Quality Assurance and Safety of Crops & Foods, 2014; 6 (3): 263-279
SPECIAL ISSUE: *Food chemistry and safety*

Wageningen Academic Publishers

Safety assessment of engineered metallic nanoparticles in foodstuff

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Received: 18 December 2013 / Accepted: 27 May 2014

REVIEW ARTICLE

Abstract

The rapid proliferation of nanotechnology and the increasing use of nanomaterials and nanoparticles in many different fields (ranging from electronics, construction, medical and pharmaceutical products, cosmetics, household appliances, textile, motor vehicles, food, etc.), has led to the production of a variety of consumer products currently available on the market. In spite of such a widespread presence, the safety of such products for human health and for the environment has not yet been fully established. This issue is of particular concern for the food sector, especially for metallic nanoparticles that are not biocompatible and tend to accumulate in the organism. These concerns seem to arise from the current lack of knowledge of the potential toxic effects of engineered nanoparticles on human and environmental health as well as from the lack of standardised methodologies to test such effects and the lack of specific regulations. This review presents an overview of the current way to assess the safety of nanoparticles and nanotechnological applications, with a special focus on metallic engineered nanoparticles and their application in the food sector. Specifically, it illustrates the methodological gaps and as well as the current regulatory and ethical issues related to this branch of technology.

Keywords: analytical methods, food, metals, nanoparticles, toxicity

1. Introduction

In the past few years nanotechnology has become a significant component of the food industry. The use of nanoparticles (NPs) and nanomaterials (NMs) is present in all steps of the food chain, from the design of new ingredients or additives, to the development of systems for controlling food quality as well as for packaging, demonstrating the great potential of this new technology in a sector as traditional as food (Pérez-Esteve *et al.*, 2013). The number of patents describing nanotechnological applications in the food sector has also increased significantly over the past 10 years (Grobe and Rissanen, 2012), indicating that the use of NPs and NMs is growing rapidly within the food industry (Chaudhry *et al.*, 2008).

The major applications of nanotechnology in the food industry are basically directed at the following areas of interest: (1) food packaging; (2) food ingredients or additive of nano-size; nano-encapsulation and specific delivery systems for functional molecules; (3) nano-structures producing the modification of the structure and consistency of the food matrix; and (4) design of instrumentation used to establish food safety (nano-sensors, nano-filters, etc.).

Metallic NPs and NMs are used mainly because of their antimicrobial properties that have been demonstrated in a number of studies (see for example Senior *et al.*, 2012; Shi *et al.*, 2014). The metals mostly used as NPs or NMs in the food sector are: titanium oxides, silver, gold as well as copper, zinc, and magnesium. As shown in Figure 1 food and beverage is the second largest sector to introduce nanotechnology-based consumer products containing metallic NPs (Project on Emerging Nanotechnologies, 2014).

Silver is undoubtedly the metal most widely used in the development of nano-based consumer products, as it is considered one of the most effective antimicrobials among metals (Khurana *et al.*, in press). The other metals also display such property though to a lesser extent. However, copper has also been shown to be an efficient sensor for humidity and titanium dioxide has resistance to abrasion

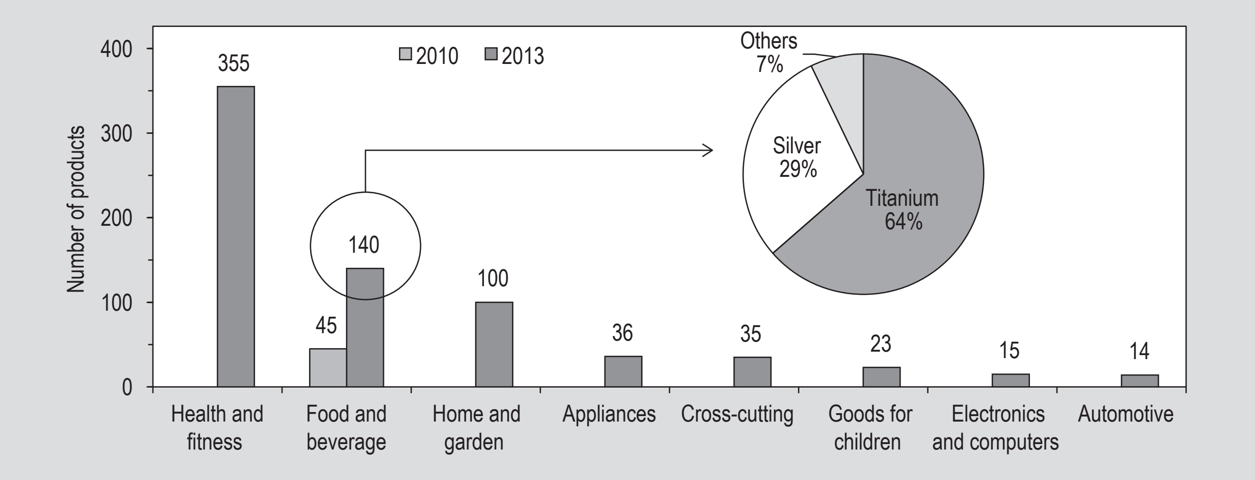

Figure 1. Number of consumer products containing metallic nanoparticles.

and a UV-blocking effect (Llorens *et al.*, 2012). The most interesting applications of metallic NPs in the food industry are in food packaging components; in the development of food additives and nano-encapsulation agents as well as in the design of instrumentation used to establish food safety. Since the latter application does not include direct contact between the metal and the foodstuff, it will not be discussed in the present work. A brief overview of metal NP applications in the food sector is given below and in Table 1.

Nanotechnology applied to food packaging

Nanotechnology-derived food contact materials (FCM) used for food packaging can incorporate NPs and NMs to improve packaging characteristics such as flexibility, gas barrier properties and stability. Polypropylene and nanoclay-based nanocomposites are some of the materials most frequently employed in this area (Lei *et al.*, 2006). Food contact materials of this type often contain up to 6% by weight of NPs, often metal-based. Due to their lower viscosity and to their barrier, optical and recycling

Table 1. Main applications of metallic nanoparticles in the food industry.

Application	Products	Functionality
Food packaging	packaging with improved properties	higher flexibility gas barrier durability and stability temperature and flame resistance processability UV-blocking/optical properties
	active packaging	antimicrobial oxygen scavenging ethylene scavenging
	intelligent packaging	detection of food spoilage monitor storage conditions
New food products	nano-sized ingredients	higher dispersability enhance tastes and flavours better quality and oxidation resistance enhance the uptakes
	nano-encapsulation	improved stability of ingredients control of release masking unpleasant tastes and flavours enhance the uptakes

properties these NMs can improve the durability, temperature resistance, flame resistance and processability of the FCM (Llorens *et al.*, 2012).

A further application of nanotechnology to food packaging is the development of active FCMs. These materials incorporate metallic NPs with antimicrobial or oxygen scavenging properties. Several NPs are used for this purpose (i.e. silicate, silver (AgNPs), magnesium oxide and zinc oxide NPs), which provide the FCM with antimicrobial effect or with properties against degradation (Fernandez *et al.*, 2012). Some examples of such applications are shown in the work of Busolo *et al.* (2010), who incorporated AgNPs in polylactic acid to obtain film packaging which was highly transparent, with an enhanced water barrier and good biocide property. Conte *et al.* (2013), on the other hand, embedded copper NPs in polylactic acid and obtained biofilms with good antibacterial activity that could preserve the sensory attributes of fresh dairy products, whereas Jiang *et al.* (2013) used alginate AgNPs coating materials to preserve the sensorial characteristics of shiitake mushroom and demonstrated that this material could expand the product shelf-life and improve the preservation of its characteristics.

Intelligent food packaging systems are also based on nanotechnology. This type of FCM incorporates nanosensors to monitor and report the condition of the food over time. For this application, NPs are applied as reactive particles, which will respond to environmental changes, degradation products or microbial contamination (Bouwmeester *et al.*, 2009). This application is useful for quality control, to ensure that consumers are able to purchase products which are at their peak of freshness and flavour, as well as for improving food safety by preventing and reducing the frequency of food-borne diseases. Many of these systems are based on the observation of colour changes that occur to metal NPs in the presence of specific analytes. Nanosensors are able to detect small organic molecules, microorganisms or gases. For example, Au NPs functionalised with cyanuric acid groups selectively bind to melamine, an adulterant used to artificially inflate the protein content of pet foods and infant formulas. The melamine-induced aggregation causes Au NPs to undergo a reproducible, analyte-concentration-dependent colour change from red to blue, which can be used to precisely measure the melamine content in raw milk and infant formula at concentrations as low as 2.5 ng/ml with the naked eye (Duncan, 2011).

Food ingredients or additive of nano-size and nano-encapsulation of functional molecules

Nano-ingredients are functional compounds which have been manufactured in the nano-size range and can be added to food. Due to the increased surface area, a very small amount is needed for the development of their function. The main advantage of reducing to the nano-size functional molecules is the improvement of the dispersability of water-insoluble compounds without the need to use additional fat or surfactants. Due to the large surface areas, these compounds usually have the ability to enhance taste and flavours. Moreover, they can be absorbed and taken up by the body more easily in comparison to their conventional equivalents. Metals can also be used to this end. One example is the use of nano selenium (SeNPs) in animal feed to improve meat quality, and oxidation resistance (Cai *et al.*, 2012). As a matter of fact, Ungvári *et al.* (2014) demonstrated with a rodent study that lamb meat enriched with selenium by feeding lambs with SeNPs provided high functional dietary benefits. Metallic NPs largely found as additives in foods are TiO_2NPs, mainly due to their whitening properties (Weir *et al.*, 2012). This compound is especially evident in chewing gums (Chen *et al.*, 2013).

Bioactive substances can be encapsulated by specific nanostructures as nano-micelles, liposomes or protein-based carrier systems. This application is specifically useful for those functional molecules which are sensitive to environmental conditions such as light, temperature and pH (such as β-carotene; Gutierrez *et al.*, 2013), present functional properties associated with unpleasant taste, or present absorption problems. Nano-encapsulation allows the preservation of nutrients and supplements during processing and storage, the masking of unpleasant tastes and flavours, the control of the release in the body thus assuring an optimal functionality of the compound, and enhancing the uptake of the nutrients (Chaudhry and Groves, 2010). Choi *et al.* (2010a) for example, improved the thermal stability of conjugated linoleic acid by encapsulating the compound with zinc oxide NPs.

From this brief overview it is possible to appreciate that there are important benefits associated with the application of nanotechnology to the food sector, such as the improved uptake, absorption and bioavailability of nano-size ingredients, the improved nutritional and organoleptic characteristics of nanostructured foods and the guarantee of food safety and quality through the development of specific packaging materials. In spite of all these advantages, the use of nanotechnology in the food industry raises important safety concerns that are similar to those that were raised in the case of genetically modified foods. Such concerns are well justified and have also been addressed in other works (Card *et al.*, 2011; Chaudhry *et al.*, 2008; Wang *et al.*, 2013). However, it is important to distinguish between organic NPs and inorganic NPs such as for example metallic NPs. Many food proteins and carbohydrates, for example, exist naturally at the nano-scale, moreover when proteins, carbohydrates and lipids are digested they are broken down into nano-scale elements before being assimilated. This means that the human body is already used to dealing with NPs in the gastrointestinal tract. The nanostructures made of lipids, proteins or carbohydrates used for encapsulation or for the development of delivery systems are also known entities for the human body. In spite of this, there is no certainty that the artificially engineered NPs (ENPs) even if they are based on natural substances, can maintain the same structure as the NPs occurring naturally and can therefore be totally harmless (Chaudhry *et al.*, 2010). Nevertheless, a greater problem originates from the use of inorganic bio-persistent NPs such as the metallic NPs described earlier. These particles are not biocompatible or biodegradable and for this reason they can easily accumulate in the human body with the potential to cause serious health problems.

It is important to stress that both natural and engineered NPs might occur in food through several pathways: naturally, intentionally or by contamination (for example, by combustion events) (Pérez *et al.*, 2009). In this article we will focus our attention on intentionally added NPs.

In spite of the rapid growth in the use of NPs in the food sector and the many doubts concerning their safety for human and environmental health, there is not yet a clear-cut conclusion on the possible health hazards that these new entities may present. As a matter of fact, the toxicity testing of NPs is a very complex issue, as the properties of these new entities can highly complicate the experimental procedure as well as the interpretation of the results.

In this review we report the state-of-the-art of how to perform toxicological studies with NPs including the analytical gaps as well as the current legislation. Due to their possible health impact we focus mainly on metallic NPs, although most of the toxicological and analytical methods described as well as the respective gaps are applicable to any type of NP.

2. The toxicology of nanoparticles

From the earliest days of nanotechnology, it became clear that the unique properties of NPs, besides allowing important technological advantages in many fields, could also represent a potential hazard for human and environmental health (Arora *et al.*, 2012). In fact, as reported in meetings of the UK Royal Society and the UK Royal Academy of Engineering a few years ago (RS/RAE, 2004), there was a concern that ENPs may lead to unexpected exposure routes, may have access to unanticipated biological compartments and may exhibit unconventional biological behaviour associated with their size (Maynard *et al.*, 2011). A number of authors claimed that the standard toxicity parameters might not be suitable for assessing the toxicity of these entities (Bouwmeester *et al.*, 2009; Rushton *et al.*, 2010). Given the specific characteristics of NPs and as a consequence the possible need to develop alternative toxicology testing strategies, Donaldson *et al.* (2004) proposed the creation of a sub-category of toxicology and named it nano-toxicology.

Nano-toxicological studies, in fact, differ from classical toxicological studies because the testing approach must also satisfy and consider the following issues (Figure 2):

1. Performance of a full physicochemical characterisation of the compound under study. This is necessary since toxicity might be affected by the specific properties of the NPs.
2. Establishment of a correct dose metric and a correct dosimetry for each compound and for each study, since these parameters might differ greatly in comparison to the correspondent macro-scale material.
3. Performance of *in vivo* long-term experiments, especially when testing the toxicity of food.

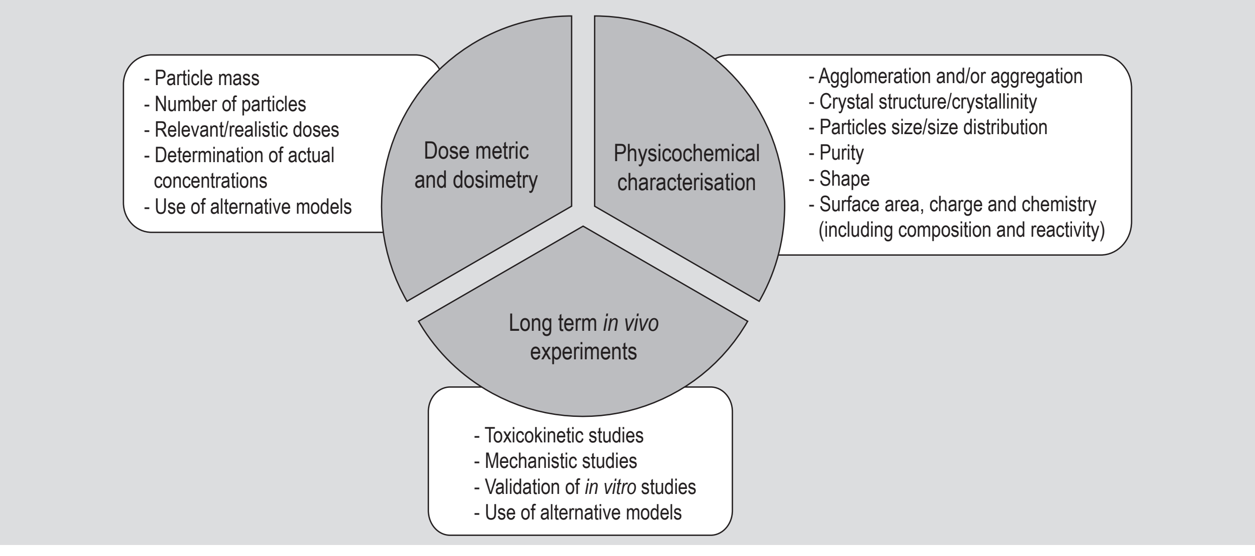

Figure 2. Specific key issues to consider in nano-toxicological studies.

These issues have been identified. However, they are not yet fully resolved and further studies are still needed to optimise current nano-toxicological testing.

Physicochemical characterisation

The physicochemical characterisation of the NPs is the first essential step of any toxicological study as reported by several Authors (Oberdörster *et al.* 2005; Silbergeld *et al.*, 2011; Tsuji *et al.*, 2006; Warheit, 2008). Among the different parameters that it is advisable to test (Oberdörster *et al.*, 2005), the following are probably the most relevant (Card and Manguson, 2007):

1. Agglomeration and/or aggregation. These parameters are extremely important as the size, dynamics and properties of an agglomerated/aggregated cluster of particles can be extremely different with respect to the same characteristics of an individual particle. These parameters influence the possibility of transport between organs and across cell barriers, and finally can greatly affect toxicity.
2. Crystal structure/crystallinity. Internal particle structure can affect toxicity. Several experiments studied the toxic effect of different crystal phases of TiO_2NPs. Jiang *et al.* (2008) for example, demonstrated that the oxidant reactivity exhibited by TiO_2 particles with similar size but different crystal structures was highest for amorphous samples followed by anatase, and then anatase/rutile mixtures, and lowest for rutile samples.
3. Particle size/size distribution. The size of the particles greatly affects the toxicity. It has been shown that smaller particles are generally more toxic than bigger ones. However, this depends on the endpoint analysed. Some pulmonary toxicity studies in rats, for example, have demonstrated that exposures to ultrafine NPs produced enhanced toxicity responses when compared with larger particles of similar chemical composition (Donaldson *et al.*, 2001).
4. Purity. It is important to establish the purity level of the NPs as in many cases the toxic effect is exerted not by the NP itself but by the chemical impurities present in the compound as a result of the manufacturing procedure.
5. Shape. The shape of NPs can affect toxicity. For example, polyhedral particles are generally considered more toxic than spherical particles because the polyhedral shape increases the surface area of the particle and consequently its reactivity and possibly its toxicity (Chae *et al.* 2009).
6. Surface area, charge and chemistry (including composition and reactivity). NPs do not move freely into all biological systems but can pass through one cell, tissue organelles or another depending on the molecules added on their surface (functionalisation). For this reason it is important to define their surface chemistry.

The way to perform the physicochemical characterisation as well as the methodological gaps still existing in this sector is discussed further.

The physicochemical characteristics of NPs can modulate the biological response of the testing organisms differently with respect to what is expected from the chemical composition alone. Furthermore, such characteristics are very variable and may change depending on the production batch, on the medium they are dissolved in and on the experimental conditions (i.e. exposure time, temperature, presence of light, presence of ions, etc.) (Murdock *et al.*, 2008). For these reasons Oberdörster *et al.* (2005) recommended performing such characterisation on the compound at three different levels: (1) as it is purchased; (2) as it is administrated; and (3) *in situ*, that is, throughout the experiment. However, the majority of the nano-toxicological studies do not report an accurate analysis of the particles properties in experimental conditions, and this might reduce the reliability of the results. Moreover, even in a well-characterised experiment, it is likely that the experimental conditions will not match the realistic ones. For example, a study of human toxicity via ingestion should evaluate the characteristics of the NPs in the conditions in which they would be inside the body once they are swallowed: i.e. the condition of the gastrointestinal tract, the conditions of the systemic post-translocation, the dissolution, the bioaccumulation, etc. Currently such evaluation is not routinely performed and this could lead to data misinterpretation or incorrect results.

Dose metric and dosimetry

In the majority of traditional toxicological studies the dose of the compound to test is expressed as mass per volume (e.g. µg/ml). This approach is not always valid for NPs dose metric. Contrary to their corresponding macro scale compounds NPs present a different relationship between mass and number of particles and this can affect the toxic effects differently, especially if the toxic response depends on the size of the particles. Moreover, in contrast to soluble compounds, NPs generally tend to diffuse, settle and agglomerate to different degrees in the exposure media, modifying the actual concentration that enters in contact with the biological system chosen for the toxicity test. If all these issues are considered, establishing a correct dose metric is a critical factor for evaluating the toxicity of NPs (Maynard *et al.*, 2011). Several studies have indicated that a surface-based concentration correlates better with the effects and endpoints of NPs with respect to the weight-based concentration, especially for insoluble particles such as metals. However, this is not always true, as reported for example by Warheit *et al.* (2006) in a study performed on TiO_2.

It is very probable that the dose metrics will have to be different depending on the nature of the NP and that more than one parameter should be used in order to define with exactitude the actual concentration of exposure. Further studies will surely be needed to define this concept more closely. In any case, we believe it is important to stress the fact that a measurement of the concentration of the NPs after exposure is crucial for determining the actual concentration that eventually comes into contact with the biological system used for the testing. Teeguarden *et al.* (2007) for example, found discrepancies between the amount of material introduced to *in vitro* cell cultures (the dose) and the amount of material cells were able to interact with. This suggests that future work is needed to characterise particle doses *in vitro* as well as *in vivo*.

Another issue that needs to be considered in nanotoxicology is the selection of an exposure concentration that matches the realistic exposure concentrations relevant for human health. Most of the earliest nano-toxicological studies were performed *in vitro* using excessive concentrations not representative of the realistic concentrations found in nature. *In vitro* data with high, unrealistic exposure doses can be useful for understanding NPs mode of action. However, they are difficult to interpret and to extrapolate to humans for appropriate risk assessment studies. Moreover, such data are not useful for predicting nanotoxicity (Oberdörster, 2010). It is therefore necessary in any nanotoxicological study to establish a correct dosimetry and define toxicological relevant exposure concentration for humans before designing the experiments.

In vivo long-term experiments

The toxic effects of NPs on human health have been studied mainly *in vitro* specifically using cell cultures. *In vitro* assays are simple and cost-effective methods, ideal for the screening of molecules, studying biological pathways or to identify the role of specific genes. However, these types of assay present some limitations in the case of NPs toxicological studies. This is partly due to the fact that the behaviour of NPs once they enter the human body is still not fully understood. Proper toxicokinetic studies need to be performed if we are to begin to understand the mode of action and the type of toxicity that NPs might lead to and these can only be performed *in vivo*. As stated by Fischer and Chan (2007): 'the combined results from multiple studies of different cells *in vitro* cannot be assumed to capture the same behaviour as the same cells arranged *in situ* in an organ'. Besides toxicokinetic studies, in order to understand and properly address the danger that NPs present it is important to establish the influence of the transport systems (lymph, blood and bile) as well as the immune system and these can only be studied on a whole animal. The fact that the results obtained with nanotoxicological *in vitro* experiments do not always agree with results obtained *in vivo* (Sayes *et al.*, 2007) supports the need for *in vivo* research. *In vitro* studies can be used but they should always be validated or supported by corresponding *in vivo* studies.

In spite of its importance *in vivo* experimentation with animal models can be expensive, time consuming and ethically demanding. To solve this problem, the use of non-animal models that are able to provide similar responses is currently expanding. A recent report from the Transatlantic Think Tank for Toxicology (t^4), for example, recommended the development of methods using alternative whole animal models such as zebra fish (*Danio rerio*), medaka (*Oryzias latipes*) at their earliest developmental stages when they are not yet considered experimental animals (Strähle *et al.*, 2012), or nematodes (*Caenorhabditis elegans*). As a matter of fact, a variety of studies on NP toxicity have been published using such models in the past few years (see for example Asharani *et al.*, 2008, 2010; Bohnsack *et al.*, 2012; Chae *et al.*, 2009; Zanni *et al.*, 2012). This approach is very effective as it allows screening of a large variety of molecules (or experimental conditions) to detect the toxic effects of NPs on an entire organism. As a matter of fact, all these models (but especially zebra fish) are used as a predictive toxicology model for humans and can be considered ideal systems for the intermediate steps between *in vitro* cell-based tests and mammalian assays. This approach does not eliminate the use of animal experimentation (rodents, or other animals), but it can help in the application of the principle of the 3Rs as it fully complies with the replacement action (Embry *et al.*, 2010; Strähle *et al.*, 2012).

The most agreed-upon strategy for assessing the safety of NPs is the tiered approaches of *in vitro* and *in vivo* screening studies followed by proper *in vivo* studies and supportive mechanistic studies (Oberdörster *et al.*, 2005; Warheit *et al.*, 2006). However, most of the investigations that have been performed to date have mainly been focused on defining acute toxicity and mortality rather than chronic exposure and morbidity. As a matter of fact, NPs are thought to have long-term effects, due to the DNA damage they cause and the fact that they might accumulate in the human body. In spite of this, only a few long-term studies have been performed so far (Naddy *et al.*, 2007; Sung *et al.*, 2009) and this issue has not yet been properly addressed (Luoma, 2008; Silbergeld *et al.*, 2011).

3. Toxicity of metallic engineered nanoparticles

Metallic NPs are those that raise major concerns in the food sector. In fact they potentially show both the toxic effects attributed to metals and those attributed to the nano-size; moreover they are generally not biocompatible and tend to accumulate in the human body. Among the commonly used metals in the food sector are silver, titanium and to a lesser extent zinc and iron. These compounds are mainly used as antimicrobials and are incorporated in FCM to protect the foodstuff from contamination and for increasing its shelf life. In some cases they are also used as food additives. One of the great challenges for nanotoxicologists is to understand the mechanism of action of NPs. In the case of metal NPs, the toxic effects detected are mainly oxidative stress, inflammation, apoptosis and cell metabolic disorder (Choi *et al.* 2010b; Kim and Ryu, 2013; Kim *et al.*, 2009; Park *et al.*, 2011; Shaw and Handy, 2011; Stone *et al.*, 2007). However, it is still debatable whether such effects are due to the size of the metal NPs, to the release of metal ions or to both factors and the results published on this topic are still rather controversial. Such studies have mainly been performed on AgNPs. According to the results of Kim *et al.* (2009), who studied the cytotoxicity of AgNPs on human hepatoma HepG2 cells, the toxicity of nanosilver is due to an intrinsic effect of the NPs independent of the presence of free Ag ions. In contrast, the results reported by Bouwmeester *et al.* (2011), who carried out a transcriptomic study on Caco-2 cells comparing the exposure to nanosilver and ionic silver, indicated that the genes expressed were the same in the two conditions. These authors concluded that the effect could be attributed only to the presence of Ag ions. Zhao and Wang (2011) also observed that the toxic effect of AgNPs was due to the release of Ag ions. Other studies pointed out that the toxic effect of AgNPs could not be explained solely by the release of ions but rather by the combined effect of ions and NP size (Griffitt *et al.*, 2008; Kawata *et al.*, 2009; Laban *et al.*, 2010; Navarro *et al.*, 2008). Beer *et al.* (2012) also drew the same conclusion. However, these authors observed that the effect of the NP size depended on the concentration of ions released in the media, that is, at low ion concentrations the effect of NP size was predominant, whereas at high silver ion concentrations the toxic effect of Ag in ionic form dominated. Olasagasti *et al.* (2014) also supported these findings. Moreover these authors suggested that the solubilisation medium affects the toxicity of the NPs. As a matter of fact, the ion composition of the suspension solution can greatly affect the availability of the metals ions released from the NPs and as a consequence their toxicity.

4. Current methodologies for the determination and characterisation of nanoparticles

Methods for the characterisation of metal nanoparticles

The physicochemical property of a metal particle, which is a key parameter for determining their application, mainly depends on particle size and shape (Liu *et al.*, 2012). However, 'size' and 'shape' should be accurately defined (e.g. geometrical, hydrodynamic, or optical radius) in order to be able to compare results from the different methods available (Zattoni *et al.*, 2014). Table 2 gives an overview of the techniques usually applied to the characterisation of metallic NPs.

Electron microscopy-based techniques are the most common ones for the visualisation and characterisation of NPs. They can provide data on size, shape, distribution, agglomeration, presence, thickness, etc. In some cases even chemical composition can be achieved. (Luo *et al.*, 2013). For this purpose the most common techniques are scanning and transmission electron microscopy (SEM and TEM) which are suitable for solid samples and in the absence of water. However, food is a very complex matrix and samples should be prepared adequately but with minimum alteration. In this regard, much effort has been made to develop new preparation methods such as chemical fixation, drying, sedimentation or freezing and to adapt these technologies to the characterisation of liquid samples (Dudkiewicz *et al.*, 2011).

Despite the useful information obtained by these techniques, there are several drawbacks for routine analysis of food samples. Amongst them, it is important to mention the high cost of analysis and the time needed to carry them out. Only a very small part of the sample is used for analysis and then a lot of samples need to be analysed to get representative results. Nevertheless, once NPs are detected in the sample the information provided is highly valuable. EM can also be combined with other techniques for the determination of the elemental composition such as energy-dispersive X-ray spectroscopy (EDS) (Gatti *et al.*, 2009). Another useful tool for NP imaging is atomic force microscopy (AFM) (Balnois *et al.*, 2007; Lacava *et al.*, 2001). The main advantage of AFM is that it images sub-nanometer structures under wet or moist conditions. However, sometimes it generates severe overestimations of the lateral dimensions of the nanoparticles (Tiede *et al.*, 2008).

Light-scattering techniques are also very useful for the evaluation of the particle size of NPs. Although NPs are too small to be imaged directly by visible light, when illuminating with a laser beam, the light scattered by them can be detected and used for localizing them. Light-scattering techniques are of two principal types: classical and dynamic. Classical light scattering can yield the molar mass, radius of gyration, and second virial coefficient, whereas dynamic light scattering (DLS) may provide information about not only the particle size (hydrodynamic radius) and distribution but also the zeta potential that is related to the electrostatic stability of NP surfaces (Brar and Verma, 2011; Murdock *et al.*, 2008). These techniques can be used in batch mode and also coupled to fractionation techniques enabling the analysis of complex samples (Schmidt *et al.*, 2011). Despite the great capabilities of these techniques, some limitations arise when analysing polydisperse materials (need for fractionation) or concentrated solutions (need for dilution) and when selecting the adequate solvents. Another scattering technique is the nanoparticle tracking analysis capable to track the movement of the particles on a particle-by-particle

Table 2. Most common techniques used for metallic nanoparticles characterisation in food samples.

Principle	Technique	Characteristics and parameters
Microscopy	SEM	single particle technique typical range: 5 to >1000 nm. size and shape, size distribution
	TEM	single particle technique typical range: 1 to >1000 nm. size and shape, size distribution
	AFM	single particle technique typical range: 0.5 to >1000 nm. morphology, size and shape, surface structure, size distribution, 3D imaging
Light scattering	classical	ensemble technique typical range: 40 to >1000 nm radius of gyration, molecular weight
	dynamic	ensemble technique typical range: 3 to >1000 nm. hydrodynamic diameter, size distribution, diffusion coefficient, zeta potential[1]
	nanoparticle tracking analysis	single particle technique typical range: 10 to >1000 nm. hydrodynamic diameter, size distribution
Fractionation methods	centrifugation	ensemble technique typical range: 10 to >1000 nm size, size distribution
	filtration	ensemble technique typical range: 1 to >1000 nm size, size distribution
	chromatography	ensemble technique typical range: 5 to 1000 nm size, size distribution
	field flow fractionation	ensemble technique typical range: 1 to 1000 nm size, size distribution

[1] When coupled to electrophoretic light scattering.

AFM = atomic force microscopy; SEM = scanning electron microscopy; TEM = transmission electron microscopy.

basis, determining its diffusion coefficient and therefore the hydrodynamic diameter (Gallego-Urrea *et al.*, 2011; Malloy and Carr, 2006). Due to the complexity of food samples the application of these techniques requires the appropriate sample preparation and they are usually combined with fractionation methods such as chromatography or field flow fractionation (FFF) (Bandyopadhyay *et al.*, 2013).

Fractionating techniques can also provide information on NP size. For this purpose centrifugation (Wohlleben, 2012), filtration (Wu *et al.*, 2013) and chromatography (Pergantis *et al.*, 2012) and related technologies such as FFF (Poda *et al.*, 2010) have been applied. These techniques will be reviewed in the section about methodologies for the sample treatment.

Methods for identification and quantification

A summary of techniques for metallic NP identification and quantification is given in Table 3 and those based on atomic spectroscopy are the most commonly used. In particular, inductively coupled plasma-mass spectrometry (ICP-MS) provides an element specific detection that can also be applied to the identification of the chemical composition of metal NPs (Beltrami *et al.*, 2011; Blasco and Picó, 2011; Fernandez *et al.*, 2010; Scheffer *et al.*, 2008). The adaptation of this technology to the monitoring of single particles has allowed the estimation of the number of particles and their size (Mitrano *et al.*, 2012; Tuoriniemi *et al.*, 2012). In contrast to traditional ICP-MS, NPs are introduced at low flow rates providing a signal for each single particle and therefore the number of NPs can be derived from the

Table 3. Most common techniques used for the identification and quantification of metallic nanoparticles in food samples.

Principle	Technique	Characteristics
Atomic spectroscopy	ICP-MS	qualitative and quantitative on-line analysis broad range of elements can be detected it can be adapted to single particle analysis destructive dynamic range: ng/kg-µg/kg
Mass spectrometry	MS	qualitative and quantitative on-line analysis of ligands broad range of elements can be detected destructive dynamic range: ng/kg-µg/kg
Light scattering	NTA	off-line quantitative analysis (number of particles) non-destructive 10^6 to 10^9 particles/ml
X-ray spectroscopy	EDS	qualitative and quantitative analysis broad range of elements can be detected non-destructive usually integrated into SEM and TEM dynamic range: >1000 mg/kg
	XRF	qualitative and quantitative analysis broad range of elements can be detected usually integrated into SEM and TEM dynamic range: 10-10,000 mg/kg
Others	ion selective electrodes	quantitative analysis of ions highly selective destructive dynamic range: µg/kg-mg/kg
	sensors	quantitative analysis (nanoparticle and ions) highly selective (depend on receptors) it can be non-destructive dynamic range: µg/kg-mg/kg

EDS = energy-dispersive X-ray spectroscopy; ICP-MS = inductively coupled plasma-mass spectrometry; MS = mass spectrometry; NTA = nanoparticle tracking analysis; SEM = scanning electron microscopy; TEM = transmission electron microscopy; XRF = x-ray fluorescence.

time-resolved signals. In order to achieve this information highly diluted samples are needed and the efficacy is limited to particles larger than 20 nm. The size of the NP can be estimated assuming a spherical geometry (Ho *et al.*, 2013; Olesik and Gray, 2012). ICP-MS can not only be used directly on the sample but can also be combined with separation techniques such as chromatography or FFF in order to be able to distinguish between particles sizes in a complex mixture such as food or environmental samples (Helfrich and Bettmer, 2011).

Mass spectrometry is a well-known and versatile technology for obtaining accurate mass information on many organic compounds. In the case of metal NPs it can be a complementary technique to ICP-MS due to its capacity to determine entire clusters or to characterise ligands bound to the surface of NPs (Helfrich and Bettmer, 2011). Furthermore, if particle density is known, size information can be obtained from the mass spectra (Guan *et al.*, 2007).

X-ray spectroscopy comprises a range of techniques useful for characterising NP surfaces and coatings. Some of them, such as EDS or x-ray fluorescence, have been applied to determine the elemental composition of NPs (Gatti *et al.*, 2009). Although they are less sensitive than ICP-MS and are not capable of distinguishing between ionic, bulk metallic and nanoscale particles, they are valuable for the rapid screening and quantification of elemental content when combined with appropriate fractionation and separation methods. For this purpose, portable equipment has been developed and used in many fields (Sánchez-Pomales *et al.*, 2013).

The quantification of NPs is a challenging task because not only should the total amount of metal be determined but also the number of NPs. With this aim, some of the aforementioned techniques such as single particle ICP-MS or electron microscopy can be used. Moreover, methodologies should differentiate the variety of forms in which metals can occur (ionic form, several valences may exist, NPs, etc.). None of the methods available can cope with all the desirable characteristics, therefore a combination of methods are needed.

As alternative methods, electroanalytical techniques can provide multi-elemental detection capability of metals in their ionic form. The use of ion selective electrodes permits the quantification of ions at very low concentrations and usually requires low-cost and low-maintenance instrumentation (Koch *et al.*, 2012). Another approach is the use of sensors (Sadik *et al.*, 2009). Raz *et al.* (2012) developed a surface plasmon resonance-based sensor based on the affinity of silver to certain binding proteins such as methallothionein. If silver ions were removed from the sample, AgNPs were also able to bind the biofunctionalised surface of the chip.

Methods for the sample treatment

The determination of NPs in food matrices requires the extraction, isolation, removal of interferences or artefacts, and, in the case of low amounts of NPs, a pre-concentration step prior to their characterisation and quantification (Figure 3). These are critical procedures because they might change the properties of NPs and thus should be minimised as much as possible. In comparison to other techniques electron microscopy imaging usually needs softer preparation procedures and much effort has been made to improve the conservation of the original state of NPs. Nevertheless, in most cases and with most analytical techniques, these steps are unavoidable and should be recorded (Tiede *et al.*, 2008).

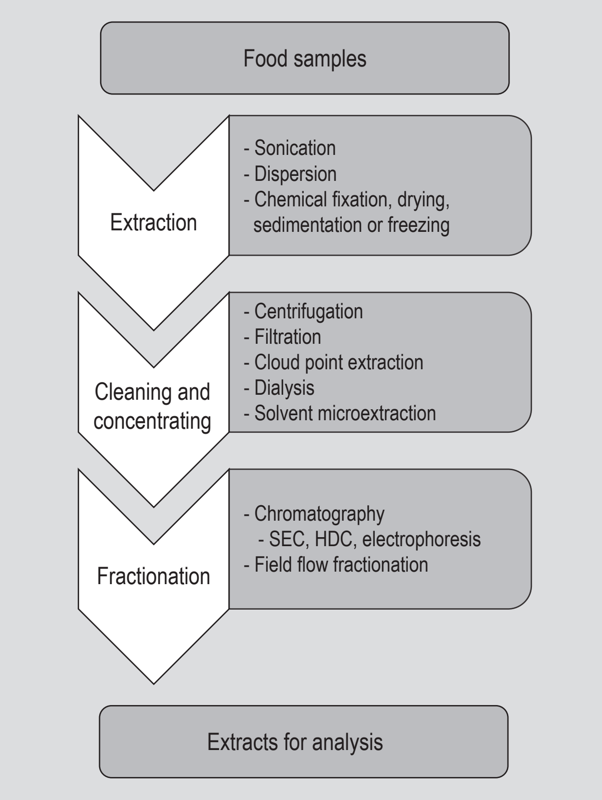

Figure 3. General workflow of the sample treatment for the determination of metallic nanoparticles in food matrices. HDC = hydrodynamic chromatography; SEC = size exclusion chromatography.

Traditional analysis of metals from foods involves a sample preparation based mainly on digestion with strong acids resulting in the determination of the total amount of metal (sum of NPs and the ionic fraction). In order to measure the NPs, very few methods have been reported for their extraction and isolation. Sonication and dispersion with chemicals have shown to be useful for the release of NPs from the matrix (Hassellow *et al.*, 2008; Peters *et al.*, 2011). However, solvents and dispersants may alter the properties of NPs, so these steps should be described in detail within the experimentation section of a publication (Bouwmeester *et al.*, 2011).

Once NPs are released different techniques (e.g. centrifugation, filtration, cloud point extraction) might be applied to get a cleaner and more concentrated extract or separate metal ions before fractionation. Centrifugation is considered to cause the least perturbation, but it can lead to the formation of aggregates due to the differential settling velocities (Bouwmeester *et al.*, 2011). In combination with an optical detector (absorbance, fluorescence or interferometer) the sedimentation process can be monitored, which is the basis of the analytical ultracentrifugation (Carney *et al.*, 2011). The application of this technique for size characterisation has some limitations and certain assumptions are required but centrifugation greatly helps reduce the complexity of the sample. Membrane filtration with pore sizes of 100 nm or greater is one of the most common techniques due to its simplicity of operation (Kowalczyk *et al.*, 2011). In theory the retention and elution of NPs will depend on the size of the membrane pores. On the other hand, the polarisation of the membrane may lead to the deposition of metal NPs and in the end the formation of filter clogs (Hassellow *et al.*, 2008; Wu *et al.*, 2013). In order to minimise these effects, cross flow filtration has been developed (Kim *et al.*, 2006). Other preparation methods reported to remove interfering components in the extract are cloud-point extraction, dialysis or solvent microextraction (Liu *et al.*, 2012; Majedi *et al.*, 2013).

Fractionation methods, already used for metallic ENPs, are mainly chromatography and related technologies. They can be divided into two groups based on the use or not of a stationary phase for the separation. Amongst the first group size exclusion chromatography (SEC), hydrodynamic chromatography (HDC) and electrophoresis can be highlighted.

SEC is one the most popular techniques for size fractionation. It uses porous packing material as stationary phase and is based on the differences in the particles' hydrodynamic volumes and not on the interaction of these particles with the stationary phase (Kowalczyk *et al.*, 2011). Small particles will pass through the pores while large particles are excluded, eluting more rapidly. In the case of HDC, the stationary phase is a non-porous material that forms capillary routes and the separation is achieved by flow velocity (Tiede *et al.*, 2008). The elution profile is similar to SEC. The separation efficiency of HDC is often poorer, but the operating range is very good (Silva *et al.*, 2011). Electrophoretic separation is based on the particle size and also on the surface charge. To be separated NPs should be stable and not change their charge along the electrophoretic systems. This is especially important for metal NPs and usually a chemical functionalisation or the addition of a surfactant is needed to stabilise NPs (Arnaud *et al.*, 2005; Lopez-Lorente *et al.*, 2011).

FFF comprises a family of techniques for the separation and sizing of particles that is increasingly being used to characterise NPs. In contrast to the use of a stationary phase, FFF is performed in an open capillary channel, avoiding the interactions of NPs with a stationary phase and reducing the mechanical stress applied to the NPs (Kammer *et al.*, 2011). The separation mechanism is based on the diffusion coefficient of particles and the elution time can provide information about the hydrodynamic diameter of NPs. The FFF procedure involves the injection of samples onto a flowing mobile phase (longitudinal flow) and separation is achieved by means of an external field applied perpendicularly (Hagendorfer *et al.*, 2011; Zattoni *et al.*, 2014). Different types of field are available (e.g. electric fields, magnetic fields, thermal gradients, solvent flow), determining the physico-chemical properties of NPs responsible for the separation.

These chromatographic and FFF techniques can be coupled to several detectors such as MS, DLS or ICP-MS enabling a more comprehensive characterisation of NPs. However, even in the case of FFF, which is considered to cause the minimum sample alteration, the stability and properties of NPs along the sample preparation steps should be checked in order to make an accurate and reliable assessment of NPs in real conditions.

Validation of methodologies

As for other analytical methodologies, the methods applied to the determination of NPs should also be validated and parameters such as limit of detection, linearity, selectivity, etc., should be assessed (Linsinger *et al.*, 2013). In this sense, the ideal situation is to obtain results making the minimum theoretical assumptions and comparing them with reference materials. Recently, some certified reference materials have been developed for the measurement of different physico-chemical properties such as size and other non-certified reference materials are commercially available (Braun *et al.*, 2012). However, due to the lack of these materials for NPs in food, validation studies should be carried out using spiked samples. This validation step is crucial for standardisation purposes and required for the implementation of future regulations on NPs.

5. Regulation of nanoparticles and nanotechnologies applied to the food sector

In spite of the increasing applications of NPs and nanotechnology in the food sector (as well as in a variety of other fields mentioned above) and the acknowledgment of the hazard they might represent for human and environmental health, no standard methodology specifically directed at assessing the risk represented by NPs has currently been developed. As a consequence no standard regulated toxicity testing strategy has been established. Basically, the regulations of the use of NPs and nanotechnology in all their applications are currently based on the adaptation of the current legislation and not on specific regulations designed for nanotechnology (Coles and Frewer, 2013; Hansen and Baun, 2012) and it is often unclear whether the current regulations are appropriate to specific NPs and their diverse applications.

The main regulation that covers all food products is the CE 178/2002 (EC, 2002) which lays down the general principles and requirements of food law, establishing the European Food Safety Authority (EFSA) and laying down procedures in matters of food safety. According to article 14 of this regulation, foodstuff that is not safe or that contains substances that are dangerous for human health cannot be marketed. This regulation is very general but it also includes food containing NPs, NMs or produced by nanotechnology.

Among other more specific regulations that can be relevant for the food sectors are regulation (EC) no. 1333/2008 (EC, 2008) of food additives, regulation (EC) no. 258/97 (EC, 1997) on novel foods and regulation (EC) no. 1935/2004 (EC, 2004) on materials and articles intended to come into contact with food. The regulation on food additives (EC, 2008) states that 'A food additive already approved under this regulation which is prepared by production methods or using starting materials significantly different from those included in the risk assessment of the authority, or different from those covered by the specifications laid down, should be submitted for evaluation by the authority.' The concept of 'significantly different' includes the use of nanotechnologies.

In 2011, regulation (EU) 1169/2011 of the European parliament and of the council of 25 October 2011 about food information to consumers changed the existing legislation on food labelling (EC, 2011). This change aimed at informing consumers of the presence of engineered nanomaterials (ENMs) in food, and at providing a definition of ENMs, as well as providing information on the properties of nanoscale compounds. This regulation reported that 'taking into account the possibility of food containing or consisting of ENMs being a novel food, the appropriate legislative framework for that definition should be considered in the context of the upcoming review of regulation (EC) no. 258/97 of the European Parliament and of the Council of 27 January 1997 concerning novel foods and novel food ingredients'. This regulation, amended at the end of 2013, also states that 'all ingredients present in the form of ENMs shall be clearly indicated in the list of ingredients. The names of such ingredients shall be followed by the word 'nano' in brackets'.

Food Contact Materials are subject to the requirements of regulation (EC) no. 1935/2004 of the European Parliament and the Council of 27 October 2004 (EC, 2004). Article 3 requires that all FCMs should be manufactured in accordance with good manufacturing practice so that, under normal or foreseeable conditions of use, they do not transfer their constituents to food in quantities that could endanger human health, bring about an unacceptable change in the composition of food, or deteriorate the organoleptic characteristics of food. Nanomaterials when used in food contact applications do fall under these same requirements. As stated before such regulations are simply adapting the concept of NPs and nanotechnology but were not developed deliberately for such a purpose.

In 2011, EFSA published some basic guidelines on how to perform a risk assessment for NPs in the food chain (EFSA, 2011). That guidance, based on a recompilation of scientific studies on NPs toxicity stated that food containing NPs or produced by nanotechnology should be tested for their safety following an approach which should include: (1) the physico-chemical characterisation of the requirements of ENMs used; and (2) toxicological testing which, in general, should include information on genotoxicity, absorption, distribution, metabolism and excretion and repeated-dose 90-day oral toxicity studies in rodents. EFSA will be revising this document periodically, with the NP application in the food sector under fast development.

In April 2012 the US Food and Drug Administration (FDA) published the following (draft) guidance for the industry (FDA, 2012): 'Assessing the effects of significant manufacturing process changes, including emerging technologies, on the safety and regulatory status of food ingredients and food contact substances (FCSs), including food ingredients that are color additives'. This guidance includes the use of nanotechnologies in the manufacturing process of foodstuff.

In conclusion, the current frameworks aim to control the risks of the use of nanotechologies in the food chain and to define the general safety requirements assuring that only safe foods are put on the market. Although there is not sufficient scientific information to warrant the application of the precautionary principle to nano-food, it is beneficial to develop adequate initiatives to test those nanotechnological applications that might carry a risk for consumers.

6. Conclusions

In spite of the widespread use of nanotechnology and NPs in many sectors including the food industry and the presence in the market of several NP-based products, there are still concerns about their safety for humans and for the environment. These concerns rise from the numerous gaps existing at different levels: toxicology testing, compound detection and characterisation and legislation. From the data reviewed from the literature it is possible to extrapolate that it is vital to establish a correct characterisation of the material at each stage of the experimental procedure; a correct dose metric and dosimetry for NPs, to potentiate *in vivo* long-term studies. Once proper consistency data on the toxicology of NP-based products are available, the regulators will be able to establish standardised methodologies for the testing of toxicity of NPs and develop specific legislations for NP-based food and products.

Acknowledgements

This work was supported by the Spanish Ministry of Research and Innovation (project ATP-Toxbio, grant code CTQ2011-28328-C02-02) and by the Department of Agriculture, Fisheries and Food of the Basque Government (projects DARETEST and Segurfish).

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