., 2005; Buly et al., 1992). Although TiO_2 can enter humans through several routes, in the workplace, the exposure routes with a high toxicological significance are through inhalation (Shakeel et al., 2015).

The toxicity of nanoparticles is higher when compared to fine particles (FPs) in inhalation studies. Severe mice pulmonary damage after

an exposure to titanium dioxide nanoparticles has already been reported (Oberdörster, 2010; Oberdörster et al., 2005; Bermudez et al., 2004). An increased sensitivity in rats when compared to hamsters and mice has also been noticed for TiO_2 (Hext et al., 2005; Bermudez et al., 2004). Warheit et al. carried out an *in vivo* intratracheal study with rats comparing TiO_2 NPs and FPs of different sizes, crystalline structures and surface areas. The results have indicated that the toxicity of particles through lung inhalation is dependent upon surface properties, rather than on size and surface area (Warheit et al., 2006). A recent review on TiO_2 nanoparticles has revealed that long-term inhalation studies by rats have caused lung tumours (Shi et al., 2013). TiO_2 nanoparticles on the pulmonary system have seemed to induce local systemic effects and increased eventual pre-existing symptoms (Warheit et al., 2006; Thompson et al., 2016). TiO_2 NPs that are inhaled through the lung are more inflammatory than fine particles (FPs) of comparable chemistry, while at similar mass concentrations. However, if the particle surface areas were equal, no significant differences have been observed on pulmonary inflammation when comparing NPs and FPs. In all different types of toxicity (acute, sub-acute, sub-chronic or chronic), TiO_2 NPs have exhibited a moderate toxicity in the respiratory system. At relatively high doses, TiO_2 NPs have stimulated the pulmonary inflammatory responses and they have increased the pulmonary cell proliferations (Warheit et al., 2006; Thompson et al., 2016). By decreasing the size of TiO_2 NPs (20 nm), particle transportation from the airway lumen to the interstitial tissues has been observed, and consequently, they have entered the systemic circulation in rodent studies (Shi et al., 2013).

Results from epidemiological studies (with no particle size defined), did not demonstrate any kind of associations between TiO_2 exposures and a risk of lung cancer, or a reduction in ventilatory capacity (Hext et al., 2005). In terms of occupational exposure, populations that are already affected by asthma and cardiovascular disease may be more sensitive to TiO_2 exposures.

Regarding the exposure limits of TiO_2, the National Institute for Occupational Safety and Health (NIOSH) has determined that TiO_2 with a primary particle diameter < 100 nm is a potential occupational carcinogen and NIOSH recommends an exposure limit of 0.3 mg/m^3 (including nanoparticles), for up to 10 h per day, during a 40-h working week (Schulte et al., 2013; Shi et al., 2013). These suggestions represent levels that are not expected to increase the risks of lung cancer. However, it is important to reflect that NIOSH established these occupational standards based upon the studies of tumours in the lungs of rats after exposures to TiO_2. Rodents are known to be more sensitive to the effects of poorly soluble particles, such as TiO_2, when compared to other species. A report developed by the New Energy and Industrial Technology Development Organisation (NEDO) in Japan has revealed that the tolerable exposure concentration of TiO_2 NPs is estimated to be 1.2 mg/m^3 for an 8 h workday and a 40 h working week (Morimoto et al., 2010). Several toxicological studies have been carried out in order to investigate the potential effects of inhaled TiO_2 nanoparticles. They have observed adverse respiratory effects, thus suggesting, the potential of causing respiratory diseases in humans (Bermudez et al., 2004; Warheit et al., 2006; Rahman et al., 2013; Grassian and O'Shaughnessy, 2007; Schulte et al., 2010; Thompson et al., 2016).

Besides inhalation, humans can be exposed to TiO_2 NPs through oral (food ingestion) and dermal (through cosmetics and sunscreen applications) routes. It is important to refer that TiO_2 that is used in food applications (as a pigment grade) is not available in the nanometer size range (Oberdörster, 2010; Shakeel et al., 2015; Takeuchi et al., 2014; Janer et al., 2014). Little or no toxicity has been observed in rats after exposures to different forms of TiO_2 nanoparticles, when following the Organisation for Economic Co-operation and Development (OECD) test guidelines (Oberdörster et al., 2000). A mixture of TiO_2 nanoparticles (anatase/rutile) exhibited a medium lethal dose (LD_{50}) in rats > 2150 mg/kg, leading to low acute oral toxicity, as reported by the Scientific Committee on Consumer Safety (SCCS) Opinion (National Institute for Occupational Safety and Health, 2011).

The probable health risks for the ingestion of food containing nanoparticles transferred from packaging is not yet fully comprehended, however, it will depend on the particle toxicity, the size, the morphology, together with the rates of migration and ingestion (Weir et al., 2012; Khang et al., 2014; Souza and Fernando, 2016). High doses of TiO_2 have induced oxidative stress and alterations in the cell signalling transduction pathways that can lead to carcinogenesis and other diseases (Foroozandeh and Aziz, 2015; Baan et al., 2006; Landsiedel et al., 2012; Y. Wang et al., 2014). A recent review focusing on NPs in food has reported that ingested NPs have the potential to cause inflammatory reactions and inflammation-associated diseases, such as Crohn's disease and ulcerative colitis, or to induce allergic diseases (*e.g.* food allergy) (Shi et al., 2013; Dell'Orco et al., 2010).

The anatase crystal structure of TiO_2 nanoparticles has been able to induce dermal fibroblast cell death at a LC_{50} and it has also decreased the human lymphoblastoid cell viability (Dell'Orco et al., 2010). Studies have revealed that upon exposures TiO_2 NPs, can enter the systemic circulation and be distributed to the liver, spleen, lungs, kidneys, and even the brain (Pelaz et al., 2012; He et al., 2015). However, upon oral ingestion, there is no clear evidence of an uptake of particles in the blood circulation of humans or in the studies of rodents (Shi et al., 2013; Shakeel et al., 2015).

The effects of TiO_2 on the skin have been also studied. TiO_2 nanoparticles can be present in the crystal structures of rutile, anatase, or a mixture of both. Anatase is considered to be more reactive and rutile seems to possess a high refractive index giving it the high capacity of spreading UV-radiation. The literature has suggested that anatase has a superior *in vitro* toxic potential when compared to rutile, since it generates a higher quantity of reactive oxygen species (ROS) under UV radiation (Shi et al., 2013). However, the size of the NPs has also had a strong influence on ROS generation. Most of the *in vitro* and *in vivo* studies of TiO_2 exposures in skin applications have not provided data on the NP physicochemical properties, such as size, size distribution, surface area, or even particle numbers, making it difficult to correlate the particle toxicity with physicochemical properties (Weir et al., 2012; Shakeel et al., 2015; Lademann et al., 1999; Schulz et al., 2002).

TiO_2 has been tested when following the OECD guidelines for skin irritation, skin sensitisation and ocular irritation. No significant skin irritating or skin sensitising effects were detected (Kumar et al., 2014). The cornea was also not affected. Most *in vivo* or *in vitro* dermal exposure studies have demonstrated that titanium nanoparticles are not able to penetrate the *stratum corneum*. However, most of the analyses were conducted with agglomerated TiO_2 nanoparticles in the micron scale (Oomen et al., 2000; Lademann et al., 1999; Crosera et al., 2015; Schulz et al., 2002). The quality and the integrity of the skin seem to be very important for the penetration of NPs of different sizes. Although there is already some evidence of the penetration of TiO_2 nanoparticles and micron-particles in burned and damaged skin, but up to now, there is no evidence of the potential health risks to humans (Shi et al., 2013). As there is a current increase in the use of cosmetic products containing TiO_2 NPs, more efforts should be carried out in order to evaluate the chronic exposure of topically applied products (Shi et al., 2013). Epidemiological studies regarding dermal exposures have evaluated the carcinogenicity of TiO_2, but still no significant conclusions can be drawn, since there is a lack of information regarding such exposures.

3.2. Copper oxide

Copper oxide is a reactive metal oxide particle that is used in a variety of applications, including catalytic processes, solar cells, electronics, lithium batteries, bioactive nanocomposites for biomedical applications (*e.g.* wound dressings), as well as in textiles (socks) (Vinardell and Mitjans, 2015; Bondarenko et al., 2013). It can be used as a bioactive coating in order to inhibit the adhesion of target microorganisms such as *Escherichia coli* and *Staphylococcus aureus* and it is

also used to exert antiviral properties (Bondarenko et al., 2013; Cioffi et al., 2005; Magaye et al., 2012). Copper oxide NPs are known to release small amounts of copper ions. From a biological point of view, copper is an essential micronutrient that is necessary for growth, development, as well as for the maintenance of connective tissues, the brain, the heart, bones, and numerous other organs. It is correlated to the improvement of the immune system, tissue healing and regeneration (Magaye et al., 2012; Bondarenko et al., 2012).

Recent studies have shown the effects of various physicochemical CuO NPs, such as reactivity, aggregation, and suspension stability on alveolar type-I cell interactions. Interestingly, it has been found that the shape and the size of CuO NPs affected cell viability, as well as interleukin IL-6 and IL-8 secretions (Misra et al., 2014). The effects of CuO NP size were also evaluated by Lanone et al. when they compared the toxicity of 24 different NPs with a similar size evaluating lung carcinoma (A549) and human monocytic (THP1 cell lines) cell lines (Lanone et al., 2009). The investigation of different endpoints, such as the inhibition of growth rate, a reduction in cell viability, and the production of reactive oxygen species (ROS), has suggested that CuO toxicity seems to be size dependent. CuO NPs were found to be more toxic when compared to CuO on the micrometre scale. These characteristics were also observed in yeast and eukaryotic cells (Kasemets et al., 2009). Similar results were observed in *in vivo* experiments when using different sizes of CuO that were administrated orally to rats (Chen et al., 2006). Indeed, Wongrakpanich et al. reported contradictory results, where 24 nm CuO NPs were more cytotoxic than 4 nm CuO NPs. The authors suggested that the bigger NPs potentially influenced the amount of intracellular ion dissolutions, resulting in higher cytotoxicities (Wongrakpanich et al., 2016).

It is well known that CuO NPs are highly toxic when compared to Cu ions, however, the potential hazardous effects of CuO NPs have not been fully elucidated (Wang et al., 2012). The higher toxicity is related to the internalisation of the CuO NPs by the cells and the subsequent intracellular dissolutions, which are also called a Trojan horse-type mechanism, resulting in a high internal concentration of Cu ions (Studer et al., 2010; Cronholm et al., 2013). Once internalised, the CuO NPs will dissolve inside of the lysosomes, promoting oxidative stress, inducing catalase and superoxide dismutase, as well as with the production of ROS and DNA damage (Studer et al., 2010; Cronholm et al., 2013).

The effects of copper dust are conceivable to be harmful to human health (De Olivera et al., 2012). In fact, when testing the human epithelial cell line H292, Ko et al. evidenced increased levels of IL-6 and IL-8 mRNA expression, in addition to protein, as well as the increased phosphorylation of mitogen-activated protein kinases (MAPKs), ERK, JNK, and p-38, after the CuO NP treatments, in a concentration-dependent manner (Magaye et al., 2012; De Olivera et al., 2012). *In vivo* studies using rats that were nose-only-exposed to CuO NPs (~11 nm) showed lung inflammation and cytotoxicity, which were characterised by alveolitis, bronchiolitis, as well as vacuolations of the respiratory epithelium and emphysema, but they were almost completely resolved after a 3-week post-exposure period (Agarwal et al., 1990; Bhunya and Jena, 1996). The liver is the organ where Cu is mostly accumulated. The stomach and the small intestines normally absorb the majority of copper. Regarding excretion, Cu is released *via* bile into the gastrointestinal tract with a minimal amount of copper reabsorbed by the intestinal cells. This allows for the conservation and the tight regulation of the Cu body content (Magaye et al., 2012).

Genotoxicity and carcinogenicity studies of water soluble copper compounds have shown that they are genotoxic, with characteristics that comprised of chromosomal aberrations and micronuclei in the bone marrow cells of White Leghorn Chicken, as well as chromosomal aberrations in Swiss mice (Agarwal et al., 1990; Bhunya and Jena, 1996).

3.3. Gold

The publications on gold nanotechnology have been increasing exponentially over the last two decades. Gold is considered to be an inert material, with regard to both ion dissolution and reactivity. The unique characteristics of Au NPs are quite different from bulk gold materials. These characteristics include optoelectronic properties, a high stability, a tunable size and shape, as well as a relatively easy surface functionalisation, allowing for a wide diversity of Au NPs (Sperling et al., 2008; Murphy et al., 2005). Gold applications range from the electronics industry to the pharmaceutics and cosmetology industries (Khan et al., 2013; Murphy et al., 2008; Dreaden et al., 2012a). In the golden age of nanomedicine, the major applications of Au NPs have been in bio diagnosis and imaging, as well as in drug delivery and gene therapy (Bondarenko et al., 2013). Furthermore, in 2013, the FDA approved the first clinical trials that were focused on a new treatment for lung cancer when using Au NPs in photothermal therapy. Beyond applications in the biomedical field, the presence of Au NPs in consumer goods within the health, fitness, food, and beverage categories (*e.g.* cosmetics, supplements, food packaging and beverages) is increasing all the time, according to the Consumer Products Inventory (CPI) (Vance et al., 2015).

Despite the exciting and promising multifunctionality presented by Au NPs in these different areas, as discussed above, the potential toxicity of these particles is still a matter of concern for regulatory agencies.

The safety of Au NPs in cells/organisms is highly controversial. Au NPs are considered as being toxic or non-toxic, depending upon the study considered. *In vitro* studies have reported a general trend for Au NP toxicity, with particles smaller than 5 nm being more toxic than the larger particles, suggesting a size-dependent cytotoxicity (Turner et al., 2008). In particular, particles < 2 nm diameter have been demonstrated to have a chemical reactivity that has not been observed in larger sizes (Kasemets et al., 2009; Eshed et al., 2012; Borkow, 2014; Shukla et al., 2005). Pan et al. (2007) noticed that 1.4 nm Au NPs (spheres) induced oxidative stress pathways and toxicity in different cell lines, while no toxic effects were observed after 15 nm Au NPs were exposed on cell exposures with similar chemical compositions (The Au NPs were stabilised by triphenylphosphine derivatives) (Pan et al., 2007). Lui et al. evaluated the cytotoxic potential of citrate-capped Au NPs. A toxic effect of 5 nm Au NPs was observed as a result of cell proliferation inhibitions, cell apoptosis increments, and cell cycle arrests, in the lung cell lines. No cytotoxic effects were observed after cell exposures of 10, 20 and 40 nm particles (Liu et al., 2014).

A recent study has explored the effects of thiolate-capped Au NPs (1.5, 4 and 14 nm) in human embryonic stem cells (HESCs). They showed a significant cell death in both HESCs and HESC-derived neuronal progenitors after 1.5 nm-particle exposures. None of the other Au NPs triggered toxic effects. However, 4 nm Au NPs have led to a decrease in global DNA methylation, exhibiting the potential to affect the epigenetic parameters (Senut et al., 2016). In contrast, the studies that were conducted by Goodman et al. (2004) and Connor et al. (2005) when using cationic 2 nm and citrate-capped 4 nm Au NPs (spheres), respectively, reported no toxic effects in the different cell lines. Indeed, most *in vitro* studies have focused on acute toxicity assessments at very high doses of Au NPs, quite far from realistic scenarios for human exposure. The high variance and the contradictory results that have been observed in *in vitro* studies may be partially resulting from the different surface coatings or the stabilisers that were used in the Au NP toxicity assays. This is because surface modifications will influence the stability and the uptake into the cells. In order to provide biocompatibility and specificity, the surfaces of Au NPs are frequently modified by conjugation, with a rich variety of biofunctional molecules. Since the surface coating regulates both inter-particle and cell-NP interactions, it plays an important role in NP internalisation and cytotoxic responses as well. Results have shown that there is a complex relationship between surface coating and toxicity mechanisms. In addition, other variable

parameters, such as cell-type sensitivity, exposure time and dosimetry, should also be taken into account in any Au NP toxicity risk evaluations. *In vivo* studies that have focused on the toxicological effects of Au NPs are less common than their *in vitro* counterpart. The distribution of Au NPs throughout the body seems to be dependent upon particle size, shape, surface coating, as well as the route of exposure. Intravenous injections are the most common route of exposure that is reported in *in vivo* studies. After intravenous administrations of citrate spherical gold nanoparticles (10, 50, 100, 250 nm) in rats, De Jong et al. observed a particle size-dependent organ distribution. The highest amount of Au NPs was noticed in the blood, the liver and the spleen, at 1 day post-injection. The 10 nm particles were the most widespread throughout different organs, including the brain (de Jong et al., 2008). Similar results were also found by Sonavane et al. after intravenous injections of 15, 50, 100 and 200 nm citrate Au NPs in mice (Sonavane et al., 2008). The maintenance of high levels of citrate Au NPs (20 nm) in the liver and the spleen of rats, throughout 2 months after the injections, was shown in a long-term study that was carried out by Balasubramanian et al. (2010). However, the authors did not observe the presence of particles in the brain, suggesting that the passage through the blood–brain barrier is NP size-dependent.

Inhalation is the main route of Au NP exposures for workers, R & D researchers, and consumers as well. A study that was performed according to Test Guideline 143 from the OECD (subchronic doses/90-days exposure) identified a dose-dependent accumulation of Au NPs (< 10 nm), but only in the lung tissues of both male and female rats, leading to an inflammatory infiltration of the cells (Sung et al., 2011). A short-term exposure study reported a size-dependent clearance of Au NPs from the lungs after inhalation, suggesting that smaller particles can be eliminated in a shorter time and that they can translocate faster from the lungs into other organs (liver, spleen, brain and blood) than when compared to larger Au NPs (Han et al., 2015). Although dermal and oral pathways also represent significant routes for Au NP exposures, the data is almost absent. The questions regarding the impact of Au NPs on human health remain unanswered, since the understandings of the potential consequences for human exposure to gold nanoparticles are still limited and controversial.

3.4. Zinc oxide

Zinc oxide is a metal oxide with a wide range of applications, including food additives, an absorption base for ointments, skin treatments, together with sunscreens, among many others. Contrasting to other metal oxide nanoparticles, ZnO is highly soluble in aqueous solutions and it tends to release zinc ions into the physiological medium (Song et al., 2010). However, it has a good solubility in an alkaline medium and it has very favourable ZnO-terminated polar surfaces. In the nanoform, in a range from tens to hundreds of nanometres, a number of studies have indicated a relevant toxicity in the liver, the spleen, the heart, the pancreas and the bone tissues, although no consensual opinions have been presented (Saptarshi et al., 2015; Annangi et al., 2015).

Even though robust evidence has indicated that the interactions between ZnO and biological matter have induced the formation of reactive oxygen species (ROS), and consequently, cell death, the physical-chemical mechanisms that are involved have not yet been fully clarified.

In the present literature, a direct or even an indirect generation of ROS induces oxidative stress and this is associated with genotoxicity, mutagenesis and carcinogenesis. Alongside a ROS formation, a Zn^{2+} release, as well as an internalisation of the ZnO nanoparticles into biological matter, electrostatic interactions also play a role (Yang et al., 2010; Choi and Choy, 2014). Moreover, as recently reviewed, severe toxicological effects may also be associated with both ionic and particulate forms. Furthermore, it seems as if there is a direct relationship between cytotoxicity and the size and the shape of ZnO nanostructures.

Although size effects are straightforwardly associated with surface area, it seems that particle shape is a more relevant parameter for the induction of toxicity (Heng et al., 2011). Several contributions in the specialised literature have attempted to associate the surface physico-chemical properties of ZnO NPs to a cellular uptake. A combination of different internalisation routes seems to play a role in this process. There is some evidence that has indicated that in the uptake mechanisms, the internalisation of positively charged ZnO nanoparticles is mediated by energy-dependent endocytosis. This would be due to strong attractive forces between the positive surface charge of the ZnO nanoparticles and the negative charge of the plasma membranes.

Commonly found in sunscreen formulations for many decades, ZnO has been known to be the most effective protection from UVA rays, when compared to any other materials (Jeon et al., 2016). Lin et al. concluded, in the last decade, that exposures to nanosized ZnO leads to dose-dependent and time-dependent cytotoxicity, which is reflected in oxidative stress, lipid peroxidation, cell membrane damage, and oxidative DNA damage (Lin et al., 2008). Since this seminal work, the dose-response patterns and the causes of cytotoxicity that are related to ZnO nanoparticle exposures have been reviewed. The relationship between ZnO nanoparticles and cancer has been extensively explored in the literature. In summing up, the current understanding of cytotoxicity relating to ZnO nanoparticles has indicated that there is a direct correspondence between the intracellular dissolutions of the nanoparticles and the subsequent release of bioavailable Zn. Furthermore, there is also a connection to intracellular reactive oxygen generation (ROS), providing more strong evidence for ZnO nanoparticle cytotoxicity in human immune cells, although ROS does not seem to be the sole cause of the induced cytotoxicity (Yang et al., 2010).

Even considering the still open questions about the hazardous effects that are related to zinc oxide nanoparticles, there are undeniable benefits linked to their use as chemotherapeutic agents, bactericide materials and other medical applications. Consequently, it has become necessary to improve those efforts of redirecting the toxic effects to target tissues, while working on synthetic approaches to control the zinc oxide nanoparticle's shape and diameter. This is together with developing surface modification strategies, in order to decrease zinc oxide nanoparticle's toxic effects and enhance its biocompatible and medical use.

As a conclusion of this literature review of ENP toxicology, it is possible to understand some conflicting results, due to the limitations of traditional cytotoxicity assays, when evaluating the huge variability of ENP's chemical and physical properties. This is together with the lack of standardised methods for assessing the toxicity of ENPs when considering common cell line models. In addition, other variable parameters, such as cell-type sensitivity, exposure time, as well as dosimetry, should be considered in the hazard evaluations of ENPs (Han et al., 2015; Annangi et al., 2015; Yang et al., 2010; Choi and Choy, 2014). In most of the cases, the assessments have been performed without knowledge of the real size, the concentrations of the ENPs, the dissolution kinetics, the chemistry of the ENPs, or the speciation of the dissolved metal ions in the biological environment. However, all of these parameters have a crucial impact on the bioavailability of ENPs for cells and animals. For all kinds of ENPs, there are articles reporting on their toxicity in different models. However, the mechanisms have not been truly elucidated. In order to predict a possible toxicity, a systematic examination of biodistribution parameters is needed for those nanoparticles with different sizes and surface chemistries. This is as well as a deep understanding of the cellular and molecular mechanisms involved in cell-nanoparticle interactions (Fig. 2). The potential for ENPs to trigger unfavourable human health effects still needs to be elucidated. Epidemiological studies are essential in order to take precautionary measures, reduce and abolish the adverse effects on health, as well as providing for a theoretical basis for the safety evaluations of nanomaterials (Laney et al., 2011). However, the major challenges include the defining of the appropriate control population

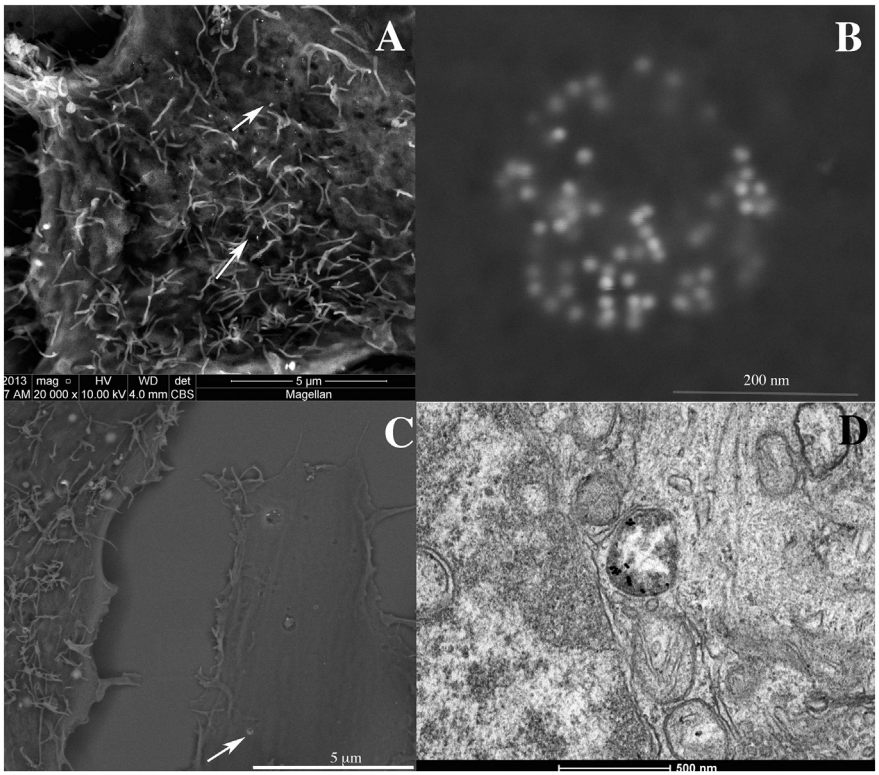

Fig. 2. Electron Microscopic images of a representative A549 cell line after incubation with 15 nm Au NPs: SEM image of a cell that was exposed to Au NPs for 24 h. It is possible to see the distribution of the NPs on the cell surface (A); high magnification demonstrating the accumulation of Au NPs in some vesicles (B); (C) transmission electron micrograph demonstrating the accumulation of ENPs in vesicles. Arrows indicate the position of the Au NPs.

and the obtainment of adequate exposure data. Of course, we should considerer that nanotoxicology is a new science, but where collaboration from a multidisciplinary team of scientists, companies and regulators is mandatory.

4. Why are the current *in vitro* experimental models for nanotoxicological evaluations a challenge?

As has been referred previously, the nanotoxicology literature contains some contradictory results, due to several factors that range from the absence of physicochemical characterisation in a biological environment, defined dose metrics, as well as standardised *in vitro* and *in vivo* methods. In order to produce robust and replicable toxicological data and to reduce contradictory data, besides the development and the implementation of specific standard operating procedures (SOPs) for the testing of ENPs, analyses should be performed following the principles of good laboratory practice (GLP). This is where the contribution of expert scientists, proficient in interpreting experimental data, in accordance with international standards, is essential.

Even if some research work is proceeding, in order to identify a minimum set of physicochemical parameters, up until now, there has been no widespread agreement on the physicochemical properties of ENPs for them to be included in health hazard studies. Without a common physicochemical characterisation approach, in terms of methodology (standard methods) and parameters (relevant physicochemical parameters), the comparability of nanotoxicology studies will still be considerably hindered.

When considering nano-specific dose-metric parameters, mass, the number of particles, as well as the surface area, these are normally the classic dose measurements in mammalian toxicity studies (Simkó et al., 2014). Currently, mass is the most commonly used dose-metric parameter that is employed in mammalian toxicity studies. However, in ENP dose-response assessment studies, it has been suggested that toxicity is not mass-dependent, but it is strongly influenced by other physical-chemical characteristics, such as surface area, and/or chemical composition (Fig. 3A) (Oberdörster, 2010; Meißner et al., 2014). In airborne exposure, for example, the metrics are considered to be particle mass per volume of air, however, metrics continue to be redefined, as new data is obtained (Chen et al., 2016). Additional inflammatory responses have been observed in TiO_2 nanoparticle exposures in the lung, when compared to fine particles at equivalent concentrations and with similar chemistry. TiO_2 nanoparticles and TiO_2 fine particles have stimulated analogous pulmonary inflammation on the basis of equivalent particle surface area (Dreaden et al., 2012b).

Fluorescent, isotopic, or radio-labelled markers, binding to the ENPs, are considered to be the best approach in order to estimate dosage. However, there are worries that the labelling process may dissociate itself from the NPs, altering the biodistribution and contributing to the toxicity as well. These particular radio-labelled markers can change the physical-chemical properties of the ENPs, with a subsequent different uptake and behaviour, when compared to non-labelled ENPs. Thus, there is a crucial need for labelled ENPs that are confirmed to possess similar properties, stabilities, and interactions, the same as their unlabelled counterparts (Schulte et al., 2013). Alternatively, single-particle-ICP-MS-based approaches (Laser ablation of tissues, single-particle ICP) have been used in order to detect and quantify unlabelled particles (Böhme et al., 2015), however these methods are laborious and are not applicable for a large amount of samples. There is already an OECD Guideline on Guidance describing sample preparation and dosimetry for the safety testing of manufactured ENPs OECD (2012). However, we suggest that whenever it is possible, to specify the dose in two different units, such as volume, surface area, and/or the number of particles, since this can be more instructive in order to express the dose-response correlations when comparing the toxic effects of different ENPs. Recently G.M Deloid et al. (2017) developed a protocol for *in vitro* dosimetry that allows for measuring the dose metrics as a function of time. It is based on NP dispersion preparation and characterisation and a computational method that derives delivered dose metrics.

Unsuitable concentrations (overload) are frequently being used, where the sedimentation of the ENPs may induce dose errors (Lozano et al., 2013; Landsiedel et al., 2012). There is a necessity for ENP stability studies and standard administration protocols. Some studies have

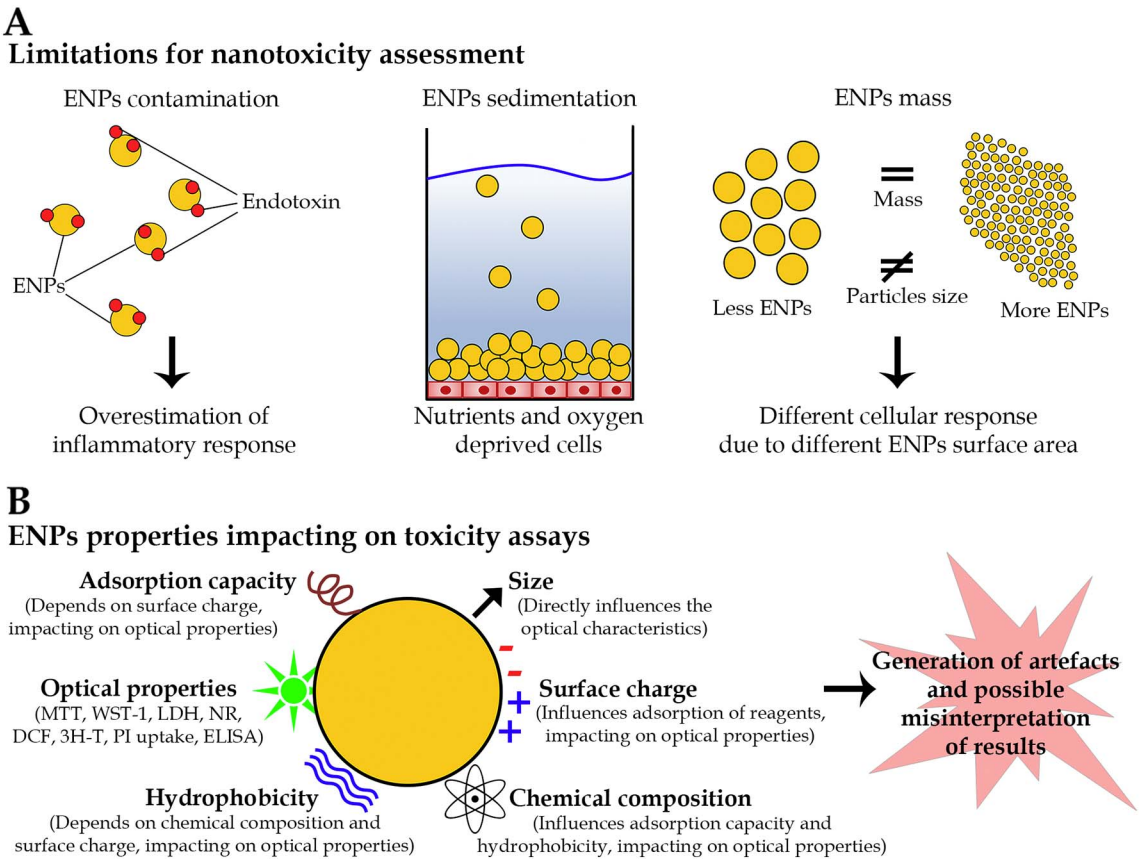

Fig. 3. Current limitations of *in vitro* assays for ENP hazard assessments: the comparability of nanotoxicology studies may be negatively impacted due to different ENP characteristics. (A) The occurrence of ENP contaminations, such as the presence of endotoxins and unsuitable concentrations, will result in an overestimation of the inflammatory responses and ENP sedimentation, promoting dose errors, respectively. Indeed, toxicity is not mass-dependent, but it is influenced by other physicochemical characteristics, such as surface area. (B) Several physicochemical characteristics have influenced the obtained results, culminating in disturbances in the optical-based methodologies, including the MTT, WST-1, LDH, NR and PI uptakes, as well as the DCF, the 3H-T and the ELISA methods. For instance, the adsorption capacity of ENPs will be influenced by their surface charge, as well as by their hydrophobicity. This adsorption capacity will depend upon the chemical composition and the surface charge. Indeed, the size and the chemical composition of ENPs were also affected by the adsorption capacity. Altogether, in order to avoid the generation of artefacts and the possible misinterpretation of the results, the appropriative method for nanotoxicity analyses should be selected, depending upon the optical properties of the ENPs.

also used very high doses of ENPs. These doses have created a physical barrier over the cells, impairing the uptake of nutrients and oxygen from the medium, leading to cell death (Chueh et al., 2014). Another topic of concern, before starting the biological evaluations, is the link between the composition of the ENPs and the observed effects, since ENP contamination (*e.g.* endotoxins, heavy metals) can generate false positive data (*e.g.* inflammatory responses, due to the presence of endotoxins) (Grzincic et al., 2014). At the moment, there is a poor choice of sterilisation methods that can be applied specifically to ENPs.

Currently, there are no validated *in vitro* testing methods for ENPs. Be that as it may, most of the currently available OECD guideline testing methods for conventional chemicals are likely to be acceptable, as a first approach for the hazard assessments of specific ENPs (SCENIHR, 2006). However, it is important to stress that the tests should be carefully assessed regarding their applicability for ENPs, by the incorporation of appropriate controls. Presently, one of the challenges for the toxicity assessments of ENPs is to overcome the interference in toxicity assays that is caused by the physicochemical characteristics of the ENPs. These interferences may generate artefacts, contributing to a misinterpretation of the results, and thus, leading to incorrect conclusions (Fig. 3B). Most of the available cytotoxicity assays are based upon optical detection *via* colorimetric and fluorimetric analyses. The ENP properties that interfere with conventional cytotoxic assays include their adsorption capacity, optical properties, hydrophobicity, chemical composition, size and surface charge (Song et al., 2010). Guadagnini et al. concluded that many of the physical and chemical characteristics of poly (lactic-*co*-glycolic acid) (PLGA) and TiO_2, together with uncoated and oleic acid coated Fe_3O_4, interfered in common toxicity methods (tetrazolium salt reduction assays (WST-1 and MTT), lactate dehydrogenase, neutral red, propidium iodide, 3H–tymidine incorporation, cell counting, oxidative stress detection and ELISA) (Guadagnini et al., 2013). A high inter-laboratory variability following colorimetric and fluorometric assays and the potential interference of silver nanoparticles (AgNPs) with IL1b and TNFα secretions on the human monocytic cell line (THP1) has also been reported by Piret et al. (2016). The same was observed with the human lung adenocarcinoma epithelial cell line (A549), as well as with caspase 3 and 7 activities. The proteolytic activities of the analysed caspases that are based upon fluorescence assays may be disturbed when using AgNPs. Silver nanoparticles also interfere with the final reaction product in an ATP determination assay based on luciferin-luciferase bioluminescence (Guadagnini et al., 2013; Ong et al., 2014). NPs, such as silver (Ag) and CuO, can interfere in analyses due to their capabilities of releasing ions, and thus, preventing the antibodies from binding to a protein, or by inactivating a reagent in a biochemical assay (Heng et al., 2011).

Indeed, nanoparticles in cytotoxicity assays optically interfere with the *in vitro* assays by scattering or absorbing light in the same spectral range of the test. They can adsorb and deplete the reagents of the biochemical reactions, or even secrete the cell products *in vitro*. These particular phenomena influence the production of the measured end-products or the detectable secreted proteins, contributing to some

misinterpretations of the results (Fig. 3B) (Guadagnini et al., 2013; Piret et al., 2016; Ong et al., 2014). In order to avoid possible interference problems, diverse adaptations should be applied to the testing methods. For instance, centrifugation, several washes, or even removing supernatants, are recommended approaches to avoid interference. It is also to be encouraged to use more than one *in vitro* assay with different detection methods and to choose adequate controls. Many works that have been published since 2010 have not reported adequate controls, in order to evaluate the interference on ENPs (Guadagnini et al., 2013; Ong et al., 2014). The absence of adequate controls makes the interpretation of the observed effects practically impossible. Caution should also be exercised with the stabilisers that are used to de-agglomerate the ENPs, since they should also be tested and introduced as a reference in the experimental set-ups. For the negative controls, it would be advisable to separately test the dispersant agents under the same conditions.

Flow cytometry is considered as one of the methods that is less sensitive to ENP interference, however, for specific analyses, charged ENPs can react with the antibodies that are sequestering them and this contributes to false cell labelling (Guadagnini et al., 2013; Fujisawa et al., 2002; Wright et al., 1994). A recent study when using flow cytometry revealed no alterations of bone cell viabilities upon exposure to TiO_2 ENPs, however, for the high concentrations, some alterations in the cell cycle were observed (Ribeiro et al., 2016). Efforts should be conducted in order to evaluate more than cell viability and cell cycle analyses should be considered as an interesting endpoint (Ribeiro et al., 2016; Medina-Reyes et al., 2014; Kim et al., 2011). To overcome the inter-experimental variations, reduced time and cost would also make substantial savings. This would allow for the use of high throughput screening (HTS) methods and high content analyses (HCA) for the toxicity of the ENPs, taking into account the testing of large numbers of different materials, at different concentrations, as well as on different types of cells (Collins et al., 2016).

Proper Reference Materials (RMs) are needed in toxicological studies in order to deliver reliable results. A reference material undergoes a procedure of validation or round-robin assessment, thereby, having specific predefined requirements for its homogeneity and stability (Orts-Gil et al., 2013). Most of the RMs produced at the moment focus on metrological characteristics, such as size and composition. Nevertheless, they are ignoring the essential parameters, such as agglomeration and the formation of protein corona in biological media (Stefaniak et al., 2013). Furthermore, most of them are not candidates for toxicological studies, since they are not compatible with the isotonic solutions at a physiological pH (Orts-Gil et al., 2013). Recent publications have demonstrated the current need of multi-parametric RMs for toxicological studies (Orts-Gil et al., 2013). Spherical gold nanoparticles, having primary particle diameters of 15 nm, have been synthesised and characterised as a reference material, while an inter-lab comparison study regarding their particle size has demonstrated a good homogeneity. Gold citrate nanoparticles are intended for two possible internal applications: an internal validation of instrument performance that is related to the dimensional characterisation of the nanoscale particles, but also as a negative control for the evaluation of *in vitro* assays in order to assess specific biological responses.

The new trend in nanotoxicology is to go further and evaluate the toxicity of ENPs under more realistic conditions, moving from 2D to 3D single cell or multi-cell cultures. Although the literature has demonstrated some encouraging results, the complexity of the tissues and the vascularisations are still a problem. The hypoxia in the centre of the spheroid that leads to cell necrosis is also an important issue to solve in order to overcome the limits of toxicological evaluations of ENPs (Chia et al., 2014; Ulusoy et al., 2016).

5. Summary

The Risk Assessment of ENPs has been challenged by significant variability and uncertainty in the data on physicochemical properties, toxicological effects, exposure doses, interspecies differences, and so forth. This is partly because as manufactured, the pristine ENP undergoes various alterations by aging processes or upon a change of the external conditions. For instance, by agglomeration or de-agglomeration processes, upon the suspension preparation, upon the incorporation into products, followed by the subsequent release into the environment.

Most ENPs are not stable in a biological milieu, where their aggregation state and their surface properties, including the formation of a protein corona, make their characterisation complicated, but at the same time, it is essential to predict the ENP hazards. In order to conduct a realistic risk assessment of ENPs, it is extremely important to identify the key physicochemical properties that can foresee different toxicological results, as well as to understand the interactions of the ENPs with biological systems. Adaptations and new methods to standardise *in vitro* testing are emerging, as well as systems mimicking the *in vivo* environment. However, additional efforts in order to provide standards and comparative methods in NP production are also essential. There is clearly the need for faster, more cost-effective methods, for assessing the toxicology of ENPs.

Scientists have considered the development of appropriate reference materials and methods in order to manage and to reduce the associated risks of extreme importance. These reference materials can be further employed on biological approaches (*in vitro*, *in vivo* and *in silico*) for assessing the toxicity of ENPs and the associated risks to human health and the environment. There is a well-defined need for cost-effective approaches for assessing the nanotoxicology of ENPs. One interesting recent finding that has been published regarding risk assessors and regulators was that significant dissimilarities in risk perceptions were found among nano-scientists, engineers and regulators (Capon et al., 2015). These results have suggested the need to involve a multidisciplinary team of professionals, with expertise at different stages along the lifecycle of ENPs, in order to support policy developments. A continued and enhanced investment on research, education, training and the dissemination of information on ENPs should now be mandatory.

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Declaration of interests

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nanomaterials is being developed within the FP7 project NanoValid. Work Packages 3, 4, and 5, provided input from the body of thought of NanoValid.

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