The Role of the Food Matrix and Gastrointestinal Tract in the assessment of biological properties of ingested engineered nanomaterials (iENMs): State of the science and knowledge gaps

David Julian McClements^{a*}, Glen DeLoid^b, Georgios Pyrgiotakis^b, Jo Anne Shatkin^c, Hang Xiao^a and Philip Demokritou^{b*}

^a*Department of Food Science, University of Massachusetts Amherst, Amherst, MA 01003, USA*

^b*Laboratory for Environmental Health NanoScience (LEHNS)
Center for Nanotechnology and Nanotoxicology, T. H. Chan School of Public Health,
Harvard University, 665 Huntington Avenue, Boston, MA 02115, USA*

^c*Vireo Advisors, LLC. PO Box 51368, Boston, MA 02205, USA*

***corresponding authors:**

David Julian McClements, Department of Food Science, University of Massachusetts Amherst, Amherst, MA 01003, USA. mcclements@foodsci.umass.edu; Tel: 413 545 1019.

Philip Demokritou, Center for Nanotechnology and nanotoxicology, T.H. Chan School of Public Health, Harvard University, Boston MA 02115, email: pdemokri@hsph.harvard.edu, Tel 617 432-3481, Web: www.hsph.harvard.edu/nano

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ABSTRACT

Many foods contain appreciable levels of engineered nanomaterials (ENMs) (diameter < 100 nm) that may be either intentionally or unintentionally added. These ENMs vary considerably in their compositions, dimensions, morphologies, physicochemical properties, and biological responses. From a toxicological point of view, it is often convenient to classify ingested ENMs (iENMs) as being either inorganic (such as TiO₂, SiO₂, Fe₂O₃, or Ag) or organic (such as lipid, protein, or carbohydrate), since the former tend to be indigestible and the latter are generally digestible. At present there is a relatively poor understanding of how different types of iENMs behave within the human gastrointestinal tract (GIT), and how the food matrix and biopolymers transform their physico-chemical properties and influence their gastrointestinal fate. This lack of knowledge confounds an understanding of their potential harmful effects on human health. The purpose of this article is to review our current understanding of the GIT fate of iENMs, and to highlight gaps where further research is urgently needed in assessing potential risks and toxicological implications of iENMs. In particular, a strong emphasis is given to the development of standardized screening methods that can be used to rapidly and accurately

assess the toxicological properties of iENMs.

Abbreviations: ENM: engineered nanomaterial; iENM: ingested engineered nanomaterial; GI: gastrointestinal; GIT: gastrointestinal tract; GRAS: generally regarded as safe; TiO₂: titanium dioxide, SiO₂: silicon dioxide; Fe₂O₃: iron (III) oxide; Fe₃O₄: iron (II, III) oxide; Ag: silver; SAR: Structure-activity relationship; SERS: Surface-enhanced Raman spectroscopy; sp-ICP-MS: single particle inductively coupled plasma mass spectrometry; FFF: field flow fractionation; XANES: X-ray absorption near edge structure; SEM: scanning electron microscopy; TEM: transmission electron microscopy.

1. Introduction

Many foods contain organic or inorganic particles that have dimensions in the nanoscale range, generally defined as having at least one dimension less than 100 nm (**Figure 1**). It is worth pointing out that the 100 nm size cut off is arbitrarily selected and that other properties in addition to size may contribute to the unique properties of such materials. These nanoparticles may be naturally present in foods, intentionally added to achieve a particular functional attribute, or unintentionally introduced through the environment, ingredients, packaging or processing operations (Bellmann *et al.*, 2015; Szakal *et al.*, 2014a; Yada *et al.*, 2014b). Naturally occurring nanoparticles in food include the casein micelles found in milk, which typically have dimensions in the range from a few tens of nanometers to a few hundred nanometers (Holt *et al.*, 2003; Livney, 2010). Organic engineered nanomaterials (ENMs) are often intentionally added to foods or beverages to encapsulate vitamins, nutraceuticals, flavors, colors, or antimicrobials (Livney, 2015; McClements *et al.*, 2015; Shin *et al.*, 2015; Yao *et al.*, 2015b). Inorganic ENMs are intentionally added to foods for numerous reasons: TiO₂ is added to provide opacity and brightness (Weir *et al.*, 2012); SiO₂ nanoparticles are added to modify food texture and powder flow characteristics (Dekkers *et al.*, 2011; Peters *et al.*, 2012); iron oxides (Fe₂O₃ or Fe₃O₄) are added to provide bioavailable iron for nutritional purposes (Raspopov *et al.*, 2011; Zimmermann and Hilty, 2011) or as colorants; and, Ag nanoparticles are added as antimicrobial agents (Hajipour *et al.*, 2012).

ENMs may also be introduced into food products unintentionally during the food manufacturing process. There may be nanoparticles present in the air or the water used within a food processing facility that find their way into the final product. Certain food ingredients containing micron-sized particles have been approved for use in food by regulatory authorities,

and are generally regarded as safe (GRAS), without the need of characterization or definition of particle size. However, these ingredients may also contain ENMs that the food manufacturer is unaware of. For example, food grade TiO₂ particles are used as lightening agents to increase the whiteness of foods, and so they are usually designed to have dimensions similar to the wavelength of light (a few hundred nanometers) so as to maximize their light scattering efficiency. However, there may be a population of nano-scale particles present within commercial ingredients (Warheit *et al.*, 2015). For example, food grade TiO₂ (E171 – European designation) contains nano-scale particles (Athinarayanan *et al.*, 2015), and the presence of nano-sized TiO₂ has been reported in dietary supplements (Lim *et al.*, 2015) and certain food products (Peters *et al.*, 2014). Likewise, nanoscale SiO₂ particles have been reported in food grade SiO₂ ingredients (E551) and at levels of up to 14.4 µg/g in commercial foods and dietary supplements (Athinarayanan *et al.*, 2015; Athinarayanan *et al.*, 2014; Dekkers *et al.*, 2011; Lim *et al.*, 2015). Furthermore, mechanical size reduction operations commonly used in the food industry, such as grinding, homogenization, or spray drying, typically produce a distribution of particle sizes, and some of these particles may also fall in the nanoscale range (McClements, 2011). In addition, packaging materials may contain functional ENMs that migrate into a food or beverage product during storage (Abdul Khalil *et al.*, 2016; Aulin *et al.*, 2012; Costa *et al.*, 2014; Dehnad *et al.*, 2014; El-Wakil *et al.*, 2015; Pezzuto *et al.*, 2015). It is also possible that solutes could migrate into a food and precipitate due to the change in their thermodynamic environment, thereby leading to the formation of nanoparticles in a food.

The physicochemical and physiological properties of particles may change considerably when their dimensions fall within the nanoscale range, *e.g.*, chemical reactivity, mechanical strength, digestibility, transport properties, and cellular uptake, accumulation, and distribution

(Acosta, 2009; Buzea *et al.*, 2007; Cohen *et al.*, 2014a; Davidson *et al.*, 2016; Konduru *et al.*, 2015; Konduru *et al.*, 2014; Konduru *et al.*, 2016; Lu *et al.*, 2016a; Lu *et al.*, 2016b; Ma *et al.*, 2015; Pirela *et al.*, 2016a; Pirela *et al.*, 2016b; Sisler *et al.*, 2015; Sotiriou *et al.*, 2014; Watson *et al.*, 2014; Yokel *et al.*, 2014; Zhou *et al.*, 2014). These changes are associated with the smaller particle dimensions and higher surface areas of nanoparticles compared to larger particles, as well as to quantum effects taking place in the nanoscale (Pyrgiotakis *et al.*, 2014; Pyrgiotakis *et al.*, 2013).

Consequently, food consumers, manufacturers, and regulators are concerned about the potential health risks associated with ENMs in foods (Bouwmeester *et al.*, 2009; Bugusu *et al.*, 2009; Yada *et al.*, 2014b). Knowledge of the impact of ingested ENM characteristics on their GI fate is currently rather limited. In addition, there is a lack of standardized protocols and methodologies to assess the GI fate of ingested engineered nanomaterials (iENMs), and their potential toxicity (Contado, 2015; Szakal *et al.*, 2014a; Szakal *et al.*, 2014c).

The purpose of this article is to provide a brief overview of the current understanding of the GI fate of iENMs, to outline major knowledge gaps and challenges in assessing their potential toxicological effects, to highlight areas where there is a lack of knowledge, and to indicate where further research is needed.

2. Intrinsic properties of iENMs

iENMs may vary considerably in their properties (**Figure 2**), which will alter their GIT fate and potential toxicity. Some of the most important ways that the properties of food-grade ENMs may vary are highlighted below:

- Composition:** ENMs may be formed from organic substances (such as lipids, proteins, and carbohydrates) or inorganic substances (such as Ag, SiO₂, TiO₂, and Fe₂O₃). The composition of an ENM has a major impact on its fate within the GIT. Many organic substances are digested by enzymes within the GIT, whereas many inorganic substances may not be digested, but may be dissolved in certain regions of the GIT due to changes in pH or dilution (Dekkers *et al.*, 2011). Nanoparticles may also impact the intestinal microbiome. For example Williams et al. reported size- and dose-dependent changes in ileal-mucosal bacterial populations, including a shift toward gram negative organisms, in rats gavaged with silver nanoparticles (Williams *et al.*, 2015). The chemical reactivity and toxicity of inorganic ENMs is also closely related to their composition, *e.g.*, gold nanoparticles tend to have a relatively low chemical reactivity and toxicity (Khalili Fard *et al.*, 2015; Pradeep and Anshup, 2009), whereas silver nanoparticles tend to be relatively highly reactive and toxic due to the release of ions (Brasiliense *et al.*, 2016; Khalili Fard *et al.*, 2015).
- Structure:** Pristine ENMs vary greatly in their initial size, morphology, crystallinity, porosity, and aggregation state, depending on the materials and processing operations used to fabricate them (Yada *et al.*, 2014b). Nanoparticle structure is likely to have a major impact on their fate within the GIT, *e.g.*, their ability to pass through biological barriers such as the mucus layer or epithelium cells, or to interact with other substances within the GIT (Bellmann *et al.*, 2015). In particular, the association (aggregation or agglomeration) of primary ENMs with each other is likely to be particularly important. It should be noted that the closely related terms aggregation and agglomeration have different meanings here, particularly in the context of inorganic ENMs. Herein we will use aggregation to indicate the fusing of primary ENM particles that occurs during their synthesis, and agglomeration to

refer to the looser associations, generally governed by Van der Waals, electrostatic, and hydrophobic forces, between ENMs that are dispersed in liquids. Whereas aggregation state is generally stable after synthesis (in the absence of dissolution or the application of extreme forces or conditions) agglomeration state is often dynamically changing, and easily altered by changes in the extrinsic properties of the suspending media (i.e., pH, ionic strength, protein content)(Cohen *et al.*, 2013). A further distinction is that aggregates are composed of the ENM material only, whereas agglomerates include media and media components trapped between or adsorbed onto the primary ENM particles (e.g. the protein corona)(DeLoid *et al.*, 2014). The effect of the protein corona on nano-bio interactions is well understood and noted in the nanotoxicology research(Kelly *et al.*, 2015; Monopoli *et al.*, 2011a; Pyrgiotakis *et al.*, 2014; Pyrgiotakis *et al.*, 2013).

- **Interfacial Properties:** The nature of the substances located at the surfaces of ENMs influence their interfacial properties, such as chemical reactivity, thickness, rheology, charge, polarity, and protein corona. These interfacial characteristics may also influence the GIT fate of nanoparticles, such as their penetration through biological barriers, interactions with other substances, and agglomeration state and stability.

The intrinsic properties of iENMs would be expected to play a major role in determining their biological fate and potential toxicity. However, there is currently a relatively poor understanding of how the initial composition, structure, and interfacial properties of food-grade nanoparticles influence their behavior within the complex environment of the human GIT (Magnuson *et al.*, 2011; Powell *et al.*, 2010; Weir *et al.*, 2012) and impact their biokinetics and toxicity. Several studies have attempted to correlate various metal oxide ENM physiochemical descriptors with measurements of *in vitro* toxicity in order to develop structure-activity

relationships (SARs). (Liu *et al.*, 2014; Liu *et al.*, 2013). Molecular SARs have been extensively and successfully used in the field of drug discovery and development (Akhtar *et al.*, 2016; Cordomi *et al.*, 2016; Monteiro *et al.*, 2016; Putz *et al.*, 2016; Sharma *et al.*, 2016). Likewise the ultimate goal of developing nano-SARs is to enable prediction of toxicity based on ENM intrinsic physicochemical properties and thus enable safer by design development of commercial ENMs. Clearly there is a need to develop SARs specifically for iENMs to understand their potential toxicological implications, and this will be an important area for systematic study in the future.

3. iENM property transformations: Impact of GIT Conditions

The initial physicochemical properties and structural characteristics of ingested engineered nanoparticles will impact their GI fate in a number of ways: (i) they may influence their interactions with other components in the GI fluids (such as enzymes, bile salts, phospholipids, and biopolymers); (ii) they may influence their agglomeration state and stability within different GIT regions; (iii) they may impact their passage through the mucus layer that coats the intestinal lining; (iv) they may influence their transport into and across the epithelium cells, *e.g.*, enterocytes and M-cells; (v) they may impact their interaction with colonic bacteria in the large intestine (Bellmann *et al.*, 2015; Bouwmeester *et al.*, 2009; Ensign *et al.*, 2012; Frohlich and Roblegg, 2012; Magnuson *et al.*, 2011; Powell *et al.*, 2010). One of the major limitations of many previous studies of the potential GIT fate of iENMs has been the use of over-simplistic cellular model systems that ignore the potential influence of GIT conditions on nanoparticle characteristics (Szakal *et al.*, 2014a). For example, the absorption of iENMs is often measured by directly incubating “pristine” nanoparticle suspensions with model intestinal cells rather than

passing them through a GIT model first. Furthermore, these pristine ENMs are usually incubated in a culture medium that contains serum proteins, which results in protein corona formation and agglomeration that will affect the bioactivity and biological properties of the nanoparticles (Cohen *et al.*, 2013; Lundqvist *et al.*, 2011; Monopoli *et al.*, 2011b; Tenzer *et al.*, 2013; Walkey and Chan, 2012). In addition, these simple cellular models ignore the effects of agglomeration on the mass transport and dosimetry of the iENMs, thereby resulting in erroneous and difficult to interpret dose-response data (Cohen *et al.*, 2015; Cohen *et al.*, 2014b; DeLoid *et al.*, 2014; DeLoid *et al.*, 2015). Consequently, the interfacial properties and agglomeration state of the iENMs are not physiologically relevant and may be very different from what the intestinal cells would encounter in real life. Commonly used *in vitro* cellular approaches have significant deficiencies and may lead to misleading or erroneous conclusions being made about the impact of specific iENM characteristics on their GI fate and biological properties.

An improved understanding of the fate of iENMs should therefore take into consideration the complex physicochemical and physiological processes that occur as they pass through the human GIT (Yada *et al.*, 2014b) and their transformations. After ingestion ENMs may move through the mouth, stomach, small intestine, and large intestine. A summary of the different environments that iENMs experience within these different regions of the GIT is given in **Figure 3**. During this journey the iENMs are exposed to: (i) changes in pH and ionic composition; (ii) surface active components (*e.g.*, bile salts, phospholipids, fatty acids, proteins); (iii) digestive enzymes (*e.g.*, amylases, proteases, lipases); (iv) biopolymers (*e.g.*, proteins, mucins); (v) biological surfaces (*e.g.*, tongue, esophagus, stomach, intestine); (vi) intestinal microbes (*e.g.*, colonic bacteria); and, (vii) complex flow/force profiles (Blijdenstein *et al.*, 2004; de Hoog *et al.*, 2006; Gwartney *et al.*, 2004; Malone *et al.*, 2003a; Malone *et al.*, 2003b; Silletti *et al.*,

2007; Vingerhoeds *et al.*, 2005). As a result, the composition, size, shape, charge, dissolution, and agglomeration state of iENMs may change considerably as they travel through the GIT (**Figure 4**). This has important consequences for determining their overall GI fate, *e.g.*, whether they are digested, dissolved, absorbed by intestinal cells, or reach the colon. Inorganic iENMs may also be distributed throughout the body after absorption and may accumulate in specific organs, which may impact their toxicity (Cha *et al.*, 2008; Kim *et al.*, 2010; Konduru *et al.*, 2015; Konduru *et al.*, 2016; Kulthong *et al.*, 2012; Park *et al.*, 2010; Zhang *et al.*, 2013).

It is often possible to carry out a comprehensive analysis of the intrinsic properties of pristine ENMs before they are intentionally added to foods because they are isolated in a simple liquid or powder form. Thus, it is possible to measure their initial particle size distribution, shape, composition, surface chemistry, and surface charge using conventional methods (Singh *et al.*, 2014; Szakal *et al.*, 2014a; Szakal *et al.*, 2014c). However, these intrinsic ENM properties may change considerably when the nanoparticles encounter the complex environment of the human GIT. Some of the potential changes of nanoparticles that may occur are highlighted in **Figure 4**, and briefly discussed below:

- **Interfacial composition and properties:** The nature of the molecular species at the surface of iENMs may change for a number of reasons. The GIT contains numerous types of surface-active molecules that may come from the ingested food (such as surfactants, free fatty acids, monoacylglycerols, proteins, peptides, and phospholipids) or be secreted by the human body (such as mucin, proteins, phospholipids, and bile salts). These surface-active molecules may adsorb to the nanoparticle surfaces and alter interfacial composition. Depending on the system, they may also adsorb to bare nanoparticle surfaces (adsorption), they may displace existing surface-active molecules from nanoparticle surfaces (competitive adsorption), or

they may adsorb between or on top of existing surface-active molecules (co-adsorption). The resulting changes in interfacial composition, structure, properties, and charge will alter the subsequent fate of the iENMs.

- ***Core composition and properties:*** The internal composition of iENMs may also change appreciably as they pass through the different regions of the GIT. The composition of digestible ENMs may be altered due to their hydrolysis by digestive enzymes in the GIT fluids. Water, salts, and other components may diffuse in or out of a nanoparticle and cause it to swell or shrink, thereby altering its composition. Nanoparticles may chemically react with other components in the GIT fluids thereby altering their composition, *e.g.*, due to oxidation reactions.
- ***Dimensions and agglomeration state:*** The size of the individual ENMs present within a food may either increase or decrease as they pass through the GIT. Particles that are susceptible to dissolution or degradation in GIT fluids may decrease in dimensions within the GIT. For example, lipid or protein nanoparticles are digested by lipases and proteases, whereas some organic nanoparticles dissolve or dissociate under the highly acidic conditions of the stomach (Axson *et al.*, 2015; Salvia-Trujillo *et al.*, 2015; Zou *et al.*, 2016). On the other hand, initially small particles may increase in dimensions as they pass through the GIT due to agglomeration, coalescence, and flocculation. Certain types of lipid nanoparticles coalesce in the GIT and form larger particles that can no longer be considered to be in the nanoscale range (Salvia-Trujillo *et al.*, 2013). Inorganic ENMs often form agglomerates in liquid suspension, typically consisting of multiple protein-coated primary particles interspersed with suspending liquid. While the surfaces of such agglomerates contain nano-scale features, the agglomerates themselves, like coalesced organic particles, are often not strictly in the

nanoscale range. In some cases, very large numbers of agglomerates can also flocculate to form macroscopic structures. Obviously, these changes in particle dimensions will impact the subsequent GI fate of the nanoparticles and should be taken into account. The dimensions of iENMs that are comprised of substances that have an appreciable solubility in water may also undergo changes in their dimensions due to Ostwald ripening processes, *i.e.*, the diffusion of molecules from small to large particles through the intervening medium (Li *et al.*, 2009; Wooster *et al.*, 2008).

- **Physical state:** iENMs may be fabricated from materials that are liquid or solid (amorphous or crystalline). The physical state of nanoparticles may change as they pass through the GIT due to alterations in temperature or solution conditions, which will impact their GI fate. A solid-liquid phase transition may occur at a temperature around the melting point of nanoparticles or at a concentration around their solubility limit. The physical state of lipid nanoparticles has been shown to impact their digestibility by lipase (Bonnaire *et al.*, 2008), as well as their stability to partial coalescence (Golding *et al.*, 2011), which altered their GIT fate. Knowledge of the physical state of nanoparticles in different regions of the GIT may therefore be important for understanding and controlling their behavior.

In vitro cellular tests developed to understand and predict the biological behavior of iENMs should therefore take these complex events into account; otherwise they will give unrepresentative results. As mentioned earlier, transformations in the properties of iENMs as they travel through the GIT are rarely considered in *in vitro* studies, preventing risk assessors from accurately assessing or quantifying potential impacts.

4. iENM property transformations: Impact of Food Matrix Effects

Foods and beverages are highly complex materials that vary considerably in their compositions, structures, and properties. They may have different pH values, ionic strengths, and ingredient types. They may be liquid, semi-solid, or solid. They may be homogeneous or heterogeneous at different length scales. They may be clear, turbid, or opaque. During processing and preparation, foods may be exposed to different mechanical forces and temperature profiles. The introduction of nanoparticles into these complex materials may alter the properties of both the nanoparticles and the foods. It is often difficult using existing techniques to characterize the properties of ENMs within foods because food matrices have such complex compositions and structures, and complete characterization of ENMs in food matrices may require development of new methods (Bellmann *et al.*, 2015; Szakal *et al.*, 2014a). Other analytical tools that might be used to provide valuable information about nanoparticle characteristics in the complex milieu of the GIT include Surface-enhanced Raman spectroscopy (SERS) (Taylor *et al.*, 2016), single particle inductively coupled plasma mass spectrometry (sp-ICP-MS) (Ma *et al.*, 2016; Montano *et al.*, 2016), Field flow fractionation (FFF) (Laborda *et al.*, 2016; Zattoni *et al.*, 2014), X-ray absorption near edge structure (XANES) (Grafe *et al.*, 2014; Kuzmin and Chaboy, 2014) and scanning and transmission electron microscopy (SEM and TEM).

In this section, we highlight some of the ways in which the food matrix may impact the properties of iENMs.

4.1. Within the food product

When pristine ENMs are mixed with a complex food matrix there may be appreciable

alterations in a number of their properties: interfacial composition and properties; core composition and properties; dimensions and agglomeration state; physical state. These factors are similar to those discussed in the previous section on the impact of GIT conditions on ENM properties, and therefore will not be considered in detail here. Another important consideration is the physical location of the iENMs within the food matrix. For example, in heterogeneous foods that contain lipid, water, and biopolymer components, the nanoparticles may be dispersed in the water phase, in the oil phase, at the oil-water interface, or they may be associated with biopolymers (Yada *et al.*, 2014a). It should be noted that many foods already contain other particles with dimensions in the nano-scale or micro-scale levels, and the presence of these particles may interfere with the isolation and characterization of the iENMs of interest.

4.2. Within the GIT

The nature of the food that is co-ingested with iENMs may also have a major impact on their GIT fate (Bellmann *et al.*, 2015). Consequently, the biological effects of a particular type of nanoparticle may change appreciably depending on the type and amount of food or beverage that it is consumed with. The interactions of iENMs with food matrices and potential effects on their physico-chemical properties is often ignored when considering their potential toxicity and should be taken into account (Cohen *et al.*, 2013; Gerloff *et al.*, 2013; Zeeb *et al.*, 2015). In this section, some of the ways that the food matrix may influence the potential GIT fate of ingested nanoparticles are discussed.

The environment of the iENMs in a food will be changed appreciably when the food matrix passes through the GIT for a number of reasons (Norton *et al.*, 2015). First, the food is diluted with GI fluids secreted from the mouth, stomach, and small intestine, which will alter the pH and ionic strength of the surrounding fluid by an amount that depends on the type and level of food

ingested. Second, macronutrients are hydrolyzed by digestive enzymes in the GIT, thereby breaking down structures present in the original food and altering the chemical composition of the GIT fluids. Third, the GI fluids contain surface-active substances arising from the food and GIT secretions, such as fatty acids, monoacylglycerols, proteins, bile salts, phospholipids, and mucin, which may adsorb to the surfaces of the iENMs. Fourth, the iENM-food matrix mixture will be exposed to complex shear flows/forces within the GIT that may alter the agglomeration state of the iENMs. Thus, the type, amount, and interactions of the ingredients present within the original food will alter the GIT environment that the iENMs are exposed to during their passage through the mouth, stomach, small intestine, and colon. Therefore, the interactions between food and GIT components and the intrinsic properties of pristine iENMs will define their fate and biological properties, and such complex transformations need to be included in cellular studies

5. Assessing the GIT Fate and Toxicity of iENMs: Challenges and knowledge gaps

Data on GIT fate and toxicity of iENMs continue to grow. However major knowledge gaps still remain prohibiting risk assessors to understand potential implications from families of iENMs. In more detail:

5.1. GIT Fate

The properties of pristine nanoparticles in a simple powder or liquid can usually be characterized using existing methodologies: light, electron or atomic force microscopy for microstructure and aggregation state (Pyrgiotakis *et al.*, 2014; Pyrgiotakis *et al.*, 2013); dynamic or static light scattering for particle size distribution; tunable resistive pulse sensing technology for particle size and charge (Pal *et al.*, 2014); microelectrophoresis for particle charge; and,

chromatography, mass spectrometry, or NMR for particle composition.

However, the analysis of the properties of iENMs dispersed in complex food matrices or GI fluids is usually much more challenging (Bellmann *et al.*, 2015; Lopez-Serrano *et al.*, 2014; Singh *et al.*, 2014; Szakal *et al.*, 2014a). Food matrices or GI fluids often contain a complicated mixture of particles and polymers that may have dimensions that overlap with those of the iENMs being studied. This often means that it is difficult to determine the particle size distribution or aggregation state of the nanoparticles using conventional light scattering or microscopy methods since it is difficult to distinguish one type of particle from another. This challenge may be overcome using a number of approaches (Grieger *et al.*, 2016; McClements and McClements, 2016; Szakal *et al.*, 2014b). First, it may be possible to isolate the iENMs from the other particulate matter in the system using selective digestion, filtration, or centrifugation methods. However, one must be careful not to alter the properties of the iENMs being studied using these approaches. Second, it may be possible to discriminate the different types of particles in a system using selective staining methods, *e.g.*, using colored, fluorescent, or radioactive probes. These techniques may enable one to determine the specific location of iENMs in a system and the type of substances that they tend to associate with. Similar problems arise with measurements of the interfacial composition or electrical charge of nanoparticles in complex foods or GIT fluids. The charge on pristine nanoparticles suspended in simple aqueous liquids (*e.g.*, cell culture media) can simply be measured by placing them within a particle electrophoresis instrument (such as a zeta potential analyzer). However, it is difficult to determine the charge on the nanoparticles when they are dispersed within a complex fluid that contains many different types of particles and polymers since they all contribute to the measured electrophoretic signal. Again, it may be possible to isolate the nanoparticles from the other types

of particulate matter in the sample prior to analysis, but one must be careful not to alter the interfacial composition during this process.

5.2. Ingested ENM Toxicity

Despite the large and growing number of ENMs used in agri-food products, nanotoxicology research and hazard assessment of iENMs lags behind that for other routes of exposure (e.g., inhalation, dermal). While evidence of toxicity associated with iENMs continues to grow, several critical knowledge gaps exist, preventing regulators and industry from assessing the potential health risks. These knowledge gaps include: 1) uncertain human exposure data; 2) limited in vivo biokinetic and toxicological studies; and 3) lack of understanding of physicochemical ENM transformations within complex food matrices and within the GIT and their effect on bioactivity. These knowledge gaps must be addressed to develop the necessary screening strategies for hazard assessment, and to help industry design safer iENMs and food products.

Although the potential toxicity of some metal oxide ENMs with substantial ingestion exposure risk have been studied, most studies have focused on inhalation and dermal exposures, or employed in vitro methods using pristine ENMs, thus ignoring important transformations of iENMs resulting from interactions with the food matrix and conditions of the GIT (Wang *et al.*, 2013). In addition, only a few studies have investigated in vivo toxicity of iENMs. Among these, studies of Ag nanoparticles are the most common, but these have often led to either conflicting or inconclusive results (Cha *et al.*, 2008; Jeong *et al.*, 2010; Kim *et al.*, 2010; Kulthong *et al.*, 2012; Park *et al.*, 2010; Zhang *et al.*, 2013). A part of the problem at this early stage in the study of the GI fate of ingested ENMs, is that the few sporadic studies in the literature vary widely in terms of ENMs tested, experimental models used, and endpoints examined. For example, Cha *et al.* reported liver inflammation and increased apoptosis in liver cells, as well as alterations in

expression of genes regulating apoptosis and inflammation in mice directly fed a single 2.5 g bolus of Ag nanoparticles (Cha *et al.*, 2008). On the other hand, Kim *et al.* reported only mild dose-dependent hepatotoxicity in rats fed ≥ 125 mg/kg-day for 90 days (Kim *et al.*, 2010). Although both of these studies are consistent with a potential hazard for liver toxicity resulting from Ag ENM ingestion, the large differences in treatment protocols and experimental models make cohesive conclusions difficult.

In the case of ingested metal oxide ENMs, the *in vivo* toxicology literature is even more sparse. Nevertheless, as with Ag, early indications suggest significant potential for toxicity. For example, TiO₂ nanoparticles were reported to have a variety of toxic effects within the GIT (Bu *et al.*, 2010; Nogueira *et al.*, 2012) and to enter the systemic circulation, accumulate and cause inflammation and oxidative damage in the liver, kidney and spleen (Cui *et al.*, 2011; Duan *et al.*, 2010; Gui *et al.*, 2011; Sycheva *et al.*, 2011). It should be pointed out that these studies focused on effects of TiO₂ nanoparticle ingestion, as distinct from GRAS forms of TiO₂, and that although nano-scale particles have been found in GRAS materials, the majority of particles are in the micron-size range (Athinarayanan *et al.*, 2015). Ingested ZnO nanoparticles were reported to undergo size-dependent intestinal absorption with accumulation in multiple organs and damage to liver and pancreas (Baek *et al.*, 2012; Choi *et al.*, 2015; Esmacillou *et al.*, 2013; Li *et al.*, 2012; Seok *et al.*, 2013). Ingested SiO₂ ENMs caused low-level hepatotoxicity in rats following a 10-week exposure period (So *et al.*, 2008).

In addition, whereas at least a small number of studies of direct toxicity of ingested ENMs are emerging, no studies of iENM toxicity to date have employed animal models of human GIT diseases (e.g. colitis), which could reveal potential human toxicity that would remain masked in healthy animals (Dixit and Boelsterli, 2007). Likewise, while growing evidence continues to

emphasize the critical role of the gut microbiome and its role in overall health (Consortium, 2012; Pflughoeft and Versalovic, 2012; Theriot *et al.*, 2014; Turnbaugh *et al.*, 2009; Weir *et al.*, 2013), potential effects of iENMs on the gut microbiome remain largely unstudied (Bergin and Witzmann, 2013; Wilding *et al.*, 2016; Williams *et al.*, 2015).

Another almost entirely neglected aspect of iENM toxicological characterization is assessment of the complex effects of food matrix and GIT conditions on ENM properties (Blasco and Picó, 2011; Wang, Du, Song and Chen, 2013). As detailed above, the resulting changes to surface chemistry, agglomeration state, and biocorona of iENMs can have profound effects on their absorption, accumulation, biodistribution, and toxicity (Bouwmeester *et al.*, 2009; Ensign *et al.*, 2012; Frohlich and Roblegg, 2012; Powell *et al.*, 2010). Most *in vitro* studies of iENM fate and toxicity have ignored these interactions, employing pristine ENMs suspended in culture media (Esch *et al.*, 2014; Faust *et al.*, 2014; Natarajan *et al.*, 2015), often leading to erroneous conclusions (Gerloff *et al.*, 2013). As discussed above, what is needed is development of *in vitro* and *in vivo* methods for studying transformations of iENMs in the GIT, and identifying structure activity relationships that influence absorption and accumulation. Methods such as simulated GIT models that include mouth, stomach, and small intestine phases that mimic the fluid compositions, incubation times, and flow/pressure conditions of the GIT are crucial for assessing ENM transformations and interactions with food matrices (Arroyo-Maya and McClements, 2016; Cho *et al.*, 2014; Lopez-Pena *et al.*, 2016; Mun *et al.*, 2007; Mun *et al.*, 2006; Ozturk *et al.*, 2015; Park *et al.*, 2007; Qiu *et al.*, 2015; Sandra *et al.*, 2008; Yao *et al.*, 2013; Yao *et al.*, 2015a; Yao *et al.*, 2014; Zou *et al.*, 2015a; Zou *et al.*, 2015b). In future work, it may be useful to employ the newly developing “omic” approaches to better understand the potential toxicity of nanoparticles, e.g., metabolomics, proteomics, and transcriptomics.

Finally, although animal studies are considered critical for hazard assessment, given the variety of iENMs, compounded by the multiplicity of possible food matrices, all of which can modulate uptake and toxicity, comprehensive animal testing of all iENMs would be extremely laborious and costly. Instead, a validated, tiered testing strategy, including in vitro, in vivo, and even in silico approaches, consistent with the US National Research Council's prescribed paradigm shift toward alternative testing strategies in *Toxicity Testing in the 21st Century: A Vision and a Strategy* (Krewski *et al.*, 2010) needs to be developed.

6. Regulatory considerations

Regulatory agencies in the US and EU have published policies and guidance on the use of nanomaterials in food (EFSA, 2011; FDA, 2014a; FDA, 2014b; Hamburg, 2012). The combination of scientific uncertainty and lack of regulatory experience has led to a situation where agencies have not yet adopted a definition of nanomaterials, nor specific processes for managing regulatory approvals for nanoscale forms of food ingredients and additives. The Food and Drug Administration (FDA), EFSA and other key international food safety agencies have collectively adopted a “case-by-case” approach to evaluation of the safety of nanomaterials in food applications. In the U.S. in lieu of a definition, FDA has developed guidance on how to assess whether a new process is a product of nanotechnology, and if so, what additional types of testing may be appropriate. The most significant change is the recommendation to measure a range of physical and chemical properties in advance of, during, and following exposure during dose response and other toxicological assays to assess the transformations that might occur in biological media and understand the characteristics of the nanoscale material. FDA's guidance is similar to that of the European Food Safety Authority (EFSA) in

recommending each physical and chemical characteristic be measured by two analytical methods due to measurement error, as well as in key experimental stages. EFSA further recommends specific types of testing depending on the persistence of the nano-form of the substance in the food article, or in the body following ingestion. The recommendation for testing of the nanospecific form is determined by whether the nano-form persists into the GI tract. Otherwise safety may be determined by testing of conventional materials.

The guidance documents indicate that regulatory agencies are concerned about potential impacts of smaller particles affecting dynamics and reactions within the GI tract, differential uptake across the gut lumen, antimicrobial effects, translocation into other organs and higher potency and/or bioavailability than conventional food additives or ingredients.

7. Conclusions

There is increasing concern about the potential adverse effects of iENMs on human health and wellbeing, as well as on the environment. Consequently, there is a need to understand the relationship between pristine ENM intrinsic properties, their physico-chemical transformations across the GIT and GI fate, and their potential toxicity. Many previous studies have used relatively simple cellular model systems where pristine iENMs are introduced into cell culture models to study their absorption and toxicity. However, these models ignore ENM property transformations that occur within foods and within the GIT. There may be pronounced changes in the interfacial properties, composition, charge, size, and aggregation state of ENMs as they pass through the GIT, which are influenced by the type of food that they are ingested with. It is therefore important to develop sophisticated in vitro model systems to take into account food matrix and GIT effects on the properties of ENMs under simulated GI conditions. The use of

these models will lead to a better understanding and prediction of the behavior of iENMs within the human body, and therefore help establish their potential toxic effects. The current methods used to characterize pristine iENMs or their aqueous or simple culture media suspensions are not adequate to be used in such complex biological media and there is a need to develop new characterization methods suitable for complex media. Furthermore, there is a need to develop a validated, tiered testing strategy, including *in vitro*, *in vivo* and even *in silico* approaches to assess potential risks from iENMs.

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FIGURE LEGENDS

Figure 1. A variety of structures can be assembled using lipid nanoparticles as building blocks, including nanoparticle filled droplets and hydrogels, nanoparticle clusters, nanoparticle colloidosomes, solid lipid nanoparticles, and nanolaminated nanoparticles (McClements, 2012; McClements, 2014) .

Figure 2. Food grade nanoparticles may vary in particle characteristics, such as size, shape, composition, and charge.

Figure 3. Schematic diagram of the physicochemical and physiological conditions in different regions of the human gastrointestinal tract. The diagram of the human body was taken from

http://en.wikipedia.org/wiki/Digestive_tract (Copyright free).

Figure 4. The properties of nanoparticles may occur in a number of different ways as they pass through the GIT. Some potential changes in particle dimensions and interfacial properties are illustrated here.