Food and Chemical Toxicology 50 (2012) 2224–2242

Contents lists available at SciVerse ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

# Approaches to the safety assessment of engineered nanomaterials (ENM) in food

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ARTICLE INFO

*Article history:*
Available online 29 December 2011

*Keywords:*
Nanomaterial
Nanotechnology
Nanoscale
Risk assessment
Diet
Food

ABSTRACT

A systematic, tiered approach to assess the safety of engineered nanomaterials (ENMs) in foods is presented. The ENM is first compared to its non-nano form counterpart to determine if ENM-specific assessment is required. Of highest concern from a toxicological perspective are ENMs which have potential for systemic translocation, are insoluble or only partially soluble over time or are particulate and bio-persistent. Where ENM-specific assessment is triggered, Tier 1 screening considers the potential for translocation across biological barriers, cytotoxicity, generation of reactive oxygen species, inflammatory response, genotoxicity and general toxicity. *In silico* and *in vitro* studies, together with a sub-acute repeat-dose rodent study, could be considered for this phase. Tier 2 hazard characterisation is based on a sentinel 90-day rodent study with an extended range of endpoints, additional parameters being investigated case-by-case. Physicochemical characterisation should be performed in a range of food and biological matrices. A default assumption of 100% bioavailability of the ENM provides a 'worst case' exposure scenario, which could be refined as additional data become available. The safety testing strategy is considered applicable to variations in ENM size within the nanoscale and to new generations of ENM.


## 1. Introduction

Although making materials smaller can generate novel and useful properties, nanotechnology has provoked public and scientific concern and debate from the perspective of safety (e.g. Aitken et al., 2009; House of Lords, 2010). One concern is that there is insufficient knowledge on how altered physicochemical properties and potentially increased systemic bioavailability of nanomaterials may influence their toxicological properties. A cautious approach to the introduction of products of nanotechnology into the manufacture and production of consumer products, including foods, has been advocated. The Task Force on Novel Foods and Nanotechnology of the European Branch of the International Life Sciences Institute (ILSI Europe) set up an expert group to develop practical guidance on how to approach the safety assessment of products of nanotechnology, aimed at scientists involved in the research and development of such products specifically for food applications.

Much of the debate on the safe use of nanotechnology for food applications has focused on the uncertainties and unknowns, and the lack of toxicological data. Nanomaterials, as with all materials added to foods, are a disparate group with individual chemical, biological, physiological, pharmacological and toxicological profiles. Several bodies and organisations have considered the terminology to be applied to this family of materials for the purposes of evaluation from different perspectives (SCENIHR, 2007; ISO,

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doi:10.1016/j.fct.2011.12.029

2008; FAO/WHO, 2009; EFSA, 2009) and there is still on-going debate to achieve working definitions to ensure consistency (JRC, 2010; SCENIHR, 2010; European Commission, 2011). There is general agreement that size, physical and chemical characteristics of nanomaterials are the key determinants of their technological, and potentially, their biological functionality. As stated by the EU Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), free and low solubility nanoparticles are a priority concern for human and environmental safety (SCENIHR, 2009).

The aim of this paper is to provide practical guidance to inform scientists on how to address potential safety issues if new food products resulting from the application of nanoscience and nanotechnology are developed, and how to identify materials that may require particular attention. To distinguish such intentionally manufactured nanomaterials from those occurring naturally, they are referred to throughout this text as engineered nanomaterials (ENMs). This guidance is not intended to be applied to nanomaterials resulting from long-established food processing practices such as emulsification, and natural biomolecules such as enzymes and other proteins.

The terms and definitions used in the discussion of nanotechnology and its applications are themselves the subject of discussion by regulatory advisory and standards-setting bodies. For consistency and clarity, where possible, the terms developed by the International Organisation for Standardisation (ISO) are used in this paper (see Glossary), supplemented by terms used by SCENIHR. While the precise definitions may evolve over time it is not considered that this will fundamentally alter the safety assessment strategy and guidance proposed in this paper.

## 2. Background to nanotechnology

The endeavour to understand materials at the atomic, molecular and supra-molecular level can be described as 'nano science'. The exploitation of this knowledge by controlling the shape and size of materials in the nanoscale is termed nanotechnology. Nanotechnology can be used to design and produce structures, devices and systems of desired specification with altered or new functionalities, as compared to the same materials in the macro scale (also termed 'bulk' materials or 'non-nano form' materials). Nanotechnology offers an opportunity to produce innovative new food products, as well as improving existing products by more precisely controlled, reproducible processing. Potential benefits for human nutrition, sensorial and technical characteristics, or improved quality and safety profiles, could be envisaged.

### 2.1. Foods naturally contain nanomaterials

Food contains many nanostructured materials. This is important to note since the distinction between natural nanostructures and deliberately engineered nanomaterials produced to a particular specification, is not always clear. Food and feed ingredients comprise many components, including biopolymers such as proteins, carbohydrates and fats, with sizes extending down to the nanoscale (e.g. casein, alginic acids and micelles/foams/colloids).

After ingestion, mechanical (chewing, peristalsis and emulsification of fats) and chemical processes (interaction with gastric, pancreatic and intestinal enzymes) during digestion in the various compartments of the gastrointestinal tract (GIT) reduces food into smaller components, ultimately in the nanoscale (small biomolecules such as sugars and amino acids are typically <1 nm in size), for assimilation by the body for growth and to maintain physiological processes. These components (monosaccharides, amino acids, inorganic salts and vitamins) are absorbed by active or passive transport through the epithelial lining of the small intestine (via enterocytes) into the capillary network which drains into the hepatic portal vein for transporting the dissolved food components to the liver and onto other sites for assimilation (or eventual clearance). The body also produces nanomaterial *de novo* in the gut lumen. For example calcium phosphate nanoparticles (20–200 nm in diameter) are formed in the mid-distal aspect of the small intestine due to homeostatic secretion and co-precipitation of calcium and phosphate ions (Powell et al., 2007). Nano particulate ferritin (approx. 12 nm diameter) ingested from plant material may also be endocytosed and utilised by gut epithelial cells as a source of iron. Regardless of the source, it is clear that the GIT is intentionally or inadvertently exposed to natural or man-made particles of all sizes, which are able to be absorbed through, or to translocate across, the intestinal mucosa (Powell et al., 2010).

Most human involvement with food also modifies foodstuff at the nanoscale. Traditional cooking practices and industrial food processing are intended to improve the nutritional and storage properties of many food products. The application of mechanical forces and heat changes the structure of foods at the nanoscale and, in doing so, modifies flavour, structure, texture and storage behaviour. Such processing is generally conducted and controlled at the macro-level without the intention of obtaining well defined nanostructures. Since "technology" implies human involvement, natural nanostructures are not regarded as products of nanotechnology, and they need to be differentiated from deliberately engineered nanomaterials when considering regulatory requirements and definitions. With advances in nano science and nanotechnology, industry can now acquire better control over processes which modify materials at the nanoscale and also can understand what specific nanostructures would provide better functionalities.

### 2.2. Production of ENMs

There are several ways to engineer nanomaterials. Processes can be categorised as '*top-down*', in which progressively smaller structures are created by physical processes, and '*bottom-up*' processes, in which nanostructures are realised by building them up from smaller building blocks.

Top-down nanotechnology is predominantly used for inorganic materials. It encompasses traditional processes like grinding, milling, sieving, heat treatments (e.g. flame spray hydrolysis) and chemical reactions (e.g. an extension of micronisation). For inorganic nanoparticles, the new properties predominantly arise from the fact that smaller structures have a much higher surface-to-volume ratio and may exhibit special quantum mechanical effects. These can range from optical properties, for example with titanium dioxide, where the optical UV reflective properties depend on the size and aggregation of the particles, to enhanced chemical reactivity as exploited in catalysts.

For food applications, bottom-up processes, such as 'self-assembly' in which larger nanostructures are formed by assembling smaller components, are more common. In self-assembly, molecules are designed in such a way that they have specific characteristics that make them spontaneously link up with other molecules (of the same or different type) to form supra-molecular structures with new functionality (e.g. John et al., 2002). Phospholipids which are components of biological membranes are an example. Phospholipid molecules have one part that is hydrophilic and one part that is hydrophobic. When put in water they spontaneously form structures (e.g. a double layer) in which the hydrophilic heads are in contact with the water and the hydrophobic tails are oriented towards each other, creating a central zone from which water is excluded.

### 2.3. Possible ENM applications in foods

Properties of nanostructures which may be altered as compared to corresponding bulk materials include optical properties,

catalytic properties, porosity, electromagnetic, mechanical (stiffness and elasticity), material and surface properties (strength, weight reduction, increased stability, altered reactivity, improved functionality and absorption potential). These properties are generally associated with specific physicochemical parameters. Such parameters and the resulting properties can also alter the behaviour of materials in different matrices and in biological systems.

Various nanomaterials linked to foods have been reported in the literature (FSAI, 2008; Chaudhry et al., 2008). Inorganic materials may have specific properties which affect, for example, their optical, solubility, and reactive properties. Globular proteins used for thermal stability and as thickening agents may take the form of nano fibres. Biodegradable polymers consisting of organic carbohydrate and protein based materials (e.g. polyglycolic-lactic acid, gelatine, collagen and milk proteins), and nano emulsions/dispersions of oil in water, can form vesicles, micelles, liposomes, poly mersomes and other assemblies which may be exploited in nano encapsulation systems (Luykx et al., 2008).The direct applications of ENMs in foods could result in improved sensorial and quality aspects such as flavour, colour and texture modification. Nutritional benefits include stabilisation of micro-nutrients in different food matrices, or targeted delivery of nutrients/bioactive compounds, leading to improved availability for absorption by the body. Consequences of such improvements could be the optimisation (reduction) of use levels required for the expected technological or nutritional effects.

Perhaps the most advanced investigations into the use of ENMs in food applications are in food contact materials for the benefit of maintaining (e.g. the use of silica nano sheets to enhance barrier properties, the inclusion of anti-microbials such as nano-silver) or monitoring (using microbial sensors) the quality and safety of foods. Such indirect food applications are not the focus of this paper, although the methodologies discussed could be applicable if significant oral exposure due to migration into the food product is suspected.

## 3. The fate of nanomaterials in the body

The physicochemical properties of any material, combined with the dynamics of the body's response to them, are the major determinants of the material's fate in the body. However, while it is predominantly the *chemistry* of bulk materials that determines their fate, it is a combination of *physical* as well as chemical attributes, the so-called *physicochemical* characteristics, i.e. large surface area to volume ratio, shape, reactivity and charge, that principally influences how the body handles ENMs.

Depending on the situation, ENMs, as with bulk materials, can enter the body via the oral, dermal, and inhalation routes (Medina et al., 2007). It has also been reported that ENM are able to enter the central nervous system (CNS) via the olfactory neuronal pathway (Oberdörster et al., 2004; Elder et al., 2006). For the purpose of this present discussion and for potential direct food applications, oral exposure is the principal route of interest.

ENMs entering the body through ingestion are subject to digestive processes in the GIT. The GIT (and its mucosal layer) can be a selective barrier to systemic exposure of materials, including particulate matter, in which case the ENM may remain in the gut lumen, perhaps with a potential for interaction with GIT surfaces or with inhabitants of the lumen (e.g. microbiota), but essentially being fully eliminated from the body via the faeces. If translocation or uptake across the gut barrier does occur, there is a potential for internal systemic exposure. In this case, the ENM could then be distributed throughout the body, gain access to other internal body compartments with further internalisation and retention in cells and organelles, and interact with bodily systems. The ENM can also be processed (e.g. by phagocytosis) for removal by the body's innate defence and clearance mechanisms. The fate of nanomaterials entering the body through ingestion are summarised schematically in Fig. 1.

In biological matrices such as food or body fluids and tissues, ENMs develop a coating of protein or lipid (described as a 'corona') (Lundqvist et al., 2008). It is in this form that the body is likely to first encounter the ENM. The corona is not a stable association and so can be removed, altered and exchanged throughout the life cycle of the ENM, dynamically changing in relation to the macromolecules and environmental conditions that it encounters. During the transit of an ENM through the body it will encounter saliva, gastric and intestinal fluids, mucosa protecting the gut barrier, lymph and extra- and intra-cellular fluids. Resulting coating(s) can affect the absorption of the ENM onto membrane surfaces and cell barriers and affect reactivity within the body. However the same particle in the same medium will reproducibly give the same protein corona. In experimental systems, it has been observed that after washing, a "hard" protein corona remains (ca. 4 nm thickness) and forms a tightly bound coating around the ENM (Casals et al., 2010; Cedervall et al., 2007; Dell'Orco et al., 2010; Lacerda et al., 2010; Lesniak et al., 2010; Lynch et al., 2009; Walczyk et al., 2010). Any biological interaction will therefore depend upon how the body (the tissues/organelles at the nano-biological interface) "sees" the ENM at a given point in time.

There have been some studies demonstrating specific effects of nanoparticles on the promotion of protein assemblies into amyloid fibrils *in vitro* by assisting the nucleation process (Linse et al., 2007). They showed that various nanoparticles (copolymer particles, cerium oxide particles, quantum dots and carbon nanotubes) enhanced the probability of appearance of a critical nucleus for nucleation of protein fibrils from human $\beta_2$-microglobulin. The same authors also however demonstrated that reduced fibrillation could also result from interaction with nanomaterials (Cabaleiro-Lago et al., 2008). Consequences of these modifications in protein fibrillation could be altered protein folding or assembly, with the *theoretical* possibility of leading to new biological interactions resulting in unanticipated toxicity; however, such effects have yet to be verified *in vivo*.

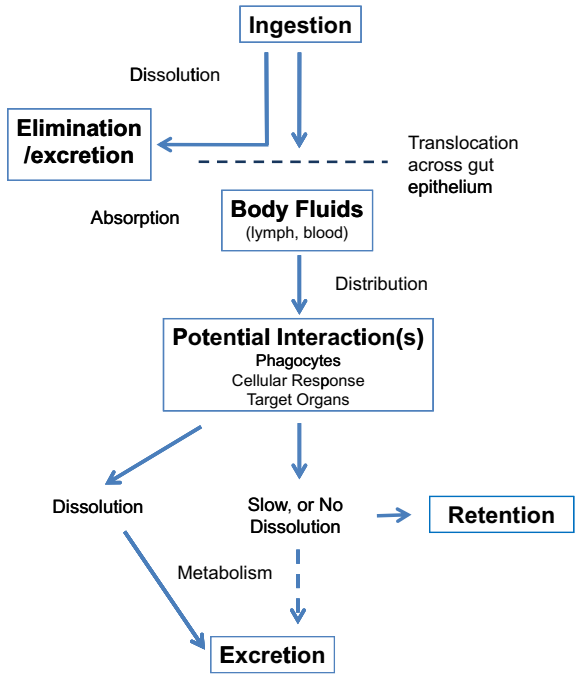


**Fig. 1.** The fate of food-associated ENMs in the body.

In water and in biological fluids nanomaterials tend to form agglomerates which may modify the biological function and toxicity potential of the free particles (Kennedy et al., 2009; Radomski et al., 2005).

### 3.1. Biokinetics

The acquisition of information facilitates an understanding of the metabolic handling and the fate of substances following ingestion (see Fig. 1).

#### 3.1.1. Absorption

The gastrointestinal tract is covered by a protective layer of mucus, a complex network of highly branched glycoproteins and macromolecules. The importance of this mucosal layer as a barrier to nanoparticle uptake (and so also for consideration in any *in vitro* testing methodology) has been demonstrated (e.g. Lai et al., 2009; Crater and Carrier, 2010). Absorption of particulate material occurs across the GIT (mainly in the small intestine) primarily via transcytosis in the M-cells of the Peyer's Patches in the gut-associated lymphoid tissue (GALT), and has been described for the uptake of large nanoparticles (20–100 nm) and small micro particles (100–500 nm) (Powell et al., 2010). Minor uptake mechanisms in gut epithelial cells include clathrin- and/or caveolin-mediated endocytosis, pinocytosis and phagocytosis. Putative mechanisms such as persorption through gaps at villous tips following loss of enterocytes, and para cellular uptake across tight junctions of the epithelial cell layer are expected to be highly inefficient, although the integrity of the epithelial layer could be compromised by disease or drugs (Powell et al., 2010). Although in some studies, a smaller particle size has resulted in a greater absorption (e.g. Jani et al., 1990; Schleh et al., 2011), the potential for systemic uptake of particles may be more affected by surface coating with protein/lipid than size. This is because the nature of the coating determines the distribution of ENMs across barriers and in target tissues or cells. Shape (aspect ratio) and flexibility are also important factors determining uptake and distribution.

#### 3.1.2. Distribution

Once translocated through the epithelium of the gut, an ENM may enter the blood-stream via portal circulation to the liver or via mesenteric lymph nodes into the lympho-reticular system, enabling transport throughout the body and to other organs.

The vascular capillary network may also be permeable to particulate ENM, whereby the ENM may extravasate to the extracellular matrix. This can be achieved by two potential mechanisms: (1) by the para-cellular route, although in the majority of cases the presence of tight junctions between vascular endothelial cells will prevent most nanoparticles exiting from the circulation; and (2) by transcytosis in which an endothelial cell encloses extracellular material in an invagination of the cell membrane to form a vesicle, then moves the vesicle across the cell to eject the material through the opposite cell membrane. The mechanism is size-dependent, therefore the smaller the particle is, the easier it passes through.

Following extravasation, particulate ENM may gain entry to cells and tissues. Most available evidence comes from pharmaceutical studies with surface-engineered particulate ENMs that were subjected to such intracellular transport because of the presence of cell-recognisable domains on the functionalized surface. These domains/ligands facilitate cell-specific transport mechanisms. Characteristics such as charge and size may be important in non-specific uptake of particulate ENMs to the cell.

#### 3.1.3. Metabolism/excretion

Particulates, including ENMs, will typically undergo an endosomal/lysosomal degradation attempt. Additionally, particulate ENMs can be cleared from the body by phagocytosis which typically involves monocytes in the blood stream and macrophages in the tissues such as the liver and spleen (Kennel et al., 2008). Phagocytosis is a cellular process by which cells envelope and digest particulate debris and micro-organisms in an attempt to remove them from the body. The ultimate fate of non-biodegradable particulate ENMs remaining in endosomes and/or lysosomes is likely to depend at least in part on the relative reactivity of the material and on its shape, in particular its aspect ratio (i.e. length to width). If a material has a low solubility and is resistant to degradation, hence bio-persistent, there is clearly a potential for bioaccumulation together with "frustrated" phagocytosis and (chronic) inflammation in the associated tissues. Inert materials may have little, if any, adverse effect at all, other than bioaccumulation. Based on studies using silica, particulate ENMs (diameter 5.5 nm) may be eliminated from the body via renal clearance (excreted in urine) and via the liver (excreted in bile and through faeces) (Burns et al., 2009; Souris et al., 2010). Size, charge, coating and lysosomal activity also appear to influence the clearance of ENMs from the body, as well as their behaviour within the body.

Overall, the handling in terms of absorption, distribution, metabolism and excretion (ADME) of ENMs which are digested, metabolised or otherwise solubilised in the gut lumen is unlikely to be different from that of the solubilised bulk material, although the uptake kinetics may be altered. For ENMs that are relatively less soluble, particle size, surface coating, reactivity and shape strongly affect the interaction with the body. Key cellular interactions observed in studies with various ENMs and their potential consequences are discussed further below.

### 3.2. Potential consequences of cellular interactions

Interactions with individual ENMs have led to one or more of the following endpoints which are not unique to nano-form materials but which may be prudent to evaluate.

#### 3.2.1. Cytotoxicity

Typically, cells attempt to internalise particulates, fibres or stable aggregates into membrane-bounded endosomes (Verma et al., 2008). The interaction of particulate ENMs with cell membrane lipid bilayers is of particular importance for membrane penetration as charged particulate ENMs (e.g. cationic quantum dots or dendrimers) induce transient poration of lipid bilayers, a process that may be associated with cytotoxicity (Lovrić et al., 2005). Not only size and charge may be associated with cytotoxicity, but also shape; for example, Yamamoto et al. (2004) showed that dendritic-shaped $TiO_2$ particles had a higher cytotoxicity than spindle- or spherical-shaped particles.

#### 3.2.2. Inflammation

Accumulation of particulate ENMs in monocytes, macrophages and tissues may result in inflammation. Opsonisation, which is the surface deposition of blood opsonic factors such as immunoglobulins and complement factors (which also contribute to corona formation), may facilitate the binding of particulate material to phagocytic cells (Owens and Peppas, 2006). Protein corona formation around particles can lead to changes in protein conformation so that they appear as "non-self", with the potential to act as adjuvants. It has been demonstrated that the uptake of such particulate adjuvants by dendritic cells activates the NALP3 inflammasome, which contributes to their enhancing effects on innate and antigen-specific cellular immunity (Sharp et al., 2009). It has also been demonstrated that after direct incubation of macrophages with

ENMs, pathways are activated leading to the production of a number of cytokines including pro-inflammatory cytokines (e.g. Yazdi et al., 2010) Particulate ENMs can not only interact with plasma proteins and immune cells, but there is some evidence (Radomski et al., 2005) that interaction with components of the vascular system (blood platelets and vascular endothelium) can also result in local inflammation. Such damage may be mediated via mechanisms such as ENM-induced oxidative (reactive oxygen species, ROS) and nitrosative (reactive nitrogen species, RNS) stress, as shown by massive production of a potent oxidant, peroxynitrite ($ONOO^-$) (Corbalan-Penas, 2010).

*3.2.3. Oxidative stress*

If cellular uptake occurs, the interaction of reactive particulates with the highly oxidatively active mitochondria may increase the generation of free radicals as reactive oxygen species (ROS). High levels of ROS can deplete natural defence antioxidants such as glutathione. This increase in oxidative stress can trigger various cell signalling pathways, in particular via the NF-kB transcription factor. NF-kB is involved in the modulation of inflammatory responses by increasing the production of pro-inflammatory cytokines, which can also lead downstream to the induction of nitric oxide synthase. The induction of this enzyme results in reactive nitrogen species (RNS), such as nitric oxide and peroxynitrite. The highly reactive ROS and RNS have been postulated to cause DNA and protein damage (Nel et al., 2006). ROS can trigger a wide variety of cellular events such as cell cycle arrest, apoptosis, inflammation, induction of signalling pathways, increased intracellular calcium and gene activation (Donaldson et al., 2010; Fubini et al., 2010; Marano et al., 2010). The increase in ROS in cells in response to stress is a common mechanism in toxicology, not a property exclusive to nanoparticles.

*3.2.4. Genotoxicity*

One potential consequence of the generation of ROS is genotoxicity. Various mechanisms of genotoxicity have been proposed (Donaldson et al., 2010). Direct primary genotoxicity is caused by a direct interaction of particles with the genomic DNA or through oxidative attack by the generation of ROS at the particle surface. Indirect genotoxicity can be brought about by enhanced endogenous generation of ROS or the impairment of antioxidant defence (e.g. depletion of glutathione). The normal innate inflammatory response is to rid the body of invading particles and restore normal function. However, in situations of chronic inflammation from the production of pro-inflammatory cytokines, persistent oxidative stress and repeated DNA insult can occur, resulting in the accumulation of genetic defects.

In some instances the shape of the ENM may influence the propensity for genotoxic effects. Carbon nanotubes (CNT, with a high aspect ratio) exhibited greater DNA damage using the Comet assay than the same dose of ZnO nanoparticles, despite the latter eliciting more oxidative stress (Yang et al., 2009). It was suggested that the DNA damage from the CNT may come from mechanical injury rather than via oxidative stress.

In conclusion, the fate of ENMs in the body depends on the characteristics of the individual ENM concerned and the ability of the body to respond adequately to any resulting insults. Fate is determined not only by particle size, but also by solubility, surface coating, reactivity, shape, flexibility and dose. In the first instance, these factors affect the potential to translocate through the GIT and to be distributed and accumulated in the body. The bio-persistence of internalised, relatively insoluble, reactive material has a greater potential to lead to cellular reactions resulting in cytotoxicity, promotion of inflammatory responses and production of intracellular reactive oxygen species, the consequences of which could be protein and DNA damage, as well as in triggering various signal transduction pathways. Such mechanisms can be screened for using *in vitro* and *in vivo* models investigating end points such as translocation, cytotoxicity, inflammation, oxidative stress and genotoxicity. It is vitally important however when using such observations for risk assessment purposes that the predictability of the assays employed for human health is known, including realistic extrapolations of dosing to anticipated exposures from food.

## 4. A strategy for safety assessment

### 4.1. Basic concepts

Steps for the safety assessment for ENMs in foods are fundamentally the same as those for conventional foods and follow the accepted risk assessment (RA) paradigm (FAO/WHO, 1995). This comprises hazard identification, hazard characterisation, exposure assessment and finally risk characterisation, which assesses the likelihood of adverse health effects occurring under specified conditions of exposure. ENMs may present behaviours of toxicological consequence additional to that of a bulk counterpart such as altered biokinetics and biological reactivity. Expert bodies however consider that the established risk assessment paradigm used for chemicals in foods is appropriate for ENMs (COT, COM, COC, 2005; EFSA, 2009, 2011a). Thus the four-stage process of risk assessment for ENMs is essentially the same as that applied to their non-nano form, bulk material counterparts.

ENMs may not be completely new structures *per se*. In general, they will either be single substances produced from raw (bulk) materials or more complex structures assembled from building blocks of different raw materials, each with known chemical identities and macrostructures. In many cases, a toxicological database will already be available for the raw materials. As nanomaterials may be considered as 'novel' forms of their bulk counterpart, it is insightful to consider the assessment processes developed for novel foods, as defined in Europe by Regulation EC 258/97 (European Parliament and Council, 1997). In the process of the safety evaluation of novel foods, a comparative approach is used which makes use of existing data on a relevant non-novel comparator (Howlett et al., 2003; Constable et al., 2007).

Such comparisons help to identify any key differences or information gaps and to inform the requirements and design of any further studies. If differences are observed which cannot be addressed by existing knowledge, then further data need to be generated. In some cases, limited, focused studies are needed to fill knowledge gaps, whereas in others, an extensive toxicological package may be required. Important limitations to this approach include where a comparator is not available or where the existing database is insufficient to allow adequate assessment of the safety of the orally ingested bulk material, especially if this material has not already been assessed for food uses. However, the advantage is that it makes full use of existing knowledge to reduce animal testing to the minimum and to focus resources on the aspects critical for the safety evaluation of the ENM. For certain toxicity testing positive or negative controls and/or reference items are useful and as an example the representative manufactured nanomaterials used in the OECD programme (OECD, 2010a) could be used for this purpose as they are well characterised and are currently being investigated with regard to human health and eco-toxicology as well as environmental fate (work on-going in OECD).

### 4.2. Evaluation of the ENM as manufactured and as used

The primary hazard identification and characterisation should be performed on the ENM as manufactured, and if possible, in comparison with its bulk counterpart and with appropriate reference

nanomaterials. However, the use of an ENM as a food ingredient may require further formulation, perhaps because of stability requirements, or because it will be associated with other components or nutrients. As the use of an ENM in matrices such as foods and packaging may considerably change its properties, notably the size distribution of the nanoparticles due to coating with proteins and other (bio)molecules, as well as its potential for agglomeration and aggregation, it is also necessary to assess the hazard potential of the ENM in the matrix in which it is delivered to the end-user. Additionally, ENMs may be elaborated into a range of other structures which may either remain intact or act as delivery systems for the ENMs. In either case it is also necessary to undertake a risk assessment of the ENMs in the food matrix as used by the consumer. Fig. 2 conceptualises this approach by illustrating the convergence of the risk assessment from the two different but complementary sides.

### 4.3. Potential for exposure

The extent of any potential risk associated with an ENM depends on the level and duration of human exposure. Thus, understanding the body's handling of the ENM and its subsequent fate as an isolated particle, (as manufactured) and/or as a result of its behaviour in a food matrix, (as consumed) is of critical importance. This key aspect is covered by biokinetics (also termed pharmacokinetics, toxicokinetics), which investigates the potential for systemic (internal) exposure after absorption and the distribution, metabolism and excretion (ADME) of substances in the body. These events occur after external exposure. In the case of ENMs used deliberately in foods, dietary intake is the more significant exposure route.

As a practical issue, because of the problems associated with quantification of nanoscale materials, one of the potential difficulties in dealing with ENMs is to achieve a good estimate of exposure. Knowledge of the extent to which ENMs dissolve or biodegrade and the rate at which this takes place is important in this context for two reasons. Firstly it determines the concentration of residual ENMs in foods and in the body and hence the potential for exposure, and secondly it impacts on the half-life and hence potential duration of residence of ENMs within organisms' systems. If no data are available on dissolution or metabolic handling, a conservative approach in the risk assessment is to assume that the entire amount of ENM added to food remains particulate, is fully bioavailable, and absorbed from the gut leading to systemic exposure. Alternatively, if it is sufficiently demonstrated that an ENM cannot persist intact in food due, for example, to its dissolution characteristics, or that it is only present in a fully stable non-nano form (e.g. as an aggregated or agglomerated ENM), then there is likely to be no ENM exposure and further risk assessment would not differ from that of a conventional substance either dissolved or in non-nano form. The only additional consideration is whether the large surface area of the stable agglomerate has any biological significance.

### 4.4. Risk characterisation

Risk characterisation combines the data on hazard and exposure in order to assess the likely risk to human health. Default fac-

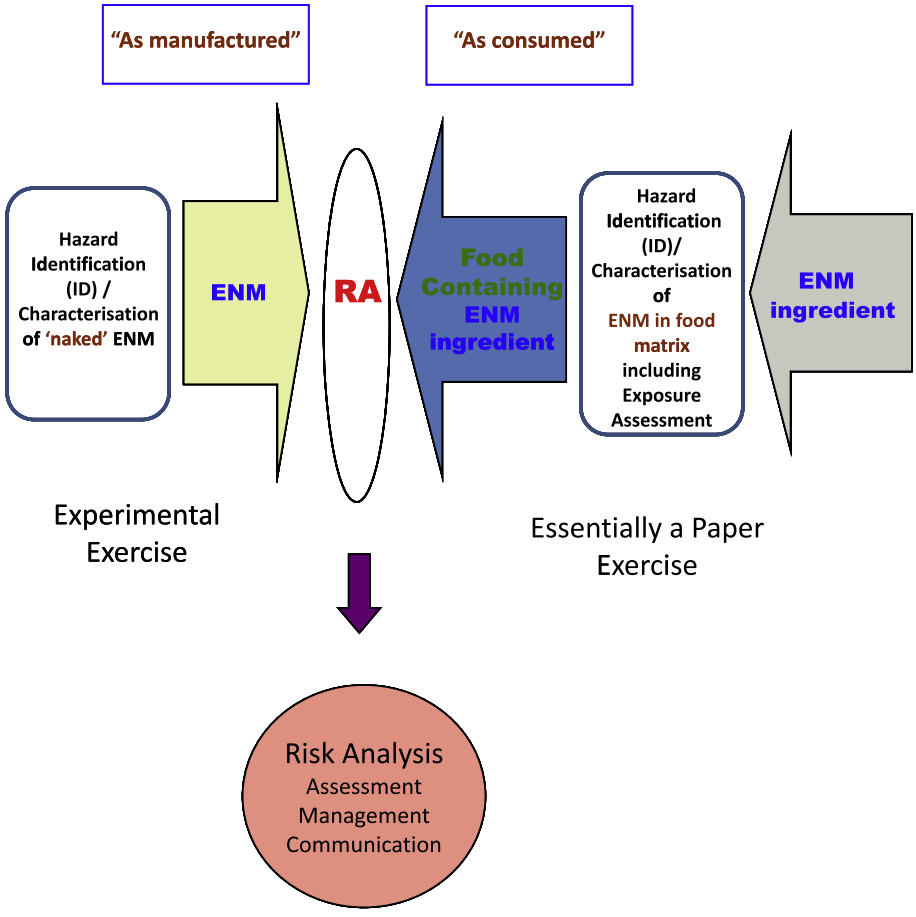


**Fig. 2.** Conceptualised 'two-sided' approach to risk assessment of ENMs in food.

tors are often used to provide an appropriate margin of safety in relation to any uncertainties involved (generally 10× for inter-species and 10× for intra-species variability are employed for extrapolating animal observations to the human situation). The adequacy of the testing procedures for different life stages, or sensitive subgroups, depending on the intended uses of the materials is also considered. The risk characterisation takes into account the probable extent, nature and duration of the exposure. Guidance values, such as an Acceptable Daily Intake (ADI) for humans might be developed against which expected human exposure from the food applications is then assessed. If the projected exposure exceeds the guidance values, risk management decisions may lead to, for example, restrictions in use levels and/or food categories.

### 4.5. A systematic approach to the safety assessment of ENMs

Taking into account the above considerations, a systematic approach is proposed for assessment of the safety of ENMs with direct food applications. This begins with a description of the material used as a comparator followed by a comprehensive characterisation of the ENM. A decision tree is then used to identify whether the ENM has properties which would trigger the need for further investigation. Where further investigation is needed, a tiered approach is followed to identify the type and extent of toxicological testing required, enabling the product developer to perform an initial profiling, followed by more in-depth analyses as necessary. The exposure assessment necessary to complete the safety assessment is carried out taking into account the behaviour of the ENM as it is intended to be used in the food matrix. These five steps are described in detail in the following sections.

## 5. Essential Information

### 5.1. The material used as a comparator

In making the choice of a comparator, and to be able to use the comparator in the risk assessment process, some basic considerations need to be taken into account. In particular:

- Is the ENM derived from a known material through a physical process (e.g. milling) or is it synthesised *de novo*?
- How do the physicochemical properties compare, including dissolution rates?
- What is known about the toxicological profile of the bulk material or comparable reference nanomaterials including effects in humans?
- How do the intended functional properties, including technological and biological properties compare?

The information requirements for the material used as a comparator, and basic information for characterisation of the ENM under evaluation are summarised in Table 1.

It should be noted that, in particular for insoluble materials, conventional dose-metrics may not be fully sufficient to compare ENMs with their bulk counterpart. The dose of a bulk material is traditionally described on a weight/weight basis, e.g. mg active substance/kg body weight, and over the years this has served as a useful metric for describing dose levels, dose response and tolerated doses. In contrast, various studies considering ENMs (SCENIHR, 2006; Brown et al., 2002; Oberdörster et al., 2000; Höhr et al., 2002) have suggested that alternative non-gravimetric characteristics such as surface area and particle number should also be taken into account in describing dose when appropriate. Recently, Kreyling et al. (2010) have suggested the use of volume specific surface area (VSSA) expressed as $m^2/cm^3$. This parameter has also been suggested by SCENIHR to be used in the definition of nanomaterials (SCENIHR, 2010). There may therefore be a need to modify some of the methodologies used for the individual elements of risk assessment, for example the development of analytical tools for the detection and characterisation of ENM in complex matrices such as food (Weigel, 2010).

### 5.2. Characterisation of the ENM

The functional properties and toxicity of an ENM or nanostructure depend on its physicochemical characteristics; its biological activity and any coating/adsorption of protein and or lipid (see Section 3 above). The physicochemical characterisation of the ENM as

**Table 1**
Information requirements on the bulk material used as comparator in the risk assessment process for ENMs.

| |
|---|
| Existing uses and functional properties |
| • Existing uses |
| • Biological and/or technological properties |
| Chemical nature of the material |
| • Identity, including CAS number |
| • Raw materials used, process technology used and potential by-products |
| • Composition and impurities |
| • Physicochemical properties |
| Specification |
| • Maximum and minimum levels of principal component and impurities |
| • Contaminants due to raw materials or introduced during processing |
| Existing risk assessments |
| • Expert opinions (e.g. JECFA, EFSA, EPA and FDA) |
| • Existing health based guidance values |
| Toxicological and nutritional data (oral data preferred, although other routes of exposure may be informative) |
| • All existing toxicological information including genotoxicity, biokinetics, animal toxicological studies (e.g. subchronic, chronic, reproductive and development, carcinogenicity) |
| • Nutritional information |
| • Information from human exposure |
| Exposure |
| • Use levels in foods, food categories, consumption patterns, target populations |
| • Non-food uses |

**Table 2**
Endpoints for identification and physicochemical characterization of ENMs (based on OECD, 2009).

| |
|---|
| ENM key information |
| • Name |
| • CAS number |
| • Structural formula/ molecular structure/wt |
| • Basic morphology (size/shape/form/simple/complex)* |
| • Composition (degree of purity, known impurities or additives)* |
| • Method of production (e.g. precipitation, gas phase) |
| • Commercial use |
| • Water solubility* |
| Physicochemical properties and characterization |
| • Particle size/ size distribution* |
| • Detailed morphology (size/shape/form/simple/complex) (e.g. by Transmission EM)* |
| • Agglomeration/aggregation potential* |
| • Surface chemistry (e.g. coating or modification)* |
| • Specific surface area* |
| • Zeta potential (surface charges)* |
| • Crystalline phase* |
| • Porosity* |
| • Catalytic activity |
| • Octanol–water partition coefficient |
| • Dustiness |

Those parameters marked with an asterisk (*) have been identified as properties of particular relevance to toxicology (Warheit et al., 2007; Oberdörster, 2010a,b).

manufactured, in food matrices as consumed, and after exposure to biological matrices such as GIT fluid, mucus, plasma, lymph, therefore plays a pivotal role in guiding the safety assessment programme. The characterisation is not simply a consideration of the raw ENM but also needs to consider whether it is further 'engineered', e.g. encapsulated to form more complex structures. An adequate characterisation of the ENM under evaluation should be conducted at an early stage. From a toxicological viewpoint it is not precisely clear what the most relevant properties are (Card and Magnusson, 2009, 2010), although Oberdörster (2010a,b), has prioritized properties of relevance to toxicology which the present authors would support. A list of such physicochemical properties has been defined by the OECD (2009). An adapted list of physicochemical parameters is outlined below in Table 2. This is not exhaustive but is considered to be a reasonable starting point for an insoluble or partially soluble ENM based on current understanding. Moreover, the parameters requiring measurement in individual cases will depend on the nature of the ENM and its intended use.

Solubility, as one of the key physicochemical properties, warrants particular consideration. OECD (2010a) has defined solubility as the degree to which a material (the solute) can be dispersed in another material (the solvent) such that a single temporally stable phase results. If an ENM is soluble or solubilises in the digestive fluids as it transits the GIT then it will be in a molecular or ionic form, and as such would have the same properties as, and would not be distinguishable from, the bulk material, other than being more likely to dissolve quicker than the bulk material (OECD, 2009). If this is the case then a detailed physicochemical characterisation is probably not warranted, as it would not add valuable information to the risk assessment. However, if the ENM is insoluble or only partially soluble in the GIT then it has the potential to cross the GIT as an intact particle. Solubility in both aqueous and lipid phases as part of the characterisation is important as a first indication of its behaviour in the GIT.

It should, however, be recognized that the precise relevance of requirements should be considered depending on the nature of the material and the data that are generated during the exploratory investigations, including those investigating the ENM in biological matrices. It is anticipated that with more examples of different types of ENMs as potential food ingredients and by applying more sophisticated approaches to physicochemical characterisation as they become available, it will become possible to build an evidence-base that better enables the recognition of those physicochemical attributes that may influence or predispose to particular functionality or toxicity (Stern and McNeil, 2008; Warheit, 2008). This in turn may enable ENMs to be categorised into different classes for hazard prediction which could facilitate the development of a range of structured testing schemes in the future.

## 6. A decision tree to guide testing/data requirements

### 6.1. ENM 'as manufactured'

Once a material has been confirmed as being an ENM, the use of a decision tree (Fig. 3) is proposed. This would include aggregates, agglomerates and also complex ENMs (e.g. incorporated into encapsulates).The tree is intended to enable the investigator to decide if i) any existing risk assessment (one which may already have

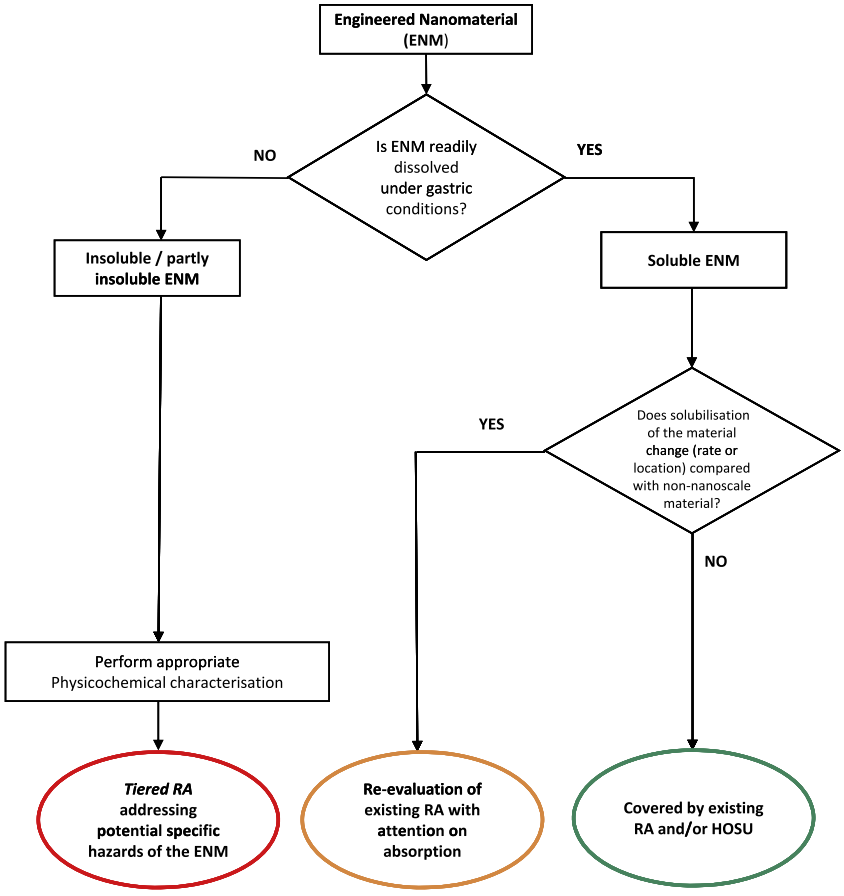


**Fig. 3.** Decision Tree.

been conducted on the bulk material) is sufficient to address the safety of the newly developed ENM, perhaps with specific, focused additional experimental data, or whether ii) additional information is necessary to address potential ENM-related hazards.

The design of the decision tree acknowledges the general consensus that an ENM which is characterised and confirmed to be insoluble, particulate, bio persistent and able to translocate systemically is more likely to pose a risk to human health compared with a readily soluble, non-bio persistent ENM (RIVM, 2009; SCENIHR, 2009).

The characterisation (Table 2) of the ENM as manufactured plays a pivotal role in guiding the safety assessment programme, as a good understanding of its physicochemical properties is a starting point from which to begin to establish its functional and toxicological behaviour. In that respect the primary question to address is that regarding the solubility of the ENM under gastric conditions. In general, the extended physicochemical characterisation can be avoided if the ENM is fully soluble.

Ultimately, for a particulate ENM, its appearance in the blood could be due to the release of the ENM from its formulation (or food substrate when used as an ingredient), its solubility, and translocation through the intestinal epithelium or Peyer's patches.

If insolubility of the ENM under gastric conditions is indicated, the question can be extended to solubility in simulated intestinal fluid. Most studies of dissolution and dissolution rate, be they for the study of the digestion of proteins from GM crops, of pharmaceuticals for process control and quality assurance, or for facilitating certain regulatory determinations (e.g. for assessing pharmaceutical formulation bioequivalence: (European Pharmacopoeia, 2001; WHO, 2003; United States Pharmacopeia, 2003), commence with a study in simulated gastric fluid. The application of several *in vitro* digestion models to investigate the bioaccessibilty of chemical contaminants from foods (e.g. Oomen et al., 2002; Van de Wiele et al., 2007; Dall'Asta et al., 2010) and of food components and formulations (e.g. McClements and Li, 2010; Tharakan et al., 2010; Hur et al., 2011) have been reviewed.

For ENMs which are insoluble/partly soluble *ex vivo* the question is whether they are readily solubilised in food and/or gastrointestinal fluids (including saliva), in which case their nano-specific properties cease to be an issue and the potential hazard depends solely on the amount of the substance entering the body and the toxicity resulting from its chemical structure. For an ENM which is insoluble/partly soluble under gastric digestive conditions, consideration could be given to studying dissolution and dissolution rate in simulated intestinal fluid and in the absence of evidence that it is readily solubilised under either or both conditions, a new risk assessment would be needed to investigate any potential for nano-specific hazards that it may have. The objective of studying dissolution rate in the stomach, and if necessary the small intestine, is to gain an understanding of the proportion of the ENM persisting in particulate-form during the digestive process, e.g. the number of particles, their size and mass, and to assess the risk of its translocation across the gastrointestinal boundary. The rate of solubilisation in relation to a relevant comparator (e.g. bulk non-nano form) should also be assessed where possible.

For ENMs that are readily solubilised or degraded in transit through the GI tract, then the question to address is whether there is a difference in the rate of release of components in comparison to the bulk material. If the ENM is more soluble, then more material per unit dose may be absorbed and potentially expose the consumer to higher levels (although it is likely that this attribute would enable lower levels of the ENM to be added to improve food functionality/safety, e.g. less sodium chloride). In the case of such increased absorption the risk assessment would need to be reviewed in this light. If the solubility is not significantly increased and/or there is no significant increase in the absorption of the ENM, then the existing risk assessment used for the bulk material can still be applied.

The decision tree needs to be considered as a guide rather than a rigid approach to be followed. It is acknowledged that there is some difficulty in defining the degree of solubility, which also involves a time component and a dynamic aspect, as this may alter in different systems (for example in food, the GIT, or in lysosomes), and in turn be modified by coating/adsorption/corona effects. However, in following the decision tree, the solubility of the ENM should be considered overall relative to the bulk material comparator wherever possible. The default position for partially soluble materials would be to consider them initially as "insoluble" when applying the decision tree process.

### 6.2. ENM 'as used'

The incorporation of an ENM into food will require consideration of any interactions between the ENM and components of the food matrix that would affect its solubility and bioavailability during passage through the digestive system.

If the outcome of those considerations is that an ENM incorporated into a food matrix is likely to exist as an insoluble ENM in the digestive system, or that it would exhibit a significantly increased solubility and/or potential for bioavailability, a new risk assessment is required. The influence of the food matrix and its impact on the testing programme is discussed in more detail in Section 8.1.

Four worked examples of how the Decision Tree might apply are provided in Box 1.

Box 1. Four examples of the application of the Decision Tree.

For those materials selected by Decision Tree analysis for an ENM-specific hazard identification and characterisation, the use of the following tiered approach (Section 7) is recommended.

## 7. A tiered approach for use in the safety assessment of ENMs

### 7.1. The rationale for a tiered approach

If the evidence gathered following a detailed comparison of the properties of the ENM with those of the bulk material indicates significant differences that could have potential impact on its safety profile, or indeed that there is no adequate reference material(s) for comparison, additional hazard identification and characterisation of the ENM is required.

Strategies for assessing the safety of ENMs have been the subject of a number of recent review articles (Stern and McNeil, 2008; Balbus et al., 2007; Davis, 2007; Warheit et al., 2007; Oberdörster et al., 2005).The general consensus is that a tiered approach is best suited for this purpose. This approach, described in detail below, proposes a range of studies which could be conducted. Initial information-mining, could determine if similar reference materials and comparators are available to use as control materials in testing systems. *In-silico* modelling (as and when available), and testing in robust, relevant and, where possible, validated (or at least standardised) targeted and non-targeted *in vitro* screening studies, and preliminary *in vivo* studies could alert to the possibility of potential toxicities in Tier 1, and provide initial information. Pivotal *in vivo* studies in Tier 2 would constitute the hazard characterisation, with supportive mechanistic studies if necessary. In contrast to a decision tree approach, which provides distinct yes/no decisions leading to a specific next step, the tiered weight-of-evidence approach is more flexible and focuses on the totality of the data in a holistic manner. Ideally, the predictive value of each study and resulting evidence would be well understood in order to give certain types of data more weight than others during the

EXAMPLE: NANO-SALT (SODIUM CHLORIDE)

Description and Purpose:

Salt produced at the nanoscale is intended to exhibit enhanced flavour properties so that a smaller amount of salt may be used in food to achieve the same flavour as conventional salt.

The ENM characterisation is taken to have been completed.

Question 1: Is the ENM readily dissolved under gastric conditions?

Answer: Yes

This classifies the ENM as soluble and the right arm of the Decision Tree applies.

Question 2: Is the release of components changed (either in time or location) as compared to non-nanoscale formulations?

Answer: No.

When nano-salt dissolves into its ionic form in the stomach, it is no different from the bulk counterpart thereafter.

Conclusion:

Covered by existing risk assessment and/or history of safe use

EXAMPLE: NANOFORM PRECIOUS METAL

Description and Purpose:

Certain precious metals are of interest for use at the nano-scale in food packaging and food utensils. Direct inclusion in food could be a possibility.

The ENM characterisation is taken as having been completed.

Question 1: Is the ENM readily dissolved under gastric conditions?

Answer: No

This classifies the ENM as insoluble or partly insoluble and the left arm of the Decision Tree applies.

Conclusion:

A tiered risk assessment is required to address the potential ENM-specific hazards.

EXAMPLE: NANO-MINERALS

Description and Purpose:

Mineral salts produced at the nano scale are intended to have improved absorption characteristics compared to conventional forms when used for the purpose of nutritional supplementation.

The ENM characterisation is taken to have been completed.

Question 1: Is the ENM readily dissolved under gastric conditions?

Answer: Yes

This classifies the ENM as soluble and the right arm of the Decision Tree applies.

Question 2: Is the release of components changed (either in time or location) as compared to non-nanoscale formulations?

Answer: Yes.

The degree of absorption, of nano-minerals is increased compared their conventional counterparts.

Conclusion:

Need to re-evaluate the existing risk assessment to take account of the increased absorption/exposure. This is particularly relevant in cases where the intention is to increase the mineral uptake. On the other hand, lower rates of use could be employed to maintain existing mineral absorption levels.

EXAMPLE: MIXED NANO FORMULATION OF A CARBOHYDRATE ENCAPSULATED ANTIMICROBIAL PEPTIDE

Description and Purpose:

The example is a nano-scale carbohydrate encapsulating material applied to an antimicrobial peptide used in food preservation. The purpose of the encapsulation is to improve the efficacy of the peptide in protecting food against microbial spoilage during the shelf-life of the food.

The ENM characterisation is taken to have been completed and it is expected that the carbohydrate capsule is insoluble under gastric conditions, but that it is broken down enzymatically under the higher pH conditions of the small intestine.

The peptide itself has not been modified and had already been subjected to a risk assessment before being permitted as a food preservative.

Question 1: Is the ENM readily dissolved under gastric conditions?

Answer: No

This classifies the ENM as insoluble and the left arm of the Decision Tree applies.

Whereas this leads into the tiered risk assessment, it is likely that the physiological fate of the nano-carbohydrate can be established relatively simply, i.e. without the need for an extensive testing programme.

However, the release of the peptide may be delayed in the small intestine and some may even reach the large intestine.

Supplementary Question: Is the absorption of the peptide changed (either in time or location) as compared to normal formulations?

Answer: Yes

Conclusions:

1. The digestion of the carbohydrate capsule should be investigated in comparison to its non-nanoform to establish if it will be covered by the existing risk assessment or whether further studies are required.

2. The release of the peptide is further down the small intestine and there is a risk that at least some of it may reach the large intestine. This means that the existing risk assessment for the peptide needs to be re-evaluated and that additional studies may be needed, for example studying the microflora.

assessment, thus leading to more confidence in the outcome. Currently, the predictive value of newly emerging tools (e.g. focused *in silico* and *in vitro* approaches) may not be clearly defined but continued experience and validation will better determine their utility for risk assessment purposes. The data gathered or generated during the Tier 1 will provide guidance and inform on study design (or appropriate further testing) in Tier 2 (see Table 3). In addition the information gathered might provide a basis for a decision to stop at Tier 1 and not to continue the development of a particular ENM, either because of too high a level of concern or because of an indication for a requirement of too great an investment in time and resource to mitigate the concern identified. This weight-of-evidence approach, in which the individual components of the safety assessment process are prioritised into two tiers of testing, enhances the efficiency and robustness of the process enabling consolidation for decision making while allowing for further relevant studies to be included at any time.

Tier 1 is designed to provide early information to the investigator regarding the potential of the ENM for local GIT effects and/or systemic penetration which can inform the scope and design of the definitive studies undertaken in Tier 2. The Tier 1 studies can be used to screen for the potential for genotoxicity, and other effects such as cytotoxicity, inflammatory responses and oxidative stress (see Section 3), as has been observed with some ENMs having particular physicochemical properties. Preliminary Biokinetics in *in vitro* studies and possibly through Physiologically Based Phar-

**Table 3**
Elements for consideration in a comparative tiered approach to ENM hazard identification and characterisation.

Tier 1
- data- and information-mining, including bulk comparator and relevant reference nanomaterials
- *In silico* modelling – PBPK, (Q)SAR, read across and grouping
- *In vitro* investigations - for mechanistic insights and alerts (dissolution, genotoxicity, inflammatory potential, ROS induction, cytotoxicity, translocation potential)
- *In vivo* – 14/28 day subacute oral repeat-dose oral rodent study including elements of AD(ME) and genotoxicity, as appropriate.
- Re-evaluation of all available data and decision on further procedure

Tier 2
- 90-day repeat-dose oral rodent study with possible additions
- Other focused studies, e.g. reproduction, if indicated
- Mechanistic studies, if indicated

Note: consideration of dosimetry is relevant to both Tiers.
Material to be tested should be the ENM compared to an appropriate bulk and nanoform reference material if available.
Testing performed to OECD protocol guidance as available (with adaptations/modification).

macoKinetic (PBPK) modelling, or built into a sub-acute 14- or 28-day repeat-dose rodent study should be considered in order to provide preliminary information on gastro-intestinal translocation and bio-persistence potential.

Such initial screening has also been proposed in schemes for identifying environmental and occupational health risks of nanomaterials (Landsiedel et al., 2010; Warheit et al., 2007; Pronk et al., 2009). Depending on the outcome of this initial toxicological profiling, alerts for potential concern may be identified (e.g. if translocation, bio-persistence, increased inflammatory responses are demonstrated), allowing the product developer to make decisions regarding continuing, modifying (e.g. re-formulation), or abandoning, further product development.

Tier 2 testing will be required to evaluate the ENM in more detail and to investigate qualitative (toxicological effects) and quantitative (dose–response) differences from the reference bulk material, if available. The studies or investigations used will ideally be a combination of (1) those targeted to detect any specific properties that have been highlighted to be of potential concern in Tier 1 and (2) those routinely employed to investigate and identify the toxicological properties of any material. A repeat dose 90-day study, modified as appropriate, is a suitable core assay for selected substances progressing to Tier 2. For all studies, the use of the bulk material and relevant validated reference nanomaterial as comparators may assist the interpretation of the results for subsequent risk assessment. The tiered approach is summarised in Table 3.

### 7.2. Tier 1

The first step in the tiered approach is to screen for potential hazards that may be qualitatively different from those seen for the reference bulk material, or similar to those for ENMs with known toxicity, and to consider whether translocation through the GIT will occur resulting in systemic (internal) exposure to the ENM. Such an initial profiling may involve a combination of information gathering, *in silico* modelling, *in vitro* cell free and cell based systems and *in vivo* investigations.

Information-mining can be useful to identify whether there are any nanomaterials which have similar physicochemical properties to those of the ENM that is being considered. If reference ENMs are available then these should be identified such that they can be used as comparators in screening and further studies. This might suggest which *in vitro* studies are reasonable to perform based on, for instance, prediction of reactivity, resistance to degradation or the shape of the ENM.

#### 7.2.1. In silico studies

Computational studies, aiming at predicting the toxicity and biokinetics of nanomaterials, such as Quantitative Structure Aactivity Relationships (QSAR) and PBPK modelling are being investigated and could eventually provide a useful screening tool in Tier 1 (Gallegos Saliner et al., 2009; Fubini et al., 2010; Li et al., 2010). SARs and QSARs are theoretical models that relate the structure or physicochemical properties of substances to their biological activities. Burello and Worth (2010), for example, have proposed a theoretical framework with which to predict the oxidative stress potential of oxide nanoparticles based on the available electronic energy levels in the nanoparticle structure. For other effects the correlated properties and their associated mechanisms of action are starting to be identified. There remains, though, the need for thorough testing and validation of this methodology to make it suitable for decision making as a recognised *in silico* method.

Information from one ENM (source) might be used to make predictions of the potential endpoints of concern of another "similar" ENM (target) by using read across and grouping. Substances with common features, showing coherent trends in their properties, which is assumed to be associated with a common underlying mechanism of action can be grouped within a category. Read-across could be performed in a qualitative or quantitative manner, depending whether the data being used are categorical or numerical in nature. It is not envisaged that read-across would be used to mitigate testing of a novel ENM but guide the assays that might be done.

Multi criteria decision making (MCDM) techniques are a means of quantifying or prioritising personal or group judgements that are typically intangible and subjective. DART (Decision analysis by Ranking Techniques) is a software tool designed to support the ranking of substances according to the level of their environmental and toxicological concern and is based on the most recent ranking theories (Gallegos Saliner et al., 2009).

However, it must be acknowledged that these modelling approaches are still at an early stage of development, both for ENMs and for bulk materials.

#### 7.2.2. Studies to Investigate Translocation Potential

*In vitro* models of the gastrointestinal tract and environment, including real or simulated digestive fluids (e.g. saliva, gastric and intestinal juices), can be utilised to investigate whether an ENM is degraded, solubilised or ionised into its molecular components, as well as the rate at which this occurs. The propensity of the ENM to breakdown in simulated gastric fluid is considered as part of the decision tree process (see Section 6.1). If only limited breakdown occurs in these assays, it is likely that the remainder of the GIT will be exposed to some ENM, and the potential for systemic translocation needs to be investigated.

Translocation potential can be screened for in *in vitro* digestion models and cell-based systems (also see later under 'Barrier Effects'). Investigation of the biokinetic behaviour of the ENM will ultimately need confirmation in *in vivo* assays. It is important to characterise the biokinetic behaviour of the ENM since it is a significant determinant of the ENM's potential toxicity and important information for the investigator.

Radio-labelled material may be needed for monitoring of absorption, distribution and excretion over several days based on estimated half-life. Techniques such as whole body autoradiography may prove helpful to visualise any unexpected concentration of the ENM in organs or tissues. Such studies are, however, resource intensive and are likely to require high specific activity labelled material because the dosages used may be low. A pragmatic approach to optimise resource and minimise use of animals would be to combine these investigations within a short term general toxicology study such as a 14–28-day dose response study,

including the examination of target tissues for evidence of pathology and/or internal exposure. Any accumulation of particulate matter within the GIT should be determined. The method of oral administration should be carefully considered, be it gavage, dietary or via the drinking water.

During evaluation of the applicability of existing test guidelines to nanomaterials, the OECD reported (OECD, 2008) that due to analytical difficulties, tracking the biokinetic behaviour of nanomaterials *in vivo* is technically challenging and therefore a guidance document is not appropriate. Instead, such studies can only be performed on a case-by-case basis, meaning that each study would contain specific elements unique to the material under test.

A review of existing toxicity testing guidelines on chemicals for applicability to nanomaterials has been published (Rocks et al., 2008) which confirms the observations of the OECD workgroups. With respect to biokinetics, a new guideline has been published recently (OECD, TG 417, 22 July 2010b). However, this only provides general guidance and therefore specific studies will need to be designed on a case-by-case basis. Studies on translocation, binding and absorption through cellular membranes may be crucial, especially in relation to the passage of ENM through tissues like the blood–brain barrier and the placenta. It is likely that sophisticated labelling and detection methods will be required to show this translocation and further distribution. Elemental analysis has been used in kinetic studies that can give indications of distribution to the organs, but due to analytical difficulties, there is not yet hard evidence that the intact ENMs themselves are distributed.

If it is possible at this stage to discount systemic exposure to the ENM, the assessment should be based on the potential for local gut lumen effects. This should initially focus on potential for uptake into GIT epithelial cells, with further studies examining irritancy, genotoxicity, cytotoxicity, inflammation and effects on barrier integrity. Peyer's patches have also been shown to have the potential to accumulate particles and the potential for immunogenicity should be considered. In addition impact on the gut microflora and influence on the availability of nutrients or xenobiotics should be considered. If the material is simply excreted, and no particular interaction with GIT cells observed, there would be no need for further studies.

If the investigation of ADME indicates that the ENM is preferentially distributed to and deposited in sensitive organs over and above the extent shown by the bulk comparator (for example, via the blood–brain, blood–testis or placental barriers), further focused investigations may be required as part of Tier 2 to enable an informed risk assessment. If evidence of bio-persistence and accumulation is established, careful consideration of long term implications would be required.

### 7.2.3. In vitro targeted assays

Targeted *in vitro* studies are based upon an evolving understanding of the potential effects of some ENMs (Section 3). Such effects can be investigated using *in vitro* techniques (Park et al., 2009) and, whilst not necessarily predictive of an *in vivo* biological outcome, they may highlight potentially important properties which would need further investigation. If such targeted profiling studies were to be incorporated into the development programme of a novel ENM at an early stage, potential issues of significance for further development and which may need particular consideration in subsequent testing may be detected. It is not suggested that these screening studies are used to make decisions on further development although it could be that the combination of results guides the manufacturer into considering that the use for direct food applications is not appropriate.

The biological significance of these *in vitro* results would depend upon the absorption and persistence of the ENM in the body or in the gut epithelia. Even if there are no obvious alerts as compared to a bulk material in this step, there is still a potential that the small size of the ENM may result in enhanced bioavailability which could alter the toxicological profile as compared to the bulk material. In such circumstances, if the exposure to the ingredient is not to be reduced, existing toxicological databases on the bulk material may not be sufficient for the ENM and non-targeted investigations looking for general toxicity and biokinetic handling in standard repeat-dose feeding studies would help to ensure that such effects are identified.

A review of current *in vitro* assays for risk assessment of nanomaterials and their limitations is described by Kroll et al. (2009). In particular it is suggested that nanoparticle features such as high adsorption capacity, hydrophobicity, surface charge, optical and magnetic properties, or catalytic activity may interfere with assay components or detection systems, thereby limiting the use of some of the *in vitro* toxicity assays. For example, the presence of ENM protein binding in *in vitro* media may deplete assay components thereby confounding assay results with false positive/negatives. Studies should be designed to take into consideration specific properties of the material under investigation. Of particular importance with all *in vitro* assays is the use of physiologically relevant dose levels in relation to anticipated human exposure, dose level responses, as well as the dynamics of time responses. In addition, the impact of traditional heat treatment against complement or other treatments (e.g. sonification) must all be addressed as important controls, if the results are to be meaningful for extrapolation to the *in vivo* situation (Oberdörster, 2010a,b). There may be substantial difficulty in the interpretation of *in vitro* data on nano form materials due to lack of a predictive knowledge-base upon which to draw. Where relevant, it is helpful to include a reference nano form material with known toxicities in the protocol (Pronk et al., 2009; Wörle-Knirsch et al., 2006), as well as the bulk reference material, for comparative purposes. The use of a relevant reference nanomaterial (as positive or negative control) will ensure the assay is working to predict the endpoint of concern. As suggested by Kroll et al. (2009) there should be flexibility in both the test conducted and adaptations to the test.

Potential *in vitro* testing methods that could be useful for screening nanomaterials are outlined in annex IV (alternative methods) of the first revision of the OECD guidance manual for testing of manufactured nanomaterials (OECD, 2010a); see below for examples of the different end points. It is not yet possible to make specific recommendations for the choice of these alternative approaches. A large number of *in vitro* assays, both cell based and cell free, may be used. The choice may be guided by laboratory experience and the availability of relevant and standardised assays with demonstrated reproducibility. Tests used must be fit for purpose and adequate to measure the intended endpoints. Although there are currently few standardised or ring-tested methodologies available, intense research is on-going into *in vitro* methods as possible alternatives to animal testing especially with respect to nanomaterials. Recently a group has been set up, the International Alliance for Nano EHS Harmonization, whose mission is to create protocols for a limited number of toxicology tests, including *in vitro* methods, on a small number of representative nanoparticles to further enhance inter-laboratory reproducibility of assessment methodologies and results. This will involve the use of nanomaterials and biological methods from a common source and a single set of protocols (http://www.nanoehsalliance.org/sections/Home).

### 7.2.4. Barrier effects

There are a number of suggestions for *in vitro* tests of barrier integrity, e.g. OECD, 2010a. Endpoints of relevance include studies measuring trans-epithelial electrical resistance (TEER), endothelial and epithelial permeability and examination of cell morphology

and effects on transport processes. As models of the GIT, primary epithelial cells from the small intestine would ideally be used for these studies. However, the most relevant available model for gastrointestinal integrity would be differentiated CaCo-2 cells. Considering that translocation in the GIT is less likely to take place via epithelial cells, it would be useful to include other cell types such as Peyer's patches or other RES cells. Other cell lines such as primary rat or porcine endothelial cells to model blood brain barrier or BeWo cell line or HUVEC cells for placental transfer may also be useful.

*7.2.5. Cytotoxicity*

*In vitro* models used for these tests are standardised cell lines; typical cells used as representative of the gut include CaCo-2, or HT-29 cells. Examples of cytotoxicity investigations (OECD, 2010a) are examination of:

- cell and cell layer;
- epithelial barrier integrity;
- cell viability/cellular metabolic activity;
- proliferation;
- cell death.

*7.2.6. Oxidative stress*

Examples of *in vitro* assays for investigation of oxidative stress are:

- ROS assays (cell-free and cell-based assays including superoxide anion, hydroxyl radical and peroxynitrite measurements)
- Nitric oxide: peroxynitrite ratio
- Modified comet assay (DNA damage)
- Glutathione (GSH) depletion assay
- Superoxide dismutase (SOD) assay
- Nrf2 reporter gene assays

Also, the induction of heat shock proteins and stress kinase activation can be determined as a stress response following exposure to ENM. However, this is not specific oxidative stress.

*7.2.7. Inflammatory response*

Inflammatory, thrombogenic and immunotoxic potential can be investigated by induction of various markers of inflammation (e.g. pro-inflammatory peptides and cytokines, mediators, prostaglandins and leukotrienes, adhesion molecules, factors for extravasation) in blood Peripheral Blood Mononuclear Cell (PBMC) and co-culture models of fibroblasts, epithelial cells, macrophages/leukocytes and other specialised cell lines.

*7.2.8. In vitro genotoxicity*

The existing standard battery of *in vitro* genotoxicity assays should establish the potential for genotoxic effects (Warheit and Donner, 2010). This battery consists of the *in vitro* tests for bacterial genotoxicity (OECD, 2010b Bacterial Reverse Mutation test), mammalian cell chromosome aberrations (OECD TG473) and mammalian cell gene mutations (OECD TG476). It has been suggested (Landsiedel et al., 2009) that the Ames test may not be appropriate because bacterial cells do not have the ability to phagocytose particles as do mammalian cells. Therefore mammalian cell assays, such as the *in vitro* micronucleus assay (OECD TG487) could be more relevant for establishing the genotoxic potential of insoluble ENMs. However, uptake of ENMs by bacteria has recently been demonstrated, which also indicates the importance of the incubation conditions and effects of protein coating in *in vitro* bacterial assays (Kumar et al., 2011a,b). Genotoxicity via a direct interaction with DNA will not be acceptable for materials added directly to foodstuffs and may have already been identified by comparison with reference materials. In such cases, further product development would typically not be feasible.

*7.2.9. Sub-acute oral repeat-dose in vivo studies*

Repeat-dose toxicity studies allow an exploration of the effects of repeated exposure to materials on fully integrated biological systems. In an initial toxicological profiling phase, a sub-acute (14/28 day) repeat dose–response rodent feeding study (OECD TG407) would provide important information on dose–response for any detected target organ effects in comparison to bulk material. Recent additions to the OECD guideline 407 also include additional immunotoxicological parameters. It can also be used as a dose range finding study to indicate suitable doses for a further toxicity study in Tier 2. The duration of the study should be decided based on available information, e.g. on potential persistence. Biokinetic parameters may be built-into gain preliminary evidence concerning the potential for systemic penetration and to investigate other aspects of ADME including distribution and accumulation. The mode of administration for *in vivo* studies needs to be considered carefully and on a case-by-case basis depending on intended applications and practicalities (i.e. gavage versus diet or drinking water). Material from such *in vivo* studies could be used, case by case, for *in vitro* investigations, e.g. bone marrow for genotoxicity assessment.

The Tier 1 studies may indicate that the ENM is not systemically available and therefore only local effects in the gastrointestinal tract specific to exposure to the ENM may need to be considered further. Alternatively the studies might indicate translocation and systemic exposure and highlight particular concerns which will need further studies to identify and characterise any hazards, including dose–response considerations.

*7.3. Tier 2*

Tier 2 is intended to determine if there is evidence indicative of qualitatively (biological endpoints) or quantitatively (dose–response) different effects compared to the bulk material or other reference material. If specific effects are observed in a sub-chronic 90-day sentinel study in the rat, further detailed studies may be indicated.

In terms of study design, a comparative *in vivo* study should be undertaken to compare the toxicological profile of the ENM against the reference bulk material. A suitable reference nanomaterial should also be considered for inclusion if relevant and available. There should be emphasis on relating the doses selected to the likely human exposure, and multiples thereof. ADME and toxicological information developed in Tier 1 may also guide the choice of suitable doses and any additional potential endpoints for special investigation. Consideration of the expected human exposure through foodstuffs is required in order to design the experiments accordingly so that a margin of exposure appropriate for risk assessment purposes is included.

Expert groups (OECD, 2010a) consider that standard *in vivo* test protocols are valid for the testing of nanomaterials (e.g. OECD TG 408 repeat dose 90-day oral study in rodents), albeit that special consideration is required for preparation of the test material, dosimetry and potential extension of the parameters investigated and methodologies used. Consideration needs to be given to enhancing the ability to detect adverse effects that may occur with ENMs and that may have been "flagged" from the targeted Tier 1 studies. Biological parameters for particular attention would be, for example, evidence of inflammation, inclusion of a wider range of organs for histological examination or electron microscopy to analyse the ENM in the test material and in biological fluids and tissues, subject to methodological verification. If there are indications of any specific target organ effect(s), supplementary studies

should be considered. An off-dose period, after cessation of dosing, could be considered to establish if effects are reversible and also to determine if particulate matter is bio persistent or leads to effects such as chronic low-level inflammation that may not have been picked up in the course of the study.

If the *in vitro* genotoxicity tests performed in Tier 1 are negative but there are indications of indirect effects from formation of reactive radical species or if it is not possible to test the ENM *in vitro,* then EFSA recommend that an *in vivo* Comet assay is performed (EFSA, 2011b). This can be included in the *in vivo* studies of Tier 1.

*In vivo* tests for somatic cell genotoxicity, such as induction of chromosomal aberrations in rodent bone marrow cells (OECD TG 475), the induction of micronuclei formation in peripheral blood and bone marrow erythrocytes (OECD TG 474) and the induction of unscheduled DNA synthesis in the liver of treated animals (OECD TG 486), are typically performed on a material which has been shown to be genotoxic in *in vitro* tests. Alternatively, these tests can be incorporated as part of a repeat-dose oral toxicity study, subject to the outcome of Tier 1. Genotoxicity testing strategies in general for food and feed assessment are currently under discussion at EFSA (EFSA, 2011b).

### 7.4. Dosimetry considerations

Where nanomaterials are insoluble, or have a low dissolution rate, their size and other characteristics such as shape and corona may have an influence on biokinetic behaviour. These characteristics may elicit a hazard profile different from the bulk or solubilised material. As particle size and surface area are only indirectly linked to dosage expressed classically as mass concentration/unit time (e.g. mg/kg bw/day), the biological effect may not be proportionally related to dose expressed in this way, resulting in a distortion of the classically expressed dose–response relationship. Therefore, the OECD has suggested that dosage could be expressed in terms of number of particles per unit volume (OECD, 2009). When this metric is given together with mass concentration, particle size and surface area are also reflected. Thus, a single measure of dosimetry may be erroneous. It is however important to note that determination of the most appropriate dose metric in any given case is based on an understanding of the parameters driving the effects observed. It is also important to note that the use of a dose-metric based on particles (e.g. number concentration) is not relevant in the case of particles which are solubilised or otherwise dispersed into their molecular components following exposure.

The OECD has provided a report on sample preparation and dosimetry which includes considerations for oral dosing (OECD, 2009) and which identifies a number of potential problems of tests with nanoparticles:

- In the case of inclusion of nanoparticles in laboratory diet a number of difficulties can be encountered:
  - Difficulties in achieving and verifying homogeneity
  - Nutritional quality of feed may be affected, for example due to oxidation.
  - Occupational risks for laboratory staff need to be taken into account.
  - For these reasons, gavage administration may often be the preferred route for *in vivo* investigations.
- Stock solutions/suspensions used for preparation of gavage dosing must be well characterised. Particle size distribution, mass concentration and aggregation must be understood to enable accurate determination of what has been administered. The composition of the solution itself also requires careful control since pH, ionic strength and composition may have an effect on particle aggregation.
- Although gavage may be the preferred route of oral administration, e.g. to ensure containment from the occupational exposure perspective as well as to ensure each animal receives its full dose, such studies should not be directly extrapolated for risk assessment of human dietary intake. It is important to consider the food matrix in which the particles are presented to the consumer and the subsequent handling in the gastrointestinal tract. Particles associated with protein may behave quite differently than those presented in a simple solution and or suspension. This is discussed further in the following section.

## 8. Exposure

### 8.1. Consideration of the food matrix

In addition to the characterisation and safety assessment of the ENM as manufactured, it is important to characterise and achieve understanding of the form of the ENM (e.g. "free" or agglomerated, soluble or insoluble, coated or "pristine", formulated or unformulated etc.) when present in a food matrix. If the latter is not possible, it should be assumed as a default that the ENM remains in its original form, i.e. as manufactured.

The potential effects on the ENM of the food matrix in which the ENM is intended or expected to be used should be addressed in an appropriate way. This does not mean that every type of food application should be tested individually, but careful consideration needs to be given to the potential effects on the ENM of the type of food(s) in which it is consumed. In particular, the physicochemical characteristics of other ingredients/components within the food may influence the degree to which the ENM is digested or translocated in the GI tract.

In addition, the possibility that components of formulations in which ENMs may be added to foods may alter the dissolution and absorption characteristics of the pristine ENM should be considered, making use of existing data on any known interactions between the ingredients used in such formulations and the parent bulk material with respect to absorption. If the ENM has already been formulated for easier incorporation as a food ingredient and has been administered as such in *in vivo* feeding studies to assess its safety, data on the absorption of the ENM from the formulation is likely to be available to assist the consideration of interactions with the final food.

Understanding the physicochemical properties of the ingredients in a food and, in particular, how they act physiologically can give indications of how they may influence the absorption of ENMs. For example, an ENM which is fat-soluble could be expected to dissolve readily when present in a high fat food. In the majority of cases the food will be unlikely to increase absorption and is more likely to result in a decrease, such as when indigestible fibre or protein may bind or adsorb onto the ENM, thereby modifying the extent or site of its absorption or other characteristics.

Thus, the foregoing information on the food should be considered carefully, taking account of its major ingredients or components which have physiological properties likely to influence the absorption of ENMs. Provided sufficient data are available to make this assessment, the expected influence of the food matrix on the absorption of the ENM may be determined without the need to engage in additional experimental work.

Only when significant data gaps are identified is it envisaged that specific additional studies may be required. Such studies should be focused and aimed at answering the specific questions identified, using a food matrix which is representative of the expected food types or ingredients requiring the additional investigation.

Similarly to the ENM formulation, *in vivo* administration of the ENM during the safety testing programme may already have used laboratory animal diets not dissimilar to the foods in which the

ENM is intended to be used. Data derived from this phase of the assessment will provide a valuable contribution to predicting the effect of the final food. Even in repeat-dose studies by gavage, the diet can be expected to influence the uptake of the ENM and its characteristics may be compared with the final food in which the ENM will be contained. Laboratory animal diets are typically based on relatively unprocessed natural ingredients, notably ground whole cereals, soybean meal, etc., affording a comparison with those unrefined foods and ingredients which are the most likely to influence ENM absorption.

### 8.2. Exposure assessment

As previously discussed, the principles for the risk assessment of ENM-containing foods is essentially the same as that for other food additives and novel foods. A critical part of the risk assessment is establishing the extent to which consumers will be exposed to the ENM when consuming a food product containing it. A number of factors need to be considered in terms of developing this understanding.

The first step is to determine the type of food product and the consumer habits associated with the ENM-containing food. A staple food product such as flour supplemented with an ENM could potentially be used in a wide range of different products, compared with an ENM delivering a specific function in a single branded product.

The next step is to understand what the maximum use level of the ENM would be in the food and the proportion of the ENM and/or formulation which remains or becomes nano form under the conditions present in the food matrix and during passage through the GIT. This would be related to the function that the ENM performs in the food and the amount that is needed to achieve this.

Finally, these two elements of information would be combined to estimate the level of consumer exposure. In most cases the exposure would be estimated on the basis of existing information on the consumption of the foods in which the ENM is proposed for use. However, the marketing and positioning of the ENM-containing food might alter its appeal and change its pattern of consumption, particularly if it has some perceived health benefit. Estimates of consumption of the food might then be based on on-pack instructions for use or worst case estimates.

The prevalence of conditions in the population of interest which might increase ENM absorption and therefore systemic exposure such as inflammatory bowel disease (e.g. Crohn's disease, irritable bowel syndrome) may require consideration.

There should also be a consideration of the appropriate dosemetric for nano form materials recognising a range of particle size distributions. It maybe that number of particles per unit of food intake is more appropriate than mass per unit of food intake. Clearly the units of this measurement have a direct effect on how the exposure assessment is reported and it is important that the exposure assessment can be directly compared to the hazard characterisation. The units utilised should be related to the parameter driving the hazard characterisation and, assuming that the physiochemical characteristics are understood, it will be possible to convert between units.

Guidance on the considerations to be weighed in the design, conduct and reporting of exposure assessments for food ingredients and food chemicals in general is available from a number of sources (Kroes et al., 2002; FAO/WHO, 2005; EFSA, 2005, 2006; WHO, 2008).

## 9. Discussion and conclusions

In order to provide guidance for the safety assessment of ENMs as used in direct food applications, a systematic approach was developed based on the conventional risk assessment paradigm used for foods, novel foods and chemicals. The approaches and testing methods used to assess the impact of ENM on human health are broadly the same as those employed for bulk materials. The question as to whether ENMs contribute unique toxicological concerns has been debated widely and the general conclusion is that this is not the case (SCENIHR, 2009).

Nanomaterials comprise a wide range of materials and key to their prioritisation for testing is a standardised definition of the term 'ENM' to ensure clear inclusion and exclusion criteria for regulatory purposes. However, whatever the final regulatory definition, it is important to acknowledge that the safety of ENMs for the purposes of food applications can still be adequately addressed. As with all safety testing, uncertainties and assumptions used in the risk assessment process must be acknowledged.

The safety assessment of ENM 'as manufactured' for food applications is divided into five steps: characterisation of the bulk material(s) from which the ENM is derived; characterisation of the physicochemical properties of the ENM; identification of ENMs requiring focused toxicological assessment (Decision Tree); toxicological assessment (Tiered Approach) and safety evaluation of the ENM 'as used' in its intended food matrix.

The decision tree sorts ENMs for toxicological testing based on solubility and dissolution rate. This approach allows for uncertainties over readiness of solubility to result in the ENM defaulting to the side of the tree requiring investigation of the potential for 'ENM-specific' effects. *In vivo* solubility is considered to be a key criterion as materials of the highest concern from a toxicological perspective are those which have the potential for systemic translocation from the gut, are insoluble or only partially soluble over time and are particulate or fibrous and bio-persistent. Although clearance pathways exist, such ENMs have the potential to accumulate which will need investigation. In contrast it is considered unlikely that the body will respond to soluble ENMs differently than to their non-nano form counterparts, other than responding to improved bioavailability where it exists. While this could lead to higher blood and or tissue concentrations with the potential for increased toxicity it is considered likely that in most situations the improved bioavailability would enable lower doses to be employed to achieve the same functional effect.

A tiered approach to subsequent hazard identification and characterisation allows decisions on further progression to higher levels of investigation to be taken logically, based on weight of evidence considerations. Tier 1 is an initial screening step designed to consider and detect the potential for any 'ENM-specific' effects using *in silico* and *in vitro* models. Coupled with a short repeat-dose toxicity study to investigate any acute or sub-acute effects *in vivo,* the combined information can contribute to a preliminary toxicological and biokinetic profile, the latter focussed towards the potential for systemic translocation and bio-persistence. Tier 2 is undertaken to investigate the effect of repeated dosing in a 90-day study which may be extended to investigate particular endpoints or aspects of ADME. If sufficient evidence indicates potential concerns from Tier 1, a developer may decide that such an ENM would not be suitable for further development, and may not progress to Tier 2.

The use of validated OECD toxicological testing methodologies combined with focused, standardised *in vitro* studies is recommended. Further studies should be conducted case-by-case as indicated. Dosimetry and internal exposure is a complex issue. As a default assumption, 100% bioavailability would provide a worst case internal exposure scenario, which could be further refined as additional data become available. As a reference point from which to guide the extent and design of toxicological testing, bulk materials and/or reference ENM(s) can be used as comparators and indeed positive and negative controls at different steps of the risk assessment process.

Key to human exposure assessment is the behaviour of the ENM 'as-used' in the food matrix, which will potentially impact the solubility and ultimately the bioavailability of the ENM.

Exciting developments are taking place on the dynamics of protein adsorption/desorption on the surface of nanoparticles which will help to provide an insight into how the body "sees" the ENM at different stages of its 'life-cycle' as it passages through the body. New areas are also likely to include research into the interaction between ENMs and the gut microflora. Significant strides are being taken with regard to analytical methodologies for ENMs, both in isolation and in biological matrices such as foodstuffs. These techniques will be relevant for the detection in biological matrices such as animal tissues. Developments in the fields of genomics and structure activity relationships and further *in vitro* research may also add new dimensions.

In summary, the proposed approach and framework allows for the safety assessment of ENMs for use in foods in a step-wise, systematic, yet flexible manner, proportionate to their physicochemical characteristics and hence potential for toxicological concern. The safety testing strategy is considered applicable to variations in ENM size within the nanoscale and new generations of ENM.

## Conflict of Interest

Andrew Cockburn acted as Chairman of the Expert Group for the duration of our consideration of the topic, through drafting and the Peer Review process when 3rd party experts were assembled to offer their critical input face to face. During the process he has made several formal presentations to interested noncommercial parties; on two occasions he received a small honorarium and/or assistance with travel and accommodation. In addition to his role as Visiting Professor to the University of Newcastle he acts from time to time as an ad hoc expert to EFSA CONTAM Panel and as an independent toxicologist. None of these activities have been related to nanotoxicology. Roberta Bradford is an employee at Unilever. Neil Buck is an employee at DSM Nutritional Products Ltd., Anne Constable is an Nestlé employee. Bernd Haber is an employee of BASF SE. Paul Hepburn is an employee of Unilever. John Howlett and Gareth Edwards are indipendet consultants in scientific and food regulatory affairs. From time to time they receive fees from food and food ingredient manufacturers for consultancy work on specific projects, none of which have had direct relevance for the subject matter of this publication. Frans Kampers, Christoph Klein, Marek Radomski, Hermann Stamm and Susan Wijnhoven declare that there are no conflicts of interest. Tanja Wildemann was employed by ILSI Europe at the time of publication.

## Glossary

*Agglomerate:* A group of particles held together by weak forces, such as van der Waals forces, some electrostatic forces and/or surface tension. It should be noted that an agglomerate will normally retain a high surface to volume ratio (SCENIHR, 2007).

*Aggregate:* A group of particles held together by strong forces such as those associated with covalent or metallic bonds. It should be noted that an aggregate may retain a high surface to volume ratio (SCENIHR, 2007).

*Bioavailability:* The fractional amount of a substance that, after ingestion, becomes available for interaction with target tissues.

*Bulk material:* In the context of nanomaterials, the term bulk material is in common use to describe the same material in other, more conventional, physical forms (SCENIHR, 2007) (same as non-nano form).

*Comparator:* The bulk material (i.e. non-nano form) for which there is a well-established history of use.

*Dissolution:* The dissolving of a material in a liquid. Pharmaceutically it is defined as the rate of mass transfer from a solid surface into the dissolution medium or solvent under standardized conditions of liquid/solid interface, temperature and solvent composition. It is a dynamic property that changes with time and explains the process by which a homogenous mixture of a solid or a liquid can be obtained in a solvent. It happens to chemically occur by the crystal breakdown into individual ions, atoms or molecules and their transport into the solvent.

*Dissolution rate:* The amount of substance that goes into solution per unit time under standardised conditions of liquid/solid interface, temperature and solvent composition.

*Dose/dose rate:* A measure of the amount of a substance administered experimentally or to which an individual is exposed through the diet.

*Dose:* For a single administration or exposure it is expressed as an amount of the substance (usually quantified as mass or number of particles) per unit body weight.

*Dose rate:* For repeated administrations or chronic exposure it is expressed as an amount of the substance (usually quantified as mass or number of particles) per unit body weight per unit time.

*Engineered nanomaterial (ENM):* Rationally designed, manufactured nanomaterial (ISO, 2010). Any material that is deliberately created such that it is composed of discrete functional parts, either internally or at the surface, many of which will have one or more dimensions of the order of 100 nm or less (EFSA, 2009). Any intentionally produced material that has one or more dimensions of the order of 100 nm or less or that is composed of discrete functional parts, either internally or at the surface, many of which have one or more dimensions of the order of 100 nm or less, including structures, agglomerates or aggregates, which may have a size above the order of 100 nm but retain properties that are characteristic of the nanoscale. Properties that are characteristic of the nanoscale include (i) those related to the large specific surface area of the materials considered; and/or (ii) specific physicochemical properties that are different from those of the non-nanoform of the same material (Council of the European Union, 2009).

*GIT:* Gastrointestinal tract.

*HOSU:* History of safe use.

*Manufactured nanomaterial:* Nanomaterial intentionally produced to have specific properties or composition (ISO, 2010).

*Nanofibre:* Nano-object with two similar external dimensions in the nano scale and the third dimension significantly larger. A nano fibre can be flexible or rigid. The two similar external dimensions are considered to differ in size by less than three times and the significantly larger external dimension is considered to differ from the other two by more than three times. The largest external dimension is not necessarily in the nanoscale (ISO, 2008).

*Nanoform:* A form of a substance with nanomaterial properties, as opposed to the bulk form of the same substance without nanomaterial properties (European Commission, 2008).

*Nanomaterial:* Material with any external dimension in the nanoscale or having internal or surface structure in the nanoscale (ISO, 2010).

*Nano-object:* Material with one, two or three external dimensions in the nanoscale (ISO, 2010).

*Nanoparticle:* A discrete entity which has three dimensions of the order of 100 nm or less (SCENIHR, 2007).

*Nanoscale:* Size range from approximately 1–100 nm (ISO, 2010).

*Nanoscience:* The systematic study, discovery and understanding of matter, properties and phenomena related to the nanoscale (ISO, 2010).

*Nanosheet:* A discrete entity which has one dimension of the order of 100 nm or less and two long dimensions (SCENIHR, 2007).

*Nanostructure:* The interrelation of the constituent parts of a material in which one or more of those constituent parts belong to the nanoscale (ISO, 2010).

*Nanostructured material:* Material having internal or surface structure in the nanoscale (ISO, 2010).

*Nanotechnology:* The application of scientific knowledge to control and utilise matter in the nanoscale, where properties and phenomena related to size or structure can emerge (ISO, 2010).

*Nanotube:* A discrete hollow entity which has two dimensions of the order of 100 nm or less and one long dimension (SCENIHR, 2007).

*RA:* Risk assessment.

*QSAR (Quantitative Structure Activity Relationship):* It is a quantitative relationship between a biological activity (e.g. toxicity) and one or more molecular descriptors that are used to predict the activity. A molecular descriptor is a structural or physicochemical property of a molecule, or part of a molecule, which specifies a particular characteristic of the molecule and is used as an independent variable in a QSAR.

*Reference nanomaterial or reference nano form (material):* A representative, standardised nano material with corresponding information and data regarding its properties allowing its use as a reference item in ENM safety assessment, see http://www.rsc.org/chemistryworld/News/2011/February/17021101.asp.

*Reticuloendothelial System:* A widely distributed system consisting of phagocytic cells able to ingest bacteria or colloidal particles.

*Solubility:* Degree to which a material (the solute) can be dispersed in another material (the solvent) such that a single, temporally stable, phase results (OECD, 2010a).

*Solubilisation:* To make or become soluble.

*Translocation:* A change of location.

## Acknowledgements

The authors would like to thank Dr. Karin Aschberger and Dr. David Carlander for their advice and contributions in the preparation of the manuscript. Furthermore, the authors would like to acknowledge the participants of the Workshop on the Safety Assessment of Nanomaterials in Food (13–15 April 2011, Cascais, Portugal) for their discussions which were taken into account in finalising this publication.

This work was commissioned by the Novel Foods and Nanotechnology Task Force of the European Branch of the International Life Sciences Institute (ILSI Europe). Industry members of this task force are BASF, Coca-Cola Europe, Danone, DSM, Kraft Foods Europe, Mars, Nestlé and Unilever. This publication was coordinated by Alessandro Chiodini, Scientific Project Manager at ILSI Europe. For further information about ILSI Europe, please email info@ilsi-europe.be or call +32 2 771 00 14. The opinions expressed herein and the conclusions of this publication are those of the authors and do not necessarily represent the views of ILSI Europe nor those of its member companies.

Karin Aschberger, Andrew Cockburn, Gareth Edwards, John Howlett, Frans Kampers, Marek Radomski, Hermann Stamm and Susan Wijnhoven received an honorarium from ILSI Europe for their participation in this publication and/or reimbursement of their travel and accommodation costs for attending the related meetings.

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