Nanoparticle toxicity by the gastrointestinal route: evidence and knowledge gaps

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Abstract: The increasing interest in nanoparticles for advanced technologies, consumer products, and biomedical applications has led to great excitement about potential benefits but also concern over the potential for adverse human health effects. The gastrointestinal tract represents a likely route of entry for many nanomaterials, both directly through intentional ingestion or indirectly via nanoparticle dissolution from food containers or by secondary ingestion of inhaled particles. Additionally, increased utilisation of nanoparticles may lead to increased environmental contamination and unintentional ingestion via water, food animals, or fish. The gastrointestinal tract is a site of complex, symbiotic interactions between host cells and the resident microbiome. Accordingly, evaluation of nanoparticles must take into consideration not only absorption and extraintestinal organ accumulation but also the potential for altered gut microbes and the effects of this perturbation on the host. The existing literature was evaluated for evidence of toxicity based on these considerations. Focus was placed on three categories of nanomaterials: nanometals and metal oxides, carbon-based nanoparticles, and polymer/dendrimers with emphasis on those particles of greatest relevance to gastrointestinal exposures.

Keywords: nanoparticles, toxicity; nanotechnology; nanomaterials; ingestion; oral; gastrointestinal; nanometals; silver; gold; titanium dioxide; silica; quantum dots; QDs; copper; carbon nanotubes; dendrimers; polymers.

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1 Introduction

Nanoparticles (NPs), defined by the US National Nanotechnology Initiative as materials having at least one diameter measuring 100 nm or less, are increasingly utilised in consumer products. This review will focus on relevant parameters and current state of knowledge regarding toxicity of ingested NP. This exposure route is highly relevant to human health as there are numerous NP applications that directly or indirectly have potential for ingestion. Such applications include flavour enhancers, food pigments, or health supplements (Hagens et al., 2007; Bouwmeester et al., 2009; Wijnhoven et al., 2009; Frohlich and Roblegg, 2012). Some non-edible products may shed NP over time – examples are nanosilver-coated toothbrushes, food and drink containers, and even baby bottles and pacifiers (Benn et al., 2010). Medical applications include oral drug delivery vehicles or therapeutic molecules (Bisht et al., 2008; Cattani et al., 2010; Dhar et al., 2010). In addition to direct ingestion, a proportion of inhaled particulate materials are eventually removed via the gastrointestinal tract (GIT), after being mobilised up the trachea via the mucociliary escalator. Additional potential exposures include consumption of fish or shellfish that have accumulated NP due to ingestion or absorption of contaminated effluent (Gaiser et al., 2009). Finally, certain NPs have been considered as alternatives to growth-promoting antibiotics in animal agriculture, which may affect tissue accumulation and microbial resistance profiles in food animals (Fondevila et al., 2009).

Specific discussion of all NP with potential for ingestion is beyond the scope of this review. Consequently, we will first discuss general features applicable to NP ingestion. These include the structure and function of the GIT and the impact of NP physicochemical parameters, dosimetry, dissolution, the gut mucus layer, and the protein corona on studies of NP toxicity. Subsequently, we will specifically discuss NPs considered to be of high likelihood for ingestion. These fall into three main categories – metals and metal oxides, carbon-based materials (fullerenes and carbon nano-tubes), and

polymeric/dendrimeric engineered nanomaterials. Of these categories, metal NPs, in particular nanosilver, have the highest potential for ingestion by the largest segments of human populations, due to their increasing inclusion in dietary supplements and food packaging materials (Wijnhoven et al., 2009; Frohlich and Roblegg, 2012). Carbon-based materials are more likely to pose accidental or occupational exposure risks and polymers/dendrimers are more likely to be utilised in biomedical applications, which are subject to pre-market regulatory scrutiny and affect a smaller population.

One important category of ingested nanomaterials that will not be evaluated with respect to toxicity in this review are nanoliposomes and related lipid-based compounds. Many naturally occurring foods, including breast milk, contain liposomes, and engineered liposomes used as flavour enhancers or facilitated nutrient delivery may be absorbed and metabolised by similar routes (Mozafari et al., 2008). Since nanoliposomes have been the topic of several recent reviews (Mozafari et al., 2008; Bouwmeester et al., 2009; Handy and Shaw, 2007; Das et al., 2009) we will limit our scope to the engineered particles described above.

2 The GIT and significant considerations for ingested NPs

2.1 The GIT

2.1.1 GIT organisation

The GIT is a selective mucosal barrier that represents a considerable surface area, estimated at 200 m^2 in the adult human, for potential interaction with ingested NP. Different species have anatomical and physiological differences that must be considered when utilising animal models for ingestion studies (Kararli, 1995; McConnell et al., 2008; Merchant et al., 2011). Each area of the GIT encompasses digestive, absorptive, secretory, and protective functions. Potential outcomes of ingestion of NP include absorption, by which NP can gain access to the blood and hence to other organs, local interaction with the GIT mucosa including deposition and/or physical effects on motility, and finally, effects on luminal components, including the mucus layer and the GIT microbiome, which have critical roles in normal gut physiologic, metabolic, and immune function (Mason et al., 2008; Hansson, 2012; Young, 2012).

All areas of the GIT are mechanically protected by epithelium and a layer of mucus of variable thickness and composition that is produced by specialised gastrointestinal epithelial cells. In the stomach, protein digestion begins by the activity of the protease pepsin. Pepsin activation is dependent upon hydrochloric acid secretion by parietal cells within the mucosal epithelium. The gastric pH varies with species and with diet and stage of ingestion. For example, the gastric pH of the human stomach varies from 1.2–2.0 in a fasted state to approximately 5.0 with a food bolus, followed by gradual re-acidification (McConnell et al., 2008). In mice, gastric pH in the fed state is lower (pH 3.0) than the fasted state (pH 4.0) (Kararli, 1995; McConnell et al., 2008; Merchant et al., 2011).

The small intestine is the site of most nutrient digestion and absorption, including carbohydrates, peptides, and fats. It also serves secretory and protective immune functions. The pH of the duodenum is between 6–7 in humans (Evans et al., 1988) and 4–5 in mice and rats (McConnell et al., 2008). The intestinal compartment is highly

chemically and physically complex. Absorption is facilitated by the increased mucosal surface area of elongated villus folds lined by absorptive enterocytes. Each enterocyte has a fine apical brush border (microvilli) that further increases surface area. The mucus layer is produced by specialised mucus-secreting goblet cells. It contains mucopolysaccharides and glycoproteins and provides a physical barrier to luminal bacteria, preventing them from reaching the enterocyte surface. In the small intestine, the mucus layer also contains enzymes for carbohydrate digestion and fat emulsion in preparation for nutrient absorption. Progressing distally in the GI tract, villus height decreases while goblet cell number and mucus production increase. While most absorption takes place in the upper small intestine, the distal small intestine and colon have specialised absorptive roles in water, B vitamin, and fatty acid absorption (Kararli, 1995).

2.1.2 Immune function of the GIT

In addition to barrier and innate defences the GIT has localised representations of the adaptive immune system in the form of gut-associated lymphoid tissue (GALT) aggregates. In the small intestine, these are termed Peyer's patches and are most numerous and prominent in the ileum, the terminal portion of the small intestine. These GALT foci represent sentinel sites for the adaptive immune system and have a highly specialised structure (Mason et al., 2008). Luminal antigens enter through specialised epithelial cells termed M cells at the surface of the mucosa. M cells transfer antigen to antigen-presenting cells which process and present antigen to T-cells in the GALT. T-cells become activated and direct B-cell generated production of antigen-specific secretory immunoglobulins (sIgA) which are secreted onto the luminal surface. Activated T-cells can also migrate to draining lymph nodes and direct development of cytotoxic T-cells which migrate into the adjacent mucosa (Mason et al., 2008). In addition to the ileum, the cecum (located at the junction of the small and large intestine) is also a significant immunologically active site.

2.1.3 Commensal microbiota of the GIT

The functions of the GIT are facilitated by microbial activity, which is tremendously increased in the cecum and colon. In humans the gut microbiome has been estimated as weighing 1 kg in an average adult human and consisting of up to 5,000 species (Zoetendal et al., 2004; Manson et al., 2008; Kim et al., 2009b; Young, 2012). In both the mouse and the human, the majority of gut bacteria belong to the phyla *Bacteroidetes* and *Firmicutes*, although there are host-species specific differences at the genus level. More than 50% of intestinal bacterial are non-culturable and, to capture these, newer techniques for evaluating gut microbial populations utilise non-culture-based sequencing technologies (Zoetendal et al., 2004; Young, 2012). In the normal gut, the overwhelming majority of bacteria are commensals that do not leave the lumen. These organisms play critical functional roles in normal digestion and in the immunological functions of the GIT. These include conjugation of bile acids, regulation of colonic enterocyte health by production of the short-chain fatty acid butyrate, production of vitamins B12 and K, detoxification (or toxification) of certain ingested drugs or plant toxins, and maturation of the immune system (Manson et al., 2008; Mason et al., 2008; Atarashi and Honda, 2011;

Young, 2012). In addition to these host metabolic or immune influences, the normal resident microbiota occupy a niche that might otherwise be exploited by a pathogenic species (Walk and Young, 2008). There is increasing recognition that altered gut microbes can influence host health in areas ranging from cancer risk (Canani et al., 2011) to xenobiotic metabolism (Clayton et al., 2009). Thus, there is a need for targeted toxicological investigations of the influence of ingested compounds, such as NP, on the gut microbiome.

2.2 Significant considerations for ingested NP

Both biological features of the GIT and physicochemical features of NP impact interpretation of NP ingestion studies. Particle size, surface area, particle number, aggregation/agglomeration state, charge, and surface coatings are all likely to influence the biological availability and effects of an administered NP (Oberdorster et al., 2005a; Abbott and Maynard, 2010). With this in mind, a set of minimum nanomaterial characterisation requirements for toxicology studies was recommended by the MINChar Initiative (2008). These parameters include particle size and size distribution, agglomeration/aggregation state, shape, chemical and surface composition, surface area/chemistry/charge, purity, and stability. Oxidant generation and rate of dissolution will also impact absorption and biological response (Castranova, 2011). For *in vivo* studies, significant variation can be introduced due to species, strain, diet, housing conditions, time of dosing, circadian rhythm variations, and endogenous microbiota. Meticulous reporting of these parameters can provide some measure of transparency and facilitate resolution of disparate inter-study results. No consensus for reporting *in vivo* experimental parameters in NP toxicity studies currently exists, however, the Animal Research: Reporting In Vivo Experiments (ARRIVE) (Kilkenny et al., 2010a, 2010b) guidelines and the *in vivo* portion of the Metabolomics Standards Initiative (Griffin et al., 2007) represent laudable efforts at standardisation of in vivo metadata.

2.2.1 GIT absorption

Translocation of particles through the intestinal barrier is a multistep process that involves diffusion through the mucus layer, contact with enterocytes and/or M-cells, and uptake via cellular entry or paracellular transport. As reviewed by Frohlich and Roblegg (2012), the most common mechanism for uptake of NP into intestinal epithelial cells appeared to be endocytosis (Frohlich and Roblegg, 2012). Mechanisms of endocytosis include clathrin-mediated, caveolae-mediated, clathrin and caveolae-independent, and macropinocytosis. It has been shown that polystyrene NPs are preferentially taken up across M cells (des Rieux et al., 2007). Size influences absorption, as shown by greater absorption of smaller (50 nm) polystyrene particles compared to larger (100 nm) particles (Jani et al., 1990). The largest particles in this study (300 nm) were not absorbed. Additionally, larger particles remained within the submucosa or GALT of the intestine and colon, while smaller particles entered the bloodstream and accumulated in the liver and spleen (Jani et al., 1990). Non-lymphoid areas can also be involved in NP uptake, particularly with conjugation of nutrients or nutrient-like compounds. For example, conjugation of tomato lectins (plant-derived glycoproteins) to polystyrene beads

increased absorption in non-lymphoid areas and decreased lymphoid detection (Florence et al., 1995). Endotoxin adherence to the large surface area of NP is a common complication of NP manufacture and may enhance pro-inflammatory pathways (Dobrovolskaia et al., 2009).

2.2.2 Dissolution

NP stability, dissolution, and release of potentially toxic ions are dependent, in part, on fluid pH, composition, and duration of exposure (Xie et al., 2012). pH variance within the gastrointestinal compartments can affect aggregation status (Peters et al., 2012) and alter surface chemistry, particularly in NPs where zeta potential is highly dependent on pH (e.g., chitosan) (Loretz and Bernkop-Schnurch, 2007). Understanding parameters of dissolution in gastrointestinal fluids may help predict uptake and blood concentrations. Limited work has been done in this area. Wang et al. (2008) exposed CdSe quantum dots (QDs) with a ZnS shell to simulated gastric fluid (SGF) and $NaHCO_3$ neutralisation. Here, SGF treatment increased QD cytoxicity, an effect reversed by neutralising the SGF-treated QDs with $NaHCO_3$. The authors postulated SGF mediated disruption of the ZnS shell, enabling dissolution and Cd++ toxicity. In contrast to most cell culture systems, pH *in vivo* varies sequentially across different gut compartments and with differing composition of ingesta. The impact of these changes are difficult to study *in vivo*. Recently, Walczak et al. (2012) used an *in vitro* human digestion model to demonstrate that, after gastric digestion, the number of 60 nm AgNPs decreased due to clustering and interaction with chlorine ions, in the presence of protein. However, the particle number increased again under intestinal conditions. Furthermore, soluble silver ($AgNO_s$) also formed silver NPs with chlorine and sulfur ions upon incubation at gastric pH. Peters et al. (2012) showed similar findings in an *in vitro* dissolution model of SiO_2NP in foods. Here, NP was present under conditions mimicking the oral cavity, but agglomerated under conditions of low pH and high electrolytes, as in the gastric compartment (Peters et al., 2012). Under intestinal pH, nanoparticulate silica reappeared, as with the Walczak AgNP experiment. Thus, it is likely that the absorptive intestinal epithelium encounters lumenal NP, at least for metals, however it is still unclear whether these are absorbed in the intestine primarily as NP or in soluble form.

2.2.3 Mucus layer

Intestinal mucus, a complex network of highly branched glycoproteins, lipids, cellular and serum macromolecules, is the first barrier through which ingested NPs must pass (Crater and Carrier, 2010). Surface charge can play a crucial role (Frohlich and Roblegg, 2012). Net neutral or positive surface charge prevents mucoadhesion, favoring penetration, whereas passage of negatively charged hydrophilic and lipophilic compounds is hindered. Small NPs also penetrate more easily than large ones. Mucin interaction with adhesive NPs and larger particulates can disrupt the 'bottle-brush' architecture of mucus, possibly enabling penetration upon subsequent exposures (McGill and Smyth, 2010; Wang et al., 2011). This is dependent upon particle type. Jachak et al. (2012) found that metal oxide NPs and two types of SWCNTs were trapped in human mucus by adhesive interactions, not steric obstruction. In contrast, ZnO NPs rapidly penetrated airway mucus layers, which may account for ZnO's general toxicity.

2.2.4 Protein corona

Whether in the GIT or a culture microenvironment, NP will develop a corona of adsorbed proteins, small molecules, and ions (Cedervall et al., 2007; Faunce et al., 2008; Lundqvist et al., 2008; Monopoli et al., 2011). This association can sequester nutrients, etc., complicating interpretation of cell culture results (Guo et al., 2008), and create an 'epitope map', or complex biologically active entity that influences the *in vivo* response (Lynch et al., 2007; Monopoli et al., 2011). The effects of the protein corona are variable. In some cases, cytotoxicity is reduced, perhaps by decreasing cellular NP uptake (Jiang et al., 2010; Casals et al., 2011; Ge et al., 2011; Safi et al., 2011) or mitigating cell membrane damage (Hu et al., 2011). Lundqvist et al. (2008) found that the protein corona on 50 nm carboxyl-modifed polystyrene NPs varied in relation to particle size and surface modification. Highly abundant plasma proteins such as inter-alpha-trypsin inhibitors, serum albumin, clusterin, and vitronectin were common to all coronas, while many less abundant proteins varied across NP size and surface. In one study of polystyrene NPs in human plasma, Zhang et al. (2011) were able to classified the NPs with respect to protein coating based on size and surface properties of the parent particles. In addition, the protein corona was demonstrated to be at equilibrium within 5 minutes of NP exposure. In an important *in vitro* study, citrate-capped Au NP-protein complexes formed in Roswell Park Memorial Institute (RPMI) media were internalised in HeLa and U937 cells to a greater extent than those from Dulbecco's Modified Eagle's (DMEM) media, resulting in higher cytotoxic effects (Maiorano et al., 2010). Thus, variable coronal protein composition may lead to some of the different biological effects of NPs that are otherwise identical (Lai et al., 2012). The above studies emphasise the need for adequate characterisation of NPs, both before and after exposure to culture media or biological fluids.

2.2.5 Dose metrics: in vitro and in vivo

The dose metrics for NP are complex, as particle number, surface area, and shape, and environmental factors (pH, chemical complexity, protein corona) all complicate traditional mass- or concentration-based metrics for *in vitro* studies. Unlike soluble chemicals, NP can settle, diffuse, and aggregate, significantly affecting the cellular dose. Defining and measuring 'dose' (e.g., dosimetry) for NP in *in vitro* systems is thus more complicated, and less comparable across NP types compared to soluble chemicals (Teeguarden et al., 2007). Furthermore, this critical aspect has been largely ignored (Teeguarden et al., 2007; Hinderliter et al., 2010), making the extrapolation from *in vitro* effects to *in vivo* studies problematic in pharmacokinetic modeling (Hinderliter et al., 2010). In most culture systems, NP deposition and consequent cellular interaction is a function of gravity and NP diffusion, both of which are influenced by NP size and density, concentration, and exposure duration (Teeguarden et al., 2007; Hinderliter et al., 2010). In their *in vitro* sedimentation, diffusion and dosimetry (ISDD) model, Teeguarden et al. (2007) demonstrated that, for liquid-based *in vitro* systems, the dose-rates and target cell doses for all NPs were not equal. In fact, differences between media NP concentrations on a μg/mL basis and actual target doses on the cell surface area were three to six orders of magnitude. Accordingly, *in vitro* hazard assessment using mass-based exposure metrics may be highly inaccurate. Accurate NP dosimetry for *in vitro* toxicology studies thus requires direct experimental quantitation of NPs interacting

with the cultured cells. As an example, the cell dose of ZnO NP to alveolar type II epithelial cells was measured as NP aggregates per unit area using scanning electron microscopy to image EM grids placed randomly over the cells before exposure (Xie et al., 2012). In this case, the dose metric used for ZnO NPs was landed-aggregates per 20 μm^2, the size of a typical cell (similar to counting red blood cells on a hemocytometer). Presumably, this clever approach for accurate dosimetry could be adapted to intestinal epithelial cell culture studies to improve *in vitro* hazard assessment.

In contrast, *in vivo* toxicity studies for ingested NP currently remain fairly traditional in the use of mass-based dosimetry (e.g., mg/kg). Although the appropriate dose-metric for ingestion studies is not yet clear, discrepancies between individual studies may become less significant when dose is compared on the basis of surface area, charge, or particle number (Drake and Hazelwood, 2005; Oberdorster et al., 2005b, 2007; Maynard et al., 2010). Knowledge of delivered NP dose on a target cell or tissue basis is also lacking in most *in vivo* studies. Although inductively coupled mass spectrometry (ICP-MS) can be used quantitatively, this technique does not discriminate between particulate and soluble forms. Recently, a combination of particle-induced X-ray emission (PIXE) spectroscopy and inductively coupled mass spectrometry was used to identify intracellular deposits of ingested TiO_2NP in the digestive epithelium of a terrestrial isopod (model invertebrate) (Novak et al., 2012). This experiment showed cytotoxicity (membrane destabilisation) on an individual cell basis but no toxicity at an organism level. Further application of this and similar techniques to mammalian NP ingestion studies might be beneficial in reconciling *in vitro* and *in vivo* findings and offer better estimation of the true delivered dose *in vivo*. The recent application of sedimentation field flow fractionation (sdFFF) to quantification of NP in tissue also represents a step forward in measurement of delivered dose in tissue, however this technique is not yet widely available for *in vivo* toxicology (Deering et al., 2008).

2.2.6 Functional genomics and proteomics

Comprehensive analysis of differential gene and protein expression can be used to derive molecular profiles indicative of exposure or effect (Lemos et al., 2010; Van Hummelen and Sasaki, 2010; Kienhuis et al., 2011; Veenstra, 2011). 'Omic approaches to the *in vitro* assessment of NPs on GI epithelial cells have been limited to only a few investigations. Using a unique Caco-2 cell/M-cell co-culture system, Bouwmeester et al. (2011) investigated the effect of Ag NP exposure on human whole-genome gene expression. Despite the absence of overt cytotoxicity, 97 genes were significantly up-regulated by at least one treatment while none were down-regulated. Up-regulated genes pertained to oxidative stress, AP1 activation, proliferation, (mitochondrial) apoptosis, unfolded protein response and ER stress, cell structure, and response to chemical stimulus. Nine metallothionein genes and genes encoding proteins essential to GSH synthesis were also up-regulated. The authors concluded that Ag NP exposure resulted in a generalised stress response and that Ag+ dissolution, not the Ag NPs, triggered the observed effects. In another study (Moos et al., 2011), SiO_2, Fe_2O_3, ZnO, and TiO_2 NP effects on RKO and Caco-2 cell monolayers were investigated using whole human genome oligonucleotide microarrays. Only ZnO NP (which underwent significant dissolution) exposures were significantly cytotoxic with up-regulation of genes related to protein folding, chaperone, and stress responses. Other up-regulated genes included those involved in transition metal binding and Zn-finger containing proteins, suggesting an

overlap between transcriptional modulation and metal metabolism. When the Caco-2 cells were exposed to soluble Zn, the genes involved in metal metabolism were induced but the genes involved in protein folding were unaffected. Using CuO NP exposure in Caco-2 monolayers, Piret et al. (2012), found increased IL-8 (rod-shaped NP greater than spherical), heme oxygenase 1 (indicative of oxidative stress), and chemokine receptor CCR4, along with several other chemokines and pro-inflammatory cytokines indicating a proinflammatory effect.

Protein expression analysis has been a staple tool for toxicologists for many years, due to the well-known disconnect between gene expression (mRNA) and corresponding cellular protein abundance (Maier et al., 2009; Yeung, 2011). Generally speaking, proteomics has been used to generate differential protein expression profiles that explain the mechanism of cellular responses to NP exposure. Most nano-related toxicoproteomic applications have used two-dimensional gel electrophoresis (2-DE) (Rabilloud et al., 2010). Due to the numerous limitations of the 2-DE approach, namely labour intensity and limited scope, innovative mass spectrometry-based techniques (Helsens et al., 2011; Xie et al., 2011) have emerged that enable broad-based relative and absolute quantitative comparisons of protein expression. Furthermore, proteomic platforms using stable isotope label-free quantitative mass spectrometry (LFQMS) (Neilson et al., 2011) have turned out to be proficient and reliable. LFQMS has recently been applied to the assessment of NP effects (Blazer-Yost et al., 2011; Teeguarden et al., 2011; Lai et al., 2012). Using an LFQMS approach, Lai et al. (2012) reported the first extensive proteomic description of biological responses to functionalised carbon nanotubes (f-CNT) in intestinal cells, where 2,282 unique proteins were identified, quantified, and statistically compared. They found that exposure to even very low levels of f-CNT (500 pg/mL) resulted in significant concentration-dependent alterations in specific pathways and molecular/cellular functions.

3 Ingested NPs: Metals and metal oxides, carbon-based, and polymer/dendrimers

3.1 Metals and metal oxides

3.1.1 Silver (AgNP)

Among the potentially ingested engineered NPs, much focus has been placed on nanosilver. Silver salts and colloidal silver suspensions were commonly used to combat infection prior to the development of modern antibiotics (CASRN, 1988; Drake and Hazelwood, 2005; El-Ansary and Al-Daihan, 2009; Varner et al., 2010). With rising concerns about antibiotic resistance, there has been a resurgent utilisation of silver as a topical antiseptic and in wound dressings and medical products (Silver, 2003; Drake and Hazelwood, 2005). Additional potential oral exposures could arise from water contamination due to run-off and accumulation within food fish (Shaw and Handy, 2011). AgNP and other metal NP are being considered as alternatives to therapeutic or growth-promoting antibiotics in animal agriculture (Fondevila et al., 2009). There is very little information available on the efficacy, safety, and environmental or human health impact of this proposed application.

3.1.2 Gold (AuNP)

Colloidal and nanoscale gold are increasingly utilised in medical applications as imaging and therapeutic agents, particularly as anti-inflammatories and drug delivery agents (Khlebtsov and Dykman, 2011). Gold has been used parenterally, topically, and enterically in medicine as an anti-inflammatory in the treatment of rheumatoid arthritis (Khlebtsov and Dykman, 2011). Currently, parenteral (injectable) administration of colloidal gold is utilised infrequently due to immune sensitisation, dermatitis, and gastrointestinal disturbance attributed to gold salts (Schuhmann, 1990; Epstein et al., 1991). Clinical improvement without toxicity was reported with an orally administered tablet form of colloidal gold (Abraham and Himmel, 1997). Over-the-counter oral preparations for nanoscale or colloidal gold intended for human consumption are currently available (http://www.nanotechproject.org/) although the true particle size parameters of these products are not clear.

3.1.3 Titanium dioxide (TiO_2NP)

TiO_2 NP are components of pigments, with a vast array of consumer applications including cosmetics, sunscreens, paints, printing, plastics, and food colorants (Trouiller et al., 2009; Weir et al., 2012). With the exception of food colorants, these materials are not intended for direct ingestion, however, small amounts of these products are likely to be ingested accidentally. Experimental evidence of pulmonary carcinogenicity in rats led to classification of TiO_2 classification as a group 2B ('possibly carcinogenic') substance for humans, although epidemiological evidence has yet to support this classification (Trouiller et al., 2009; Weir et al., 2012). TiO_2NP are comprised of varying proportions of different crystalline structures-namely rutile, anatase, or brookite. *In vitro* cytotoxicity depends on size (smaller particles being more toxic) and crystal structure, with anatase having 100-fold higher cytotoxicity than rutile (Weir et al., 2012). The reported total dietary intake of TiO_2 NP, in food varies with dietary habits and the assumptions used in modeling. Some estimates of likely TiO_2NP exposure are fairly low at 0.035 mg/kg/d (2.5 mg/person/day) (Frohlich and Roblegg, 2012). Others arrive at higher estimates with Weir et al recently calculating TiO_2NP intake in hard-coated candies and gums as 1–2 mg/kg/d for children and 0.2–0.7 mg/kg/d for adults in the USA (Weir et al., 2012). For this reason, public and regulatory attention is likely to increasingly focus on TiO_2NP consumption, and a shift of experimental emphasis to food-grade rather than the more experimentally available industrial-grade TiO_2NP has been recommended (Weir et al., 2012).

3.1.4 Other metallic NPs: silica (silicon dioxide, SiO_2), CuNP, QDs

Other metals also have potential for gastrointestinal exposure. The most prevalent is probably silica (silicon dioxide, SiO_2). Food-grade silica has traditionally been synthetic amorphous silica produced in a variety of forms (pyrogenic, gel, sol, precipitate) and used as an additive for clearing alcoholic beverages or as an anti-caking agent (Dekkers et al., 2012). Since this material encompasses primary particles in the range of 10–100 nm, with likely aggregation or agglomeration to larger sizes, some information on the likely properties of SiO_2NP may be extrapolated from studies of synthetic amorphous silica (Dekkers et al., 2012). The major difference with engineered SiO_2NP is the potential for production as a well-dispersed preparation of nanoscale particles. Whether such

preparations actually have different pharmacokinetics or distribution *in vivo* compared to traditional food-grade SiO_2 is not known. In addition to their application as food additive, SiO_2NP have been proposed as drug conjugates for improved efficacy or delivery of therapeutically active compounds, particularly for intestinal inflammatory conditions (Moulari et al., 2008).

Similarly to AgNP, copper nanoparticles (CuNP) have shown *in vitro* antimicrobial efficacy and have been proposed as antibiotic alternatives in human health and agriculture. CuNP are produced as industrial additives for lubricants, plastics, and metallic coatings, inks, and as part of the anodes of lithium batteries (Chen et al., 2006). They purportedly have antimicrobial properties when used as part of a coating (Chen et al., 2006).

QD semiconductors have been applied for research purposes as bioimaging and drug delivery vehicles (Mohs et al., 2009). QDs typically consist of a crystalline core of metals or metal complexes surrounded by a protective shell that conveys bioavailability. They can be surface functionalised to improve solubility or other properties (Mohs et al., 2009). Most literature involves *in vitro* systems or parenteral administration (Hardman, 2006). Due to quenching in an acidic environment, ingestion has not been a major route, although improved stability by surface polymerisation is beginning to show promise in this area (Mohs et al., 2009). Little is known about potential toxicity specific to ingestion.

3.2 Carbon-based nanomaterials (multi- or single-walled carbon nanotubes [MWCNTs, SWCNTs])

Carbon-based nanotubes (CNTs) have multiple electronics, aerospace, and computer technology applications and investigationally as drug delivery vehicles (Bianco et al., 2005; Lam et al., 2006). Ultrashort (< 80 nm), single-walled carbon nanotubes have potential for imaging or radiotherapeutic applications (Kolosnjaj-Tabi et al., 2010). Carbon nanotubes have been proposed for antimicrobial or antiparasitic therapeutics, some of which involve oral administration (Prajapati et al., 2012). Additionally, there is potential for indirect or accidental ingestion secondary to other exposures. Because inhaled, aggregated CNTs have been associated with granulomatous inflammation in the lung and multiple organs, their utilisation in biomedical applications has met with some concern. By manipulation of their length and surface characteristics, it is hoped that adverse effects may be avoided. For example, functionalisation of the surface of CNTs increases their solubility and decreases manifestations of cytotoxicity (Lam et al., 2006).

3.3 Polymer/dendrimers

Engineered polymeric or dendrimeric NPs hold promise as drug delivery devices and have been the subject of recent reviews (Patri et al., 2002; El-Ansary and Al-Daihan, 2009; Malik et al., 2011). These applications differ from environmental exposures in that they are likely to have a smaller exposed population and are less likely to represent a chronic exposure. Furthermore, because particles intended for biomedical use will need to undergo formal regulatory evaluation and approval, they represent a lower overall risk to the general public. Nevertheless, questions remain as to whether traditional toxicology and safety assessment protocols for investigational new drugs or medical devices are suitable for the evaluation of nanodevices as therapeutics (DeJong and Borm, 2008).

4 Study selection criteria for *in vivo* and *in vitro* toxicity

In light of the previous information, pertinent toxicology-related studies of NP oral exposure were identified in the recent literature, focusing on metal NPs, carbon-based NPs, and polymeric NPs. Available data from *in vitro* and *in vivo* studies were compiled after exhaustive searches of publicly available databases including PubMed, Web of Science, and the European Commission's Nanohealth and Environmental Commented Database (NHECD) using the names of the nanomaterials discussed above and the keywords ingestion, gastrointestinal, oral, toxicity, nanotoxicity, *in vitro*, *in vivo*, and NP. Names of particular *in vitro* cell culture systems were also queried. Criteria for study inclusion were toxicity-focused studies using *in vivo* or *in vitro* mammalian systems.

The primary selection criterion was a focus on toxicity and tissue distribution with respect to ingested NP. A few efficacy studies were included where the endpoints were pertinent to toxicology (i.e., effects on gut microbiome). Most information was available for AgNP and this material will accordingly be given most coverage. Other NP will be represented to the extent permitted by the existing literature. For polymeric NP, most literature focused on therapeutic efficacy, therefore for these materials only studies pertaining to toxicity were included. Significant findings are presented below by study type (*in vivo* or *in vitro*) and class of material.

5 *In vivo* studies of NP toxicity by ingestion.

5.1 Metal NPs

5.1.1 AgNP

Ten studies in rodents using AgNP exposure by the oral route were identified (Table 1). Nine of the rodent studies assessed tissue distribution and/or toxicity in healthy animals (Cha et al., 2008; Kim et al., 2008, 2009a, 2010b; Jeong et al., 2010; Park et al., 2010a, 2011; Loeschner et al., 2011; Hadrup et al., 2012) and one focused on modulatory effects on inflammation in a colitis model (Bhol and Schechter, 2007). Six additional studies of AgNP in domestic livestock (poultry and pigs) from agricultural science literature focused specifically on AgNP as an alternative to growth-promoting antibiotics (Ahmadi, 2009; Ahmadi et al., 2009; Fondevila et al., 2009; Ahmadi and Kurdestany, 2010; Ahmadi and Kordestany, 2011; Ahmadi and Rahimi, 2011). These were included because alterations of the gut microbiome have toxicological implications due to their effects on host health and xenobiotic metabolism (Bjorkholm et al. 2009). There are many reports on AgNP in non-mammmalian species (Griffitt et al., 2008, 2009; Gaiser et al., 2009; Bilberg et al., 2010; Hinther et al., 2010; Bilberg et al., 2011; Cowart et al., 2011; Croteau et al., 2011; Posgai et al., 2011; Bilberg et al., 2012; McLaughlin and Bonzongo, 2012). These studies are informative for environmental risks and accumulation in food species (e.g., food fish), however, these studies were not directly analogous to ingestion exposures in mammals and will not be discussed here.

The experimental animal data for AgNP ingestion should be considered in light of the considerable historical information on ingested colloidal Ag [reviewed by Varner et al. (2010)]. Colloidal silver generally ranges, on average, from 250–500 nm but consists of aggregated smaller particulate Ag, some of which may be in the ultrafine range (< 100 nm) (Varner et al., 2010). Historically, bioavailability of ingested colloidal Ag has been estimated at 10%, with retention of < 2% to 3% in body tissues. Elimination of the majority of ingested material is via the faces, either directly or following biliary excretion (Armitage et al., 1996; Drake and Hazelwood, 2005). Urinary excretion is typically very low (< 1%), except at exceedingly high doses (Drake and Hazelwood, 2005). The adverse effects classically associated with excessive Ag ingestion in humans are argyria and argyrosis. Argyria refers to a bluish-grey pigmentation of the skin associated with absorption of soluble Ag and its reduction and precipitation in skin and connective tissue. Argyrosis is a similar pigmentation of the ocular tissues. Argyria has been associated with a calculated total dose retention of between 1–8 g of Ag in tissue (CASRN, 1988; ATSDR, 1990; Brandt et al., 2005; Varner et al., 2010). With consideration of bioavailability, this translates to a lifetime oral exposure of 0.014 mg/kg/day as the lowest dose associated with argyria (CASRN, 1988). This lifetime dose was used by the EPA to extrapolate an oral reference dose (RfD) of 0.005 mg/kg/day (CASRN, 1988; ATSDR, 1990; Varner et al., 2010). The oral RfD represents the maximal tolerated amount of silver that can be ingested on a daily basis over a lifetime without adverse effects (argyria/arygyrosis). It should be noted that, although argyria and argyrosis are considered 'adverse' effects, in most cases their clinical implications are primarily cosmetic, as no major disease or functional deficits have been convincingly associated with their occurrence. In a recent case report and literature review of clinically evident argyria among users of over-the-counter dietary Ag supplements, there were 15 case reports of argyria associated with ingestion of colloidal Ag or Ag salts (Bowden et al., 2011). Since these were clinical case reports involving over-the-counter products, information about size, dispersion, or even an accurate dose level was typically unavailable. Interestingly, the median onset of argyria in cases stemming from colloidal silver was 20 months, while in a case arising from soluble Ag ($AgNO_3$), the time of onset was only 8 months (Bowden et al., 2011). This is consistent with experimental evidence from animal studies that highly soluble Ag has higher bioavailability.

AgNP: tissue distribution

There are multiple AgNP ingestion studies focused on tissue distribution (Kim et al., 2008, 2009a, 2010b; Park et al., 2010a; Loeschner et al., 2011). Only one study (Park et al., 2011) was specifically designed to assess bioavailability, or the fraction of administered dose reaching the systemic circulation. Additionally, these studies are really assessing distribution or bioavailability of silver, as standard methods cannot distinguish between Ag and AgNP in tissue. Nevertheless, the weight of evidence suggests that both tissue distribution and bioavailability of ingested AgNP are low. There is indirect evidence that AgNP may have lower bioavailability than ionic Ag. In a 28 day study of 14 nm PVP-coated AgNP administered at 12.6 mg/kg, 63% of the daily dose was

eliminated in the faces, compared to 49% of the daily dose of ionic Ag in the form of silver acetate (AgOAc) (Loeschner et al., 2011). The one study designed to assess bioavailability found that the majority of a one-time dose of citrate-capped AgNP (7.9 nm) in rats was eliminated via the feces (Park et al., 2011). Bioavailability assessed directly using blood levels was found to be < 5% of the daily dose. In both of these studies, there was evidence for biliary excretion of AgNP.

With respect to distribution, Jeong, Kim, and Loeschner found histochemically detectable Ag in the lamina propria of the intestine and along the small intestine surface using autometallographic techniques (Jeong et al., 2010; Kim et al., 2010b; Loeschner et al., 2011). This intestinal distribution corresponded to the highest Ag concentrations detectable by ICP-MS (Loeschner et al., 2011). Thus, a portion of enterically absorbed Ag may actually remain in the submucosal tissue of the intestine and never reach the systemic circulation or visceral organs. This is a significant observation since intestinal concentrations are not typically measured in most standard toxicity-focused distribution studies. Distribution of ingested AgNP in extraintestinal tissues was very low in all studies (ppb levels). The most common target tissues for accumulation in repeated dose studies were kidney and liver. Although absorption seemed inversely proportional to particle size (Park et al., 2010a), no studies directly compared particles of the same size with different coatings. In studies using PVP or carboxymethlycellulose (CMC)-coated AgNP ranging from 14 to 60 nm, kidney levels were slightly higher than levels in liver (Kim et al., 2008, 2009a, 2010b; Loeschner et al., 2011). Conversely, 7.9 nm citrate-capped AgNP showed slightly higher accumulation in the liver, although all tissue levels were low (Park et al., 2011). Of interest, Kim et al. (2008, 2009a, 2010b) repeatedly demonstrated that kidney accumulation in female rats was higher than that in males, although the mechanism of this finding is uncertain. Notably, in all studies the Ag detection methods used (ICP-MS, autometallography) cannot distinguish the physical state of Ag, so whether it is absorbed predominantly as intact AgNP or as ionic or molecular Ag is unknown.

In studies of tissue distribution in chickens administered up to 15 ppm AgNP in feed, the highest tissue Ag concentrations were in muscle, at low concentrations (ppb range) (Ahmadi and Kordestany, 2011; Ahmadi and Rahimi, 2011). A similar experiment in poultry given up to 25 ppm of AgNP in water had comparable low Ag concentrations in muscle and liver (Ahmadi and Kurdestany, 2010). Percent retention of total administered dose was not evaluated. In contrast, in pigs administered up to 40 ppm AgNP in feed for 5 weeks there was no retention in muscle and only minimal retention in liver (Fondevila et al., 2009). The possibility of muscle retention in chickens is an important consideration, given that distribution into muscle is not typically evaluated in rodent toxicity studies and is a potential source of human exposure in food animal species. Whether this finding is specific to avian species and whether it is repeatable or relevant to human health risk bears further investigation (Hadrup et al., 2012).

AgNP: toxicity

Adverse effects reported in AgNP oral dosing studies were mild and were only evident at doses of 125 mg/kg and above. A lowest observed adverse effects level (LOAEL) of

125 mg/kg in one 90d study using 60 nm AgNP in 0.5% CMC corresponded to elevated cholesterol and cholestatic enzymes (alkaline phosphatase) and was accompanied by biliary hyperplasia (Kim et al., 2010b). The same group found similar cholestatic enzyme effects and slight hemoconcentration at 300 mg/kg with the same material in a 28d study in rats (Kim et al., 2008). This suggests that the biliary system may be a target for Ag accumulation or metabolism. The significance of these results is difficult to determine as the elevated enzymes may simply represent increased biliary excretion activity (i.e., an adaptive physiological response). Only one study evaluated adverse effects at a lower, more physiologically relevant dose (Park et al., 2010a). In mice given 0.25–1 mg/kg citrate-capped AgNP for 28 days, there were mild, dose-dependent increases in both serum pro-inflammatory (IL-1, IL-6, IL-12) and anti-inflammatory (IL-10, TGFß) cytokines. B-cells and IgE were also mildly increased. No histological alterations were found in this study and the biological significance of these cytokine alterations is uncertain. No evidence of genotoxicity was detected using micronucleus assay on bone marrow cells in rats administered up to 1,000 mg/kg of 60 nm AgNP in 0.5% CMC over 28d (Kim et al., 2008).

Overall, evidence suggests that the likelihood for adverse effects on host tissues caused by acute or subchronic oral administration of AgNP is quite low. This was true even in studies using doses up to 100–1,000 mg/kg/d, which are 20,000–200,000× the EPA oral reference dose (recommended maximal ingestion) of 0.005 mg/kg/day. Evidence of biliary hyperplasia (Kim et al., 2010a) and distribution studies suggesting biliary secretion support that, where adverse effects are present, the hepatobiliary system may be a focus. No adverse renal effects were noted, despite the renal accumulation found in female rats as discussed under tissue distribution (Kim et al., 2008, 2009a, 2010b).

Of note, only one rodent study evaluated effects of ingested AgNP on the gut microbiota. Hadrup et al. showed 28 days gavage administration of 14 nm Ag-PVP or silver acetate did not alter the balance and number of the two major bacterial phyla in the gut (*Bacteroidetes* and *Firmicutes*), as determined by quantitative PCR of bacterial 16S rRNA genes (Hadrup et al., 2012). In contrast, pigs fed a polydisperse mixture of 60–100 nm AgNP at up to 40 ppm for 14 days, showed decreased intestinal coliforms, as measured by culture (Fondevila et al., 2009). This correlated with an increased rate of weight gain. Since the particles in the pig study were larger, a possible explanation for these disparate results are that comparatively more Ag remained within the digestive tract, in contact with the luminal bacteria, than in the rat study which used 14 nm Ag. This bears further investigation in a single study with the same species. Additional studies exploring selection pressure for Ag resistance in the face of environmental exposure or direct ingestion of AgNP would also be helpful, particularly considering that AgNP are being suggested as an alternative to antibiotics in some human health (Percival et al., 2005) and animal agricultural applications (Fondevila et al., 2009). Hadrup et al. (2012) found that the plasmid-borne silver resistance genes *sil*RS, *sil*P, and *sil*CBA were not altered in bacteria from silver-fed or control rats. Nevertheless, given that this was a 28 day study and 'real-world' silver exposures are likely to be of longer duration, additional investigation into this area is warranted.

Table 1 *In vivo* studies of AgNP exposures by the oral route

Reference	*Material (size)*	*Coating*	*Dose/endpoint*	*Species*	*Effects*	*Distribution*
Mammalian, rodent						
Bhol (2007)	Ag (NR)	5.7% PVA	0.4–4–40 mg/kg, recurring, 5 d	Rat (SD)	High dose: ↓ inflammation in colitis model	NR
Cha (2008)	Ag (13 nm)	none	2.5 g one dose (150 g/kg)/3 d	Mouse (BALB/c)	Localised lymphocyte infiltration; gene alterations in apoptosis, inflammation	NR
Hadrup (2012)	Ag (14nm)	PVP	2.25–9 mg/kg recurring, 28 d	Rat (Wistar)	No toxicological effects	NR
Jeong (2010)	Ag (60 nm)	0.5% CMC	30–300–1,000 mg/kg recurring, 28 d	Rat (SD)	Luminal and surface intestinal particle accumulation; ileal and colonic lamina propria and villus tips; increased mucus discharge into lumen from colon	NR
Kim (2010)	Ag (56 nm)	0.5% CMC	30–125–500 mg/kg recurring, 90 d	Rat (F344)	NOAEL: 30 mg/kg At 125 mg/kg liver effects: ↑alk phos and cholesterol; biliary hyperplasia	Kidney > Liver > Lung > Testes >> Brain >> Blood; In intestinal wall in male, not female; Females had higher kidney levels
Kim (2009)	Ag (60 nm)	0.5% CMC	30–300–1,000 mg/kg recurring, 90 d	Rat (F344)	Not assessed (distribution-focused)	Confirmed renal retention in females > males
Kim (2008)	Ag (60 nm)	0.5% CMC	30–300–1,000 mg/kg recurring, 28 d	Rat (SD)	LOAEL: 300 mg/kg based on ↑ alk phos, cholesterol; no genetic toxity as measured by micronucleus test on bone marrow; ↑RBC, Hgb, Hct, ↓ aPTT	Stomach > kidney > liver > lung; kidney accumulation female > male

Table 1 *In vivo* studies of AgNP exposures by the oral route (continued)

Reference	*Material (size)*	*Coating*	*Dose/endpoint*	*Species*	*Effects*	*Distribution*
Mammalian, rodent						
Loeschner (2011)	Ag (14 nm)	PVP	12.6 mg/kg recurring; 28 d	Rat (Wistar-Hannover-Galas)	Not assessed (distribution-focused study)	Small intestine >> stomach > kidney > liver >> lung; same tissue distribution as AgOAc but lower amount (by 40-50%) and higher fecal excretion (63% of daily dose); lamina propria and macrophage lysosomes'
Park (2011)	Ag (7.9 nm)	Citrate	1, 10 mg/kg, one dose, 24 hrs	Rat (SD)	Not assessed (distribution and bioavailablity-focused study)	Feces >>> Liver > Lung >kidney; evidence of biliary excretion; bioavailability of 1.2% (1 mg/kg) to 4.2% (10 mg/kg)
Park (2010)	Ag (22, 42, 71 and 323 nm)	Citrate	0.25, 0.5, 1 mg/kg recurring, 14 and 28 d	Mouse	Dose-dependent ↑ inflammatory cytokines in blood; elevated B cells and IgE	Absorption inversely proportional to size, highest size not detected; Brain/liver/kidney/testis all at low levels
Mammalian, non-rodent						
Ahmadi (2011a)	Ag	NR	5–10–15 ppm feed recurring, 42 d	Chicken	None significant	Muscle > spleen > liver > kidney > feces
Ahmadi and Rahimi (2011b)	Ag	NR	4–8–12 ppm water recurring; 42 d	Chicken	↓ body wt, feed intake	Muscle > liver > feces
Ahmadi (2010)	Ag (14 nm)	NR	5–15–25 ppm water recurring; 42 d	Chicken	Decreased lymphoid organ (bursa) weight	NR
Ahmadi (2009, 2009)	Ag (14 nm)	NR	300–600–900 ppm water recurring; 56 d	Chicken (broilers)	None significant	NR
Fondevila (2009)	Ag (60–100 nm)	Sepiolite	20–40 mg/kg (ppm) feed recurring; 35 d	Pigs	↑ growth rate; ↓ coliforms	None in muscle, low in liver (2%)

Growth promotant and anti-inflammatory effects

Seven *in vivo* studies specifically addressed efficacy of AgNP with respect to growth rate or inflammation. These consisted of several food animal studies (Ahmadi, 2009; Ahmadi et al., 2009; Fondevila et al., 2009; Ahmadi and Kurdestany, 2010; Ahmadi and Kordestany, 2011; Ahmadi and Rahimi, 2011) and one rodent study (Bhol and Schechter, 2007). In chickens, there was no effect on growth when AgNP were administered in feed and either no effects (Ahmadi, 2009; Ahmadi et al., 2009) or a negative effect (decreased body weight, feed intake, lymphoid organ weight) when AgNP were administered in water (Ahmadi and Kurdestany, 2010; Ahmadi and Rahimi, 2011). In a study using a rat model of chemically induced colitis, there was decreased inflammation and decreased gut inflammatory cytokines in rats given 40 mg/kg of AgNP orally (Bhol and Schechter, 2007). This is in contrast with toxicity studies in rodents showing serologically elevated levels of both pro- and anti-inflammatory cytokines (Park et al., 2010a). Since serological cytokine levels may be quite variable and may not reflect local activity in the gut, additional targeted studies may contribute to determining whether and under what conditions ingested AgNP may have a net pro- or anti-inflammatory effect.

5.1.2 AuNP

Only a few studies have evaluated toxicity or tissue distribution of orally administered AuNP (Table 2) (Hillyer and Albrecht, 2001; Dhar et al., 2010; Zhang et al., 2010b) .None of the identified studies specifically addressed bioavailability. One study (Hillyer and Albrecht, 2001) extensively evaluated tissue distribution and identification of a potentially novel means of NP entry into the body. AuNP were administered to mice in drinking water at concentrations of 200 ug/ml over 7 days. Particles were visualised by TEM and quantified by ICP-MS to determine distribution. Distribution was inversely related to size, as the smallest particles (4 nm) were retained in the highest amounts and the largest (58 nm) were not detectable in any evaluated tissues. There were some differences in the distribution between the smaller sizes (4–10–58 nm). For the 4 nm particles, the highest amount was in the kidneys, for the 10 nm particles, the highest amount was in stomach, and for the 28 nm particles, in stomach and small intestine. This suggests that the smallest particles were better able to transit the intestinal mucosa while particles of increasing size became adherent to or entrapped within the mucus layer or intestinal wall. TEM supported these findings as 4 nm particles were observed transiting the mucosa at the sites of dead or dying extruded enterocytes. Enterocyte apoptosis and extrusion is a normal part of enterocyte turnover and it was posited that this route represents a unique paracellular uptake mechanism by which the smallest NPs (or at least nanoAu) may be able to bypass endocytosis. This was not observed for the 10 and 28 nm particles, although some proportion must have transited the mucosa as evidenced by detectable levels in other tissues. This study was also notable for the detection of particles in the brain albeit in very low levels, which were again inversely proportional to size. Whether entry into the brain involved paracellular routes, endocytosis, or another mechanism was not evident. The tissue retention of AuNP was at very low levels, ranging from 4–75 ng/g in the tissues of the 4 nm treated animals. This could not be directly assessed as a percent of total administered dose since the AuNP were administered via *ad libitum* water consumption.

Table 2 *In vivo* studies of metal NP other than Ag by the oral exposure route

Reference	*Material (size)*	*Coating*	*Dose/duration*	*Species*	*Effects*	*Distribution*
Hillyer and Albrecht (2001)	Au (4, 10, 28, and 58 nm)	None/malto dextrin	200 ug/ml in water; 7 d	Mouse (BALB/c)	Persorption through extruding (dying) ileal enterocytes	58 nm: very low in all tissues; 28 nm: Intestine >> Stomach >> Spln >> Kidney/Liv/Heart; 10 nm: Intest/Kid/Stom > Lung/Hrt/Spleen>Liver/Brain; 4 nm: Kidney > Intest > Stom/Spln/Liv/Lung > Heart
Zhang et al. (2010c)	Au (13.5 nm)	None/water	137.5–2,200 ug/kg; 14–28 d	Mouse (not specified)	↓ body weight ; enlarged spleen at high dose; increased red blood cells at high dose	NR
Dhar et al. (2010)	Au (14 nm)	0.02% gellan-gum stabilised	75–150–300 ppm (mg/kg); 28 d	Rat (Wistar)	No alterations (body wt, hematology/clinical chemistry, histology, urinalysis, organ wts)	NR
Bu et al. (2010)	TiO_2 (< 50 nm); mixture of anatase and rutile (% not specified)	Water	160–400–1,000 mg/kg for 14 d	Rat (Wistar)	Dose-dependent ↑ liver (AST) and muscle (CK) enzymes; no histological changes; altered urinary metabolites at 1,000 g/kg	NR
Cui et al. (2011)	TiO_2 (5 nm, anatase)	Hydroxypropyl-methylcellulose (HPMC)	5–10–50 mg/kg; for 60 d	Mouse (CD-1)	↑ inflammation-associated genes (TLR 2, TLR4, TNFa, NFkB, IL-2)	Only liver assessed (dose-dependent accumulation)
Duan et al. (2009)	TiO_2 (5 nm as prepared: anatase)	HPMC	62.5–125–250 mg/kg; for 30 d	Mouse (CD-1)	↓ body wt, ↑ wt spleen, liver, kidney, thymus; hematological alterations mid-dose (125)	NR
Gui et al. (2011)	TiO_2 (5–6 nm, as prepared; 294 nm hydrodynamic diameter with HPMC: anatase)	HPMC	2.5–5–10 mg/kg; 90 d	Mouse (CD-1)	↑ kidney weight; variable ↑ inflamm cytokines, variable ↑ renal biochemical parameters	Only kidney assessed (dose-dependent accumulation)
Trouiller et al. (2009)	TiO_2 (P25: 25 nm; 25% rutile, 75% anatase; 160 nm as dosed)	In drinking water	60–100 mg/kg; 5 d	Mouse C57BL/6 transgenic	DNA deletions, strand breaks, oxidative damage	NR

Table 2 *In vivo* studies of metal NP other than Ag by the oral exposure route (continued)

Reference	*Material (size)*	*Coating*	*Dose/duration*	*Species*	*Effects*	*Distribution*
Wang et al. (2007)	TiO_2 (25–80–155 nm; crystal composition not reported)	HPMC	5,000 mg/kg; single dose; 24 hrs	Mouse (CD-ICR)	Minor ↑ liver and kidney enzymes in females for 25 nm	Liver >> lung > kidney > spleen > brain
Warheit et al. (2007)	TiO_2 (140 nm as dosed; 80% anatase, 20% rutile)	Water	175–550–1,750–5,000 mg/kg; single dose	Rat	Grey-coloured feces in high doses, no adverse effects	NR
Zhang et al. (2010a)	TiO_2 (50–120 nm); and same sizes in combination with PbAC; crystal composition not reported	Phosphate buffered saline	500, 5,000 mg/kg;7 d	Mouse (Kun Ming)	Synergistic (PbAC and TiO_2) elevation in ROS in liver; hepatic necrosis in TiO_2-PbAc groups	Liver>kidney>>brain (these were the only tissues evaluated)
Moulari (2008)	SiO_2 (140 nm as dosed)	Coupled to 5-aminosalicylate (active drug)	Not defined (5-ASA conjugate up to 100 mg/kg) × 6 d	Mouse (BALB/c)	TNBS-induced colitis model; decreased effective dose for anti-inflammatory effects of 5-aminosalicylic acid in a colitis model	Active drug accumulated in inflamed tissues; other tissue distributions not measured
Chen (2006)	Cu (23.5 nm)	Hydroxypropyl-methylcellulose	108–1,080 mg/kg; 48 h	Mouse (CD-ICR)	Renal tubular necrosis and pigmentary nephrosis; splenic atrophy; ↑ bile acids; LD50 (nanoCu): 413 mg/kg; LD50 (microCu): > 5,000 mg/kg; LD50 (ionicCu): 110 mg/kg	NR
Han (2010, 2011)	Cu-loaded chitosan (121.9 nm)	0.9% saline	80–160 mg/kg; 21 d	Rat (SD)	↓ pathogenic cecal microbes, ↑ beneficial cecal microbes, ↑ digestive enzyme secretion	NR
Loginova (2012)	CdSe/CdS/CdZnS/ZnS quantum dots	Functionalised: with MPA, poly T, or poly T-APS (silica)	200–290 pmol; single dose	Mouse (nude and CD-1)	No adverse effects	Detected only in GI tract; polyT-APS (silica) protected QDs from degradation

Two studies specifically evaluated adverse effects of oral administration of AuNP (Dhar et al., 2010; Zhang et al., 2010b). AuNP were administered to mice or rats at doses ranging from 0.138–2.2 mg/kg for 14–28 d (Zhang et al., 2010b). Adverse effects in mice included decreased body weight and enlarged spleens with decreased peripheral red blood cells beginning at 1.1 mg/kg. Gold NPs were also visualised by TEM in the red blood cells of the high dose group (other tissues not evaluated). No significant differences in other organ weight indices were noted and no deaths were reported. In this particular study, the finding of AuNP in red blood cells coupled with decreased peripheral red cells suggests AuNP-related hemolysis, particularly since hemolysis is a recognised off-target effect of therapeutically utilised solubilised gold compounds (e.g., gold III dithiocarbamate) (Ronconi et al., 2006). Rodents typically respond to red cell destruction by increasing extramedullary hematopoietic activity in the spleen and an enlarged spleen was observed in this study, although this was not evaluated by histology or hematological parameters related to hemolysis. In contrast to these findings, in a 28 day study of gum-stabilised AuNP oral administration in rats at doses up to 300 mg/kg, there were no detectable adverse effects (organ/body wt, histology, hematology, clinical chemistry) (Dhar et al., 2010). Red blood cell or other tissue uptake were not evaluated *in vivo* in the rats, although the gellan gum particles were taken up *in vitro* by fibroblast and glioma cell lines. The difference between this study and Zhang's results in mice could have several causes. Both studies used AuNP of similar size as manufactured (13.5 and 14 nm) but the size as dosed was not clear. The dose ranges were overlapping but different coatings were used – of note, the Zhang study, which showed toxicity, used uncoated gold particles while the Dhar study, showing no toxicity, used gum-stabilised particles.

All told, the limited current *in vivo* information suggests that the toxicity of orally ingested AuNP at therapeutic or biologically relevant doses is low, with only one study showing adverse effects suggestive of hemolysis in one species (mouse) at doses of 1,100 ug/kg (1.1 mg/kg) (Zhang et al., 2010b). Nevertheless, given the demonstrated ability of small gold NP to enter cells (Hillyer and Albrecht, 2001; Zhang et al., 2010b) and the known toxicity of solubilised gold therapeutic agents (Ronconi et al., 2006), hemolysis should be kept in mind. Tissue quantification, measurement of particle size as dosed, and correlation of tissue or cell distributions with adverse hematological or histological findings would be helpful in better defining risk or uptake mechanisms. Both *in vivo* and *in vitro* approaches would greatly benefit from direct comparison of AuNP with similar dose ranges of soluble gold controls. This is necessary to determine whether the NP form decreases, increases, or has no impact on toxicity of the parent element.

5.1.3 Titanium dioxide nanoparticles (TiO_2NP)

Eight studies that experimentally evaluated distribution or toxicity of ingested TiO_2NP were identified (Table 2) (Wang et al., 2007; Warheit et al., 2007; Duan et al., 2009; Trouiller et al., 2009; Bu et al., 2010; Zhang et al., 2010a; Cui et al., 2011; Gui et al., 2011). These studies vary considerably with respect to dose and characterisation of TiO_2NP. Of note, not all identified the crystal composition (anatase, rutile) of the dosed

compound. Since the anatase crystalline form has higher cytotoxicity than the rutile (Weir et al., 2012), this is a potentially significant omission. Two studies used very high doses (up to 5,000 mg/kg) to identify potential target organs for adverse effects (Wang et al., 2007; Warheit et al., 2007). One of these found no evidence of cytotoxicity using 130 nm TiO_2NP in single doses of up to 5,000 mg/kg in rats (Warheit et al., 2007). The other cited liver and kidney toxicity in female mice only when dosed with 25 nm TiO_2 at 5,000 mg/kg (Wang et al., 2007). However, this claim was based predominantly on minor elevations of ALT (liver enzyme) and BUN (urea nitrogen-renal biomarker) (Wang et al., 2007). Although statistically different from control animals, the degree of elevation does not appear biologically significant. Combined with the lack of elevation of other liver parameters (AST, bilirubin, ALP), the lack of elevation of other renal parameters (creatinine), and the lack of correlation between maximal tissue accumulation and reported histological alterations, the occurrence of true renal or hepatic toxicity in this study is uncertain. Studies of 5–6 nm TiO_2NP orally administered to mice for 60–90 d at low doses (2.5–50 mg/kg) reported dose-dependent accumulation in the kidney (Gui et al., 2011) and liver (Cui et al., 2011) with elevation of some pro-inflammatory cytokines (Cui et al., 2011). These lower dose studies are difficult to reconcile with the lack of alterations in the 5,000 mg/kg Warheit study. Potential explanations for the discrepancies in these studies could include decreased absorption in very high doses (increased potential for aggregation in the gut lumen). Additionally, the histological evidence of hepatotoxicity depicted in the images of several of the studies is not convincing, nor are the nomenclature and diagnostic criteria described (Wang et al., 2007; Duan et al., 2009).

Several TiO_2NP utilised interesting approaches to potential metabolic or genotoxic aspects of TiO_2NP toxicity. One study evaluated urinary and serum metabolite alterations in rats dosed with TiO_2NP of < 50 nm at 1,000 mg/kg (Bu et al., 2010). Several altered urinary metabolites were detected. These included compounds indicative of a ketogenic state including elevated ketone bodies (3-D-HB) and ketone body metabolites (acetate). Additionally, there were elevated gut microbe-derived metabolites (PAG, hippurate) similar to those seen in studies using antibiotic-treated animals. This suggests that gut microbes may be affected. Since TiO_2NP have been investigated as a solid-phase extraction agent for removal of lead or other heavy metal contaminants from water (Zhang et al., 2010a), TiO_2 and lead acetate (PbAC) in saline suspension were evaluated for individual and potential synergistic toxicity (Zhang et al., 2010a). There was synergistic elevation of reactive oxygen species in liver in combination with liver necrosis. Although tissue retention with respect to total dose was not calculated, the highest distribution was to the liver and was highest for the combined dose (TiO_2 and PbAC) group in comparison to the individually dosed groups. In light of the recent classification of microscale TiO_2 as a potential carcinogen, an *in vivo* genotoxicity study was performed using industrial-grade (P25) TiO_2NP in drinking water (Trouiller et al., 2009). This compound was supplied during gestation to mice genetically engineered for mutagen-induced instability in the pink-eyed dilute gene (C57BL/6Jp$^{un/un}$) (Trouiller et al., 2009). The offspring of these mice were found to have elevated DNA deletion

events and DNA oxidative damage in the high dose group. DNA strand breaks were increased in a dose-dependent manner. DNA instability in *in vitro* assays has been variable, and this *in vivo* study is difficult to interpret with respect to human health risk. Nevertheless, it suggests that potential for genotoxic effects in sensitive populations, such as foetuses in the period of organogenesis.

5.1.4 Other metal or metal oxide NPs: SiO_2NP, CuNP, and QDs

SiO_2NP

In vivo studies focused on toxicity of ingested of nanoscale silica were not available. One study focused primarily on silica NP efficacy as a drug carrier for 5-aminosalicylate (5-AS) in a chemically-induced mouse model of colitis (Moulari et al., 2008) (Table 2). The dose of silica was not defined in this efficacy-focused study, although the complex 5-AS-SiO_2NP was well toleratedat doses up to 100 mg/kg for 6 days. The complex deposited in inflamed tissue but further distribution was not reported (Moulari et al., 2008). Some information can be extrapolated from earlier work on synthetic silica in food. Dekkers et al. (2012) used information from the limited studies of SiO_2 and extrapolated information from studies of synthetic amorphous silica to suggest that less than 1% of the mass dosed is recoverable from tissues. Insufficient information is available to determine whether there is a significant difference in toxicity between orally administered SiO_2NP and traditional forms of food-grade SiO_2, or whether there is size-dependence for absorption as with Au or TiO_2. From the colitis efficacy study, there is evidence that SiO_2 may have lumenal effects and may target to sites of inflammation (Moulari et al., 2008).

CuNP

Three toxicity-focused studies of CuNP were found (Chen et al., 2006; Han et al., 2010, 2011) (Table 2). In a maximum tolerated dose study of 23.5 nm copper orally administered to mice, the oral LD_{50} for CuNP was 413 mg/kg, as compared to 5,610 mg/kg for microCu (17 um) and 110 mg/kg for ionic Cu (from $CuCl_2$) (Chen et al., 2006). The physical findings associated with CuNP were similar to those of classical Cu toxicosis in mammals and included renal tubular necrosis accompanied by dark brown to black renal pigmentation. The latter is typically associated with hemoglobinuric casts arising from intravascular hemolysis in Cu toxicosis, although hemolysis was not assessed in this study. These results indicates that ionic Cu and CuNP are most bioavailable and have similar adverse effects while micro-Cu is much less bioavailable than either CuNP or ionic Cu. Disparities in tolerated dose between microCu and ionic or CuNP may be less pronounced when compared in terms of other relevant metrics such as the proportion of ionised Cu in CuNP vs. microCu. The proportion of ionic Cu in the nanoscale prep on an as-fed basis or in the GIT is not known.

Cu has been advocated as an *in vivo* antimicrobial. One group evaluated microbial populations using Cu complexed to chitosan NPs and administered to rats in feed (Han et al., 2010, 2011). This combination was found to decrease cecal pathogenic bacteria (e.g., *Salmonella*, *E. coli*, *Clostridium*), increase beneficial bacteria (e.g., *Lactobacilli*) and, potentially, facilitate nutrient utilisation by increased secretion of digestive enzymes (Han et al., 2010, 2011). The specific dimensions of the Cu-chitosan NP were not given and is unknown if the complex remained intralumenal or was absorbed, as tissue residues were not determined. No clinically evident adverse effects were noted in the rats in this study.

Quantum dots

Only one toxicity or distribution-focused study of ingested QDs was found (Loginova et al., 2012) (Table 2). QDs, consisting of CdSe-cored particles with ZnS caps were evaluated for stability in the GIT. Standard coatings of thiol groups (polyT) or mercapto residues (MPA) resulted in rapid degradation and loss of surface characteristics. Interestingly, when coated with a combination of polythiol groups and silica (PolyT-APS), the particles were protected from degradation (Loginova et al., 2012). No adverse effects were noted (single administration) and the particles were detected only within the GIT.

5.2 Carbon-based NPs: MWCNTs, SWCNTs

Five toxicity-focused studies of ingested carbon-based NPs were identified (Table 3) (Szendi and Varga, 2008; Folkmann et al., 2009; Kolosnjaj-Tabi et al., 2010; Lim et al., 2011; Philbrook et al., 2011). In inhalation studies and in cell culture experiments, CNTs have been associated with granulomatous inflammation, oxidative damage, and mutagenicity, and the ingestion studies used one or more of these parameters as endpoints. In rats with gestational oral exposures (d6-d19) to MWCNT (10–15 nm diameter, 20 um long), the NOAEL was determined as 200 mg/kg/d (Lim et al., 2011). At higher doses, there were slight decreases in thymic weights (which can be a direct lymphoid effect or a non-specific indication of stress). No altered reproductive parameters (foetal resorption, litter sizes, malformations) were noted and there was no detection of oxidative damage markers in urine. In contrast, reproductive parameters were altered in a study of functionalised (hydroxylated) SWCNT (1–2 nm diameter, 5–30 um length) administered to mice on gestation d9. Here there were increased foetal resorptions and increased foetal skeletal and ocular anomalies at 10 mg/kg (Philbrook et al., 2011). In the same study, there were no adverse effects on reproductive parameters in mice administered a higher dose of 100 mg/kg (Philbrook et al., 2011). A postulated

cause for this seemingly anomalous result was that higher concentrations might be more likely to aggregate or agglomerate in the intestine, impeding absorption (Philbrook et al., 2011). This highlights the difficulty in comparing standard mass-based (as dosed orally) dose metrics to CNT toxicity studies, since the ideal parameter to measure would be dose delivered to target cells. The potential for different effects in the intestinal and extraintestinal compartment is illustrated in another study using much smaller doses (0.064 and 0.64 mg/kg) of SWCNT (0.9–1.7 nm, < 1 um length) administered as a one-time dose to rats (Folkmann et al., 2009). Here, there was significantly elevated oxidative DNA damage, assessed as tissue levels of 8-oxo-dG, in liver and lung but not colon. In the same study, rats given were given the same doses of C60 fullerenes (234 to 3,124 nm), resulting in elevated levels of 8-oxo-2'-deoxyguanosine(8-oxo-dG) in the liver at both doses but in the lung only at the high dose. Colon 8-oxo-dG was not observed. Of note in this study, the primary particle lengths were lower (< 1 μm) in comparison to those of the previously described studies (up to 30 μm), which may have influenced absorption. In contrast, no mutagenicity (measured by Salmonella mutagenicity test on urinary adducts) was detected in rats given higher doses (50 mg/kg) of SWCNT or MWCNT up to 30 μm diameter and 15 μm long (Szendi and Varga, 2008). Finally, an extremely high dose (1,000 mg/kg) of SWCNT administered to orally to mice showed no toxicity irrespective of fibre length (20 nm up to > 1 μm) (Kolosnjaj-Tabi et al., 2010). The same study showed granuloma formation in multiple organs when SWCNTs of all fibre lengths were administered by intraperitoneal injection. In the orally dosed mice, absorption was not measured, but the lack of histological lesions in comparison to the IP dosing suggests that absorption was very low. Although not specifically measured in this study, this would be consistent with the studies describing increased aggregation or agglomeration (and hence decreased absorption) with increasing dose concentration.

These studies suggest that oral absorption of carbon-based NPs may be facilitated by low doses, low particle length, and potentially, functionalisation. These properties could perhaps be differentially exploited in situations where absorption is desirable (certain biomedical applications) and in those where it is undesirable (environmental or technological applications). With respect to traditional toxicology testing of ingested materials, it will be challenging to precisely define the dose-responsiveness of measured endpoints for CNTs, since aggregation/agglomeration of the dosing suspension were reported in all studies. Aggregation or agglomeration depends on functionalisation, vehicle, nanomaterial, and concentration, as well as variable characteristics of the GI tract (pH, ingesta, microbiota). Additional studies directly comparing varying particle parameters (e.g., functionalised vs. non-functionalised, or 1 μm length vs. 20–30 μm length) and administered at different concentrations would be helpful.

Table 3 *In vivo* studies of carbon-based or polymer/dendrimer NP by the oral exposure route

Reference	*Material (size)*	*Coating*	*Dose; duration*	*Species*	*Effects*	*Distribution*
Mori et al. (2006)	C60/C70	CMC	2,000 mg/kg	Rat (SD)	No toxicity-body weights	NR
Shinohara et al. (2009)	C60 (33–84 nm)	CMC	22–88 mg/kg	Mice (Crl:CD1)	No genotoxicity-micronucleus test	NR
Takahashi et al. (2012)	C60 (pristine material 0.71 nm diameter, visible aggregates as dosed)	Corn oil	10–100–1,000 mg/kg/d for 29 days, 14 day recovery	Rat (Crl:CD1)	No adverse effects;	not detected in liver, kidney, spleen
Folkmann (2009)	C60 (234-3,124 nm, as dosed) SWCNT (34-1015 nm, as dosed)	CMS	0.064, 0.64 mg/kg	Rat (F344)	SWCNT: ↑ levels 8-oxodG in liver, lung but not colon at both doses; C60: ↑ levels 8-oxodG in liver both doses, lung only in high dose; no difference in corn oil or saline as vehicle (does not require dispersion to be absorbed); corn oil alone: more genotoxicity than particles	NR
Kolosnjan-Tabi et al. (2010)	SWCNT (1 nm diameter, 20 up to >2 um length)	Aqueous solution (no surfactant)	1,000 mg/kg	Mouse (Swiss)	No behavioural or histological indications of toxicity (granulomas with IP administration)	NR
Philbrook (2011)	functionalised (–OH group) SWCNT (1-2 nm diameter, 5-30 um length)	Tracaganth gum	10, 100 mg/kg at gest d9	Mouse (CD-1)	10 mg/kg: increased fetal resorption and fetal skeletal/ocular anomalies; 100 mg/kg: no alterations	NR
Szendi (2008)	SWCNT (<2 nm × 4–15 um); MWCNT (10–30 nm × 1–2 um)	Carbopol gel	50 mg/kg, single dose	Rat (F344)	Only evaluated urinary mutagenicity – none detected	NR
Lim (2011)	MWCNT (CM-95, 10–15 nm diameter, 20 um length)	CMC	8–40–200–1,000 mg/kg/d from gestational d6 to d19	Rat (SD)	No differences in reproductive parameters; no change in parameters of renal oxidative damage; no clinical or biochemical effects apart from ↓ thymic wt in high dose; NOAEL: 200 mg/kg/d	NR
Bisht (2008)	polymers: 60:20:20N-isopropylacrylamide; methylmethacrylate; acrylic acid (< 100 nm)	-	500 mg/kg	Mouse	No adverse effects, efficacy in rapamycin delivery in pancreatic cancer model	NR

5.3 Polymer, dendrimer NPs

There has been an explosion of studies exploring the feasibility of dendrimers as drug delivery devices (reviewed by Malik et al., 2011; Patri et al., 2002; El-Ansary and Al-Daihan, 2009; Malik et al., 2011).Much of the evaluation of these NPs in the academic literature is focused on efficacy, with toxicity, adverse effects, or the lack thereof remarked upon only secondarily. While safety evaluation of these NPs is conducted in the contract research and pharmaceutical arenas, these results are typically not published. An efficacy study in a mouse model of exocrine pancreatic cancer included toxicity parameters and is included here (Bisht et al., 2008) (Table 3). Experimentally, polymer-based delivery of rapamycin by the oral route was well-tolerated in mice at doses up to 500 mg/kg with no adverse effects and apparent therapeutic efficacy (Bisht et al., 2008). The safety and efficacy of orally-administered polymeric delivery systems will depend greatly on the physical properties such as surface charge and surface functional groups [reviewed by Patri et al. (2002), and DeJong and Borm (2008)]. Although *in vivo* dosing studies in intact animals focused on toxicity were scarce, in an everted intestinal sac explant system from rat, anionic PAMAM dendrimers had rapid trans-serosal absorption and deposit in tissue at low levels that correlate with increasing molecular diameter (larger dendrimers depositing at higher levels than smaller) (Wiwattanapatapee et al., 2000). This is an interesting contrast to the metallic NPs and the CNTs, which have greater absorption of smaller diameter particles. Cationic PAMAM dendrimers have lower absorption due to adherence to negatively charged cell membranes of the gut epithelium (Wiwattanapatapee et al., 2000). In a similar rat gut explant study using lipid core NP for delivery of indomethacin, the NP was degraded and the core exposed in the gut lumen, indicated by metabolic conversion of indomethacin in the lumen prior to absorption into blood (Cattani et al., 2010). Thus, for compounds in which toxicity or activity is dependent on metabolic activation, the bioavailability may be variable depending on the NP carrier and the microbial composition of the gut, which includes organisms capable of phase I and II drug metabolism.

6 *In vitro* studies of NP toxicity

6.1 In vitro model systems

In vivo studies have given us some sense of the possible target organs and potential effects of ingested nanomaterials. Nevertheless these consequences remain uncertain and impossible to predict without some investigation of mechanism, which requires relevant *in vitro* models. *In vitro* models for simulating the effects of NP ingestion will be presented here. Relevant studies are listed in Table 4. *In vitro* models do have their limitations. Because cells in isolation suffer from reduced survival, disrupted metabolic competence, reduced cell-cell interaction, disrupted organ topology, and absence of tissue communication, *in vitro* exposure systems may fail to accurately reflect *in vivo* responses (Eisenbrand et al., 2002).

Table 4 *In vitro* toxicity studies of intestinal NP exposures

Author	*NP*	*Size*	*Dose*	*Model*	*Endpoint(s)*	*Effect/conclusion*
#(Bouwmeester et al. 2011)	Ag	20–113 nm	5–50 μg/mL; 4 and 24 h	Caco-2/M cell co-culture	WST-1, TEER, microarray	No toxicity; significant changes in gene expression due to ionic Ag
(Lamb et al., 2010)	Ag	Not reported	1–10 μg/mL; 24 h	Caco-2 (MDR1.C)	MultiTox-Fluor Multiplex Cytotoxicity assay; MDR1 reporter induction response to drugs	LD_{50} 5 μg/mL; <1 μg/mL had no effect on drug metabolism
#(Gaiser et al., 2012)	Ag and CeO_2	35 nm (Ag) and <25 nm (CeO–)	3.125 and 31.25 μg/cm^2; and 24 h	Caco-2	LDH	Uptake but no toxicity
(Alkilany et al., 2009)	Au nanorod (coated)	AR 4.1	0.4 nM; 4 da	HT29	MTT, cell count	Cytotoxicity observed due to surfactant coating; not Au nanorods
(Pelka et al., 2009)	Pt	< 20, < 100, > 100 nm	0.0001–1,000 ng/cm^2; 3 and 24 h	HT29	ROS, GSH, TBET, Nrf-2 translocation, comet assay	↓ GSH level; impaired DNA integrity; no ROS; no viability effect; no Nrf-2 translocation
(Rodriguez-Luccioni et al., 2011)	FeO (magnetic)	72 nm	0.3–1.5 mg/mL; 24 and 48 h	Caco-2	CellTiter-Blue™, ApoPercentage assay	Cytotoxicity and apoptosis with exposure + magnetic field-induced hyperthermia
#(Zhang et al., 2010b)	Hematite (αFe_2O_3)	26, 53, 76 and 98 nm	100, 200 and 300 mg/L; 5, 15, 25 and 45 min	Caco-2	SEM, TEER, immunocytochemistry	↓ TEER; dynamic reorganisation and detachment of microvilli, cell junctions disrupted
(Hildebrand et al., 2010)	Magnetite (Fe_3O_4) and palladium/magnetite	255 nm and 307 nm	5, 10 and 25 μg/mL; 1 h–3 da	Caco-2	AlamarBlue/CFDA-AM/Neutral red, ROS	Minor viability effects; minor membrane and lysosomal integrity ↓; no ↑ ROS
#(Piret et al., 2012)	CuO	12 and 50–80 nm	5–100 μg/ml; 24 and 120 h	Caco-2	TEER, MTS, qPCR, TaqMan PCR array, IL-8	Monolayer integrity disrupted; cytotoxicity; ↑ pro-inflammatory cytokine/chemokine gene expression; Cu++ toxicity implicated
#(Koeneman et al., 2010)	TiO_2	< 40 nm	1–1,000 μg/mL; 1–24 h	Caco-2	TEER, TEM and confocal imaging	No effect on monolayer integrity; tight junctions; cell death; non-lethal effects on microvilli and intracellular Ca++

Note: # studies in which fully differentiated intestinal cell monolayers were used.

Table 4 *In vitro* toxicity studies of intestinal NP exposures (continued)

Author	*NP*	*Size*	*Dose*	*Model*	*Endpoint(s)*	*Effect/conclusion*
(Gehrke et al., 2012)	SiO_2	12, 40 and 200 nm	0.3–156.3 µg/cm^2; 24, 48, 72 h	HT29	SRB & WST-1 assays, LDH, ROS, GSH, Comet assay, qPCR	Cytotoxicity depends on concentration, size, and FCS content; ↓GSH; no ↑ROS; interference with MAPK/ERK1/2 and Nrf2/ARE signalling
(De Berardis et al., 2010)	ZnO	50–70 nm	10, 20 and 40 µg/cm^2; 24 h	LoVo	WST-1, apoptosis, ROS, GSH, TBET, SEM, cytokine ELISA	↓ viability; ↑ ROS; ↓ GSH; IL-8 release; may be due to ionic Zn
(Moos et al., 2010)	nZnO and mZnO	20–60 nm and 100–1,000 nm	1–100 µg/mL; 24 h	RKO	Cell Counting Kit-8, propidium iodide exclusion, Vybrant apoptosis, MitoProbe JC-1, MitoSOX	Cytotoxicity involving apoptotic pathways, dependent on NP contact with cell; disruption of mitochondrial potential; ↑ ROS
(Moos et al., 2011)	SiO_2, TiO_2, ZnO, Fe_2O_3	10 nm, 5 nm, 8–10 nm, 3 nm	1–100 µg/cm^2; 4 and 24 h	RKO, Caco-2	Cell Counting Kit-8, functional genomics via microarray	ZnO maximally toxic; TiO2 minimally toxic; changes in metal metabolism; chaperonin; and protein folding gene expression
(Gerloff et al., 2009)	TiO_2, SiO_2, MgO, ZnO	8–80 nm	20–80 µg/cm^2; 4 and 24 h	Caco-2	Comet assay, LDH, WST-1, GSH depletion	Cytotoxicity & DNA damage (ZnO)
(Rhoads et al., 2010)	VO nanotubes	15–100 nm × 500 nm	0.1–0.5 mg/mL; 4–24 h	Caco-2	Neutral red assay	Nanotubes caused significant loss in viability
(Wang et al., 2008)	CdSe QD	1.4–2.5 nm	2–200 pM; 24 h	Caco-2	MTT, cell attachment assay	Cytotoxicity at 200 pM due to Cd++
#(Koeneman et al., 2009)	CdTe QD	15 nm and 500 nm aggregates	0.01µg–1 mg/L; 24 h	Caco-2	TEER	Disruption of monolayer and cell death at 0.1 mg/L; caused by the nano-sized QDs
#(Loretz and Bernkop-Schnurch, 2007)	chitosan/pDNA	18.4–197.0 nm (hydro. diam)	5 and 10 µg pDNA; 5–120 min	Caco-2	TEER, LDH, MTT	Toxicity related to surface charge – cationic nanoparticles caused severe cytotoxic effects
(Kitchens et al., 2007)	PAMAM dendrimers	G2NH2, G4NH2, G1.5COOH, G3.5COOH	0–1 mM, 2 h	Caco-2	visualisation with TEM	Cationic surface groups on G4NH2 dendrimers disrupt membrane
(Bhattacharjee et al., 2012)	PEG tri-block copolymer	45 nm	0.1–400 µg/mL; 24 h	Caco-2	MTT, ROS	(+) PNPs more cytotoxic and induced ↑ ROS than neutral and (-); surface charge- specific interaction of clathrin with (+) PNPs and caveolin receptors with (-) PNPs

Note: # studies in which fully differentiated intestinal cell monolayers were used.

Table 4 *In vitro* toxicity studies of intestinal NP exposures (continued)

Author	*NP*	*Size*	*Dose*	*Model*	*Endpoint(s)*	*Effect/conclusion*
#(Thubagere and Reinhard, 2010)	NH and COOH funct. polystyrene (FluoSpheres)	20 and 40 nm	0.3–6.6 nM; 4, 8, 12, and 16 h	Caco-2	Live/Dead® cell assay, Vybrant apoptosis assay	Uptake efficiency and cytotoxicity higher for (-) charged and smaller PS; oxidative stress-mediated apoptosis of bystander cells
(Fröhlich et al., 2012)	Carboxyl polystyrene NP	20–1,000 nm	50–500 μg/mL; 4 and 24 h	Caco-2	Neutral Red uptake, ATP content, MTS, WST-1, MTT, SRB, leucine uptake	Small size, greater toxicity; IC50 320 μg/mL
(Ruizendaal et al., 2009)	Si NP ((+), (-) and neutral charge)	1.6 nm	0–2.2 mg/L; 24 h	Caco-2	MTT, BrdU assay,	(+) charged Si NPs are more cytotoxic than (-) charged Si NPs and require fetal calf serum in media
#(Jos et al., 2009)	SWCNT-COOH	1.4 × 4–5 nm	5–1,000 μg/mL; 24 h	Caco-2	Neutral red uptake, MTS, TBET, LDH,	Toxic effects at concentrations > 100 μg/mL
(Kulamarva et al., 2008)	SWCNT-COOH-plasmid complex	NR	0.5–2.0 μg/mL; 4–48 h	SW480	MTS	↓ cell viability at higher dose
#(Lai et al., 2012)	SWCNT-COOH, MWCNT-COOH, MWCNT-PVP	0.8–1.2 × 0.1–1 μm 20–30 nm × 10–30 μm 20–30 nm × 10–30 μm	500 pg/mL and 10 μg/mL; 48 h	Caco-2/HT29-MTX co-culture	XTT, LDH, ROS, proinflammatory cytokines, lucifer yellow flux, proteomics	No cytotoxicity; no irritation; barrier integrity normal; no ROS; significant NP- and dose-specific alterations in global protein expression and functional pathways
(Pelka et al., 2011)	SWCNT, CB	1.8 × 500 nm, 14 nm (CB)	0.05 ng/mL–0.2 μg/mL (SWCNT); 0.5 mg/mL (CB); 24, 48 and 72 h	HT29	Comet assay, sulforhodamine B assay, WST-1, LDH, ROS, GSH, Nrf-2 translocation, micronuclei count	↓ growth at 48 and 72 h; ↓ mitochondrial activity at 24 h; DNA damage; ↑ micronuclei; membrane integrity unaltered
(Chiaretti et al., 2008)	MWCNT	110–170 nm × 5–9 μm	1–100 μg/mL; 24 and 72 h	Caco-2	Cell count	No toxicity
(Ponti et al., 2010)	MWCNT	10 nm × 0.1–10 μm	1, 10 and 100 μg/mL; 72 h	Caco-2	Colony forming efficiency (CFE) assay	No cytotoxicity based on CFE
(Zhang et al., 2008)	MWCNT and phosphoryl choline	10–30 nm × 5-15 μm	40, 200 and 1,000 μg/mL; 48 h	Caco-2	MTT, WST-1	No toxicity

Note: # studies in which fully differentiated intestinal cell monolayers were used.

To overcome these limitations and improve physiological relevance with respect to the gastrointestinal system, multicell cultures that incorporate mucosa-resident cells in addition to barrier epithelia, e.g., mucus secreting goblet cells (Walter et al., 1996; Mahler et al., 2009), M cells (Bouwmeester et al., 2011), and even immunocompetent macrophages and dendritic cells (Leonard et al,. 2010) are available. These developments significantly increase the utility of the *in vitro* intestinal model (Liebsch et al., 2011).

Thirty-one studies whose primary aim was to assess the potential toxicity of many different types of NPs in intestinal cell culture models were identified in the literature and are summarised in Table 4. A limited number of *in vitro* toxicology studies of NP exposure in GI models have been identified that utilise the above approaches and other methods. Additionally, other potentially relevant studies focused on intestinal absorption and transport functions but also monitored cell viability, paracellular permeability, and/or monolayer integrity as evidence of cytotoxicity.

6.2 Metals and metal oxide NPs

With respect to nanometals, *in vitro* Ag NP exposures have been studied using concentration-based dose metrics no greater than 50 μg/mL and durations no greater than 24 h. Metal ion toxicity associated with metal NP exposure was observed for spherical Ag NPs (Bouwmeester et al., 2011) where 6% to 17% of the silver NPs were found to be dissociated into silver ions. Lamb et al. (2010) used luciferase reporter-engineered Caco-2 (MDR1.C) cells and found Ag NP to exert cytoxicity with an LD_{50} of 5 μg/mL, though Ag ion dissolution was not determined. By contrast, Gaiser et al. (2012) observed no Ag NP-related cytotoxicity in Caco-2 monolayers and Ag ion dissolution < 1%. This supports speculation from *in vivo* studies that toxicity increases with increasing dissolution.

Alkilany et al. (2009) found that Au nanorods capped with the surfactant cetyltrimethylammonium bromide (CTAB) were cytotoxic to HT29 cells. However, this effect was found to be due to free CTAB, rather than the NPs, in a manner similar to the contribution of metal catalyst contaminants in unrefined carbon nanotube toxicity. When micron-sized vanadium oxide (VO) powder and ethylene diamine intercalated vanadium oxide were compared to VO nanotubes, only the nanotubes caused a significant loss in Caco-2 cell viability.

In contrast to AgNPs or other particles where toxicity corresponded with increased ion dissolution (De Berardis et al., 2010; Piret et al., 2012), Moos et al. (2010, 2011) found that ZnO toxicity related to particle contact with the cell surface independent of soluble Zn. In other metal oxide studies, significant cytotoxicity was observed, but the contribution of free metal ions was not considered (Gerloff et al., 2009; Koeneman et al., 2010; Rhoads et al., 2010; Rodriguez-Luccioni et al., 2011).

In many of the studies mentioned above, metal NPs were found to be internalised readily by the intestinal cells. Intracellular dissolution of metal and metal oxide NPs, after internalisation, with resulting intracellular release of ions also may account for observed cytotoxic effects through a so-called ‘Trojan horse effect’ (Navarro et al., 2008; Johnston et al., 2010; Park et al., 2010b; Frohlich and Roblegg, 2012). Whereas metal ion quantitation in culture media is readily feasible and frequently determined, cytosolic levels may be more difficult to assess and largely have been ignored.

Two studies have investigated the effect of CdSe QDs on Caco-2 monolayers. In the first of these (Wang et al., 2008), acid treatment simulating QD exposure to gastric juice increased the toxicity of PEG coated QDs, by removing the coating and enabling dissolution. In the other study (Koeneman et al., 2009), QDs coated with hydrophilic thioglycolate capping ligands caused Caco-2 monolayer disruption and cell death at 0.1 μg/mL. The authors concluded that cytotoxicity was caused by the 15 nm QDs rather than the Cd++ or sodium thioglycolate. Large aggregated QDs (500 nm) had no adverse effects.

Zha et al. (2008) compared the absorption efficiency of chromium NPs, chromium picolinate, and chromium chloride in Caco-2 cells. Monolayer integrity was monitored by trans-epithelial electrical resistance (TEER) measurements and transcellular mannitol flux. Measurable decreases in TEER reflect reversible opening of tight junctions in intestinal epithelia and have been used to indicate impaired cell barrier. Using a concentration range of 0.2–20 μmol/L, the authors found that while the transport of chromium NP, picolinate, and chloride across the Caco-2 cell monolayers occurred mainly via passive transport, chromium NPs exhibited significantly higher absorption efficiency. None of the materials altered monolayer integrity.

6.3 Carbon-based NPs

As listed in Table 4, several studies investigated the effects of various carbon-based nanomaterials, including carbon black (CB), single-walled carbon nanotubes (SWCNT), and multi-walled carbon nanotubes (MWCNT). The nanotube exposures also included those with or without functionalisation, all with very different dimensions, and some using dispersants. The carbon nanomaterials were used to treat either Caco-2, SW480, and HT29 cells or Caco-2/HT29-MTX cells in co-culture. In general and in contrast to the nanometals, oxides, and QDs mentioned above, carbon nanomaterial exposure had little adverse effect in Caco-2 cells or co-culture. Using an HT29 cell monolayer, Pelka et al. (2011) dispersed SWCNTs with sodium cholate (0.02%) resulting in finely dispersed exposures. Significant cytotoxic effects were noted at low-level exposures (pg and ng/mL), although in many assays, cells were exposed after only a day or two after plating. Typically, these cells (as well as other intestinal epithelial cells such as Caco-2) require at least 14–21 d to fully polarise and form tight junctions (Chantret et al., 1988; Cohen et al., 1999), essential for TEER measurement.

At 48 h, HT29 cells form a multilayer of non-polarised cells that display an undifferentiated phenotype and are thus not representative of an intestinal epithelial barrier. It may be for this reason that they were susceptible to the effect of SWCNTs. A similar approach was taken in other studies using Caco-2 cells (Chiaretti et al., 2008; Kulamarva et al., 2008; Zhang et al., 2008; Ponti et al., 2010). Only two studies investigated fully differentiated and polarised, post confluent Caco-2 cells (Jos et al., 2009) and Caco-2/HT29-MTX co-cultures (Lai et al., 2012) where no toxic effects were observed below 100 μg/mL exposures. Studies of other NPs where fully differentiated cell monolayers were used have been indicated as such in Table 4.

Coyuco et al. (2011) explored the potential of functionalised carbon nanomaterials for potential oral drug delivery in Caco-2 cell monolayers, using polyhydroxy small-gap fullerenes (OH-fullerenes), carboxylic acid functionalised single-walled carbon nanotubes (SWCNT-COOH) and poly(ethylene glycol) functionalised single-walled carbon nanotubes (SWCNT-PEG) in a concentration range of 15.6–1,000 μg/mL and

24 h of exposure. Of the three carbon NPs studied, SWCNT-COOH was associated with greatest inhibition of efflux pump activity through inhibition of the P-glycoprotein (P-gp) efflux system, resulting in increased cellular accumulation of the pump substrate, rhodamine-123. SWCNT-COOH also caused the greatest modulation of the tight junctions through perturbation of zonulin-1 distribution, a tight junction marker protein,as indicated by fluorescence imaging, significantly reduced TEER and enhanced Lucifer Yellow flux. These findings were viewed as evidence that SWCNT-COOH NPs could be useful modulators for oral drug delivery by enhancing paracellular permeability via disruption of tight junctions. However, these alterations could also be viewed as significant breaches in monolayer integrity – a potential adverse effect. However traditional cytotoxic endpoint measurements, LDH leakage and MTT assay, showed no cellular effects at any dose.

6.4 Polymeric NPs

Most *in vitro* studies of polymeric NPs have focused on NP-assisted absorption and systemic drug delivery of orally administered therapeutics. The majority of these studies have used the Caco-2 cell monolayer model. For example, polyamidoamine (PAMAM) 'dense star' dendrimers are a class of hydrophilic polymers with both anionic and cationic surface charge, nanometer diameters, and potential as drug carriers for transepithelial transport and delivery of therapeutics. El-Sayed et al. (2002) investigated the effect of 3.5 h exposures to neutral surface charge PAMAM-OH or anionic PAMAM-COOH at concentrations of 0.1, 1.0, or 10 mM on junctional integrity as measured by TEER, paracellular permeability (mannitol permeance), and viability (membrane integrity via LDH leakage) in Caco-2 cell monolayers. TEER decreased in a dendrimer concentration- and diameter-dependent manner for only large diameter, anionic dendrimers. This apparent decline in monolayer integrity was also reflected by increased mannitol permeability and cytotoxicity as indicated by loss of cell membrane integrity and LDH leakage.

Surface modifications were evaluated in a similar study (Jevprasesphant et al., 2003). Here, PAMAM dendrimers and surface-modified cationic PAMAM dendrimers were compared with respect to cytotoxicity, permeation, and transport mechanisms in Caco-2 monolayers. After 3 h of exposure to concentrations ranging from 0 to 1 mM, permeation of dendrimers and cytotoxicity (measured by the metabolic activity assay MTT) increased with both concentration and generation (diameter). They found that the cytotoxicity of cationic dendrimers was greater than that of anionic dendrimers and was significantly reduced by conjugation with lauroyl chloride. In terms of Caco-2 barrier integrity, cationic dendrimers decreased TEER and significantly increased the paracellular permeability of mannitol. Modified dendrimers also reduced TEER and caused a greater increase in mannitol permeation. The authors concluded that lipid-modification of PAMAM dendrimers may improve the safety and efficacy as drug delivery systems. Cytotoxicity attributable to surface modifications were also detected in a study of ornithine and arginine conjugated PAMAM (200 ug/ml) in a porcine intestinal primary cell line (IPEC-J2) (Pisal et al., 2008). Here, surface modified PAMAM were more permeant, decreased TEER, and were slightly more toxic in terms of percent cell viability than unmodified PAMAM.

Other therapeutic strategies under investigation include mucoadhesive polysaccharide chitosan-based micro/nanoparticulate drug delivery systems. Chitosan ([1-4]2-amino-2-deoxy-β-D-glucan) is an abundant natural polycationic polymer, an N-deacetylated product of chitin. Chitosan derivatives stabilise and improve delivery of therapeutic peptides in the GIT. Several studies have investigated various chitosan NP formulations in Caco-2 cells with similar conclusions (Thanou et al., 2000; Silva et al., 2006; Korjamo et al., 2008; Kowapradit et al., 2008; Kudsiova and Lawrence, 2008; Martien et al., 2008; Sadeghi et al., 2008; Jia et al., 2009; Sonaje et al., 2009; Kowapradit et al., 2010; Saremi et al., 2011; Zheng et al., 2011). Using various cell viability measures, all of these studies documented chitosan particle-mediated decreases in TEER, increases in tight junction permeability, and enhanced absorption without evident adverse effects on the intestinal monolayers. Chitosan nanocapsules associate with the apical side of both Caco-2 and HT29 model cell cultures, with preference for the mucus-secreting HT29 cells (Prego et al., 2006). The physiological implications of this phenomenon are unclear. When trimethyl chitosan chloride (TMC) and TMC-goblet cell targeting peptide (0.125 to 2 mg/ml) were studied independently in Caco-2 and HT29-MTX cells (Jin et al., 2012), both exhibited dose-dependent cytotoxicity (via MTT assay). The results also indicated that modification of TMC with the targeting-peptide was associated with lower zeta potential and lower cytotoxicity suggesting that the conjugated form was less cationic. The studies cited above form a general consensus that variably modified chitosan has the potential to be used as an intestinal absorption enhancer of therapeutic macromolecules with toxicity mediated by positively charged surfaces.

In many of the studies mentioned above, enhanced absorption of therapeutics was the goal. To this end, alterations such as decreased tight junctional integrity, decreased TEER, and increased paracellular flux can be viewed as favorable. However, such alterations of intestinal membrane permeability also carry the potential for adverse effects, including enhanced absorption of pathogenic organisms, drugs, or harmful compounds (e.g., endotoxin) from the GIT lumen. This bears further investigation in models (both *in vivo* and *in vitro*) utilising a pathogen, drug, or endotoxin challenge.

7 Discussion: relevant factors for NP ingestion studies

It is clear that the GIT is a highly complex environment. Accurate understanding of the fate of ingested NPs requires consideration of multiple factors. A number of features have been identified that are important to interpretation of NP ingestion studies. These include physicochemical characterisation of NPs and reporting of metadata from *in vivo* studies. Additionally, a feature somewhat unique to the ingestion route is the potential for toxic effects related to interactions with the gut microbiome. Finally, although doses higher than 'typical' exposures are a standard and necessary part of toxicity studies for establishing dose range parameters, these doses should be logically based and critically compared to likely exposure levels,.

7.1 Physicochemical parameters and metadata

For physicochemical characterisation, a readily available guideline exists in the MINChar Initiative (2008). In addition to basic criteria of material, size and size range, shape, charge, coatings, and surface functionalisation, this should include baseline

characterisation of the degree of aggregation or agglomeration and percent of available ion in the material as dosed. This permits correlation of NP properties to biological effects. For example, for metal NPs, increasing percent dissolution, smaller size, and higher dose appeared to facilitate absorption. For CNTs, higher dose decreased absorption, possibly by facilitating aggregation (Philbrook et al., 2011). For CNTs, larger size (longer axis ratio) also decreased absorption (Philbrook et al., 2011). In contrast, for dendrimers, greater diameter (later generation) and negative surface charge correlated with greater absorption (Wiwattanapatapee et al., 2000). It has been beneficial to include a non-NP control within NP toxicity studies. For example, the description of CuNP toxic effects in a rodent model was consistent with hemoglobinuric nephrosis. This is the same toxic effect as elemental Cu toxicity, but occurred as a lower dose with NP dosing, perhaps due to higher percent dissolution (Hillyer and Albrecht, 2001; Chen et al., 2006). Of note, in the metal studies reviewed here, there was no evidence of toxicities unique to the nanoscale version of the material. Thus, the toxicity contribution of nanoscale metals might actually be enhanced (or diminished) absorption rather than unique pathology.

With respect to absorption, a knowledge gap still exists in defining whether NP are absorbed as particulate material or predominantly in ionic form. Newer visualisation and quantification techniques such as sedimentation field flow fractionation analysis (Deering et al., 2008) or particle-inducd x-ray emission in combination with inductively coupled mass spectrometry (Novak et al., 2012) may be helpful in this arena. The importance of a non-NP control is critical in such studies, however, as demonstrated by the finding in an *ex vivo* digestion model that dosing with ionic silver, in the form of $AgNO_3$, resulted in NP formation following exposure to low pH and chloride ions (Walczak et al., 2012). Thus, the observation of NP retained within tissue does not necessarily mean that the particles were absorbed in NP form.

Important requirements for metadata with respect to *in vivo* studies include specification of animal species, age, sex, strain, housing, and husbandry practices. For ingestion studies it is particularly important to report whether animals were dosed in the fasting vs. fed state and what time of day dosing took place. Although no specific guidelines for NP ingestion studies exist, the ARRIVE or Metabolomics metadata standards are a good starting point (Griffin et al., 2007; Kilkenny et al., 2010a, 2010b; Griffin
et al., 2011).

7.2 NPs and the gut microbiome

A feature that bears additional scrutiny is the potential interaction of ingested NP with the gut microbiome. It is uncertain from the existing literature whether these interactions occur and whether they are detrimental, positive, or inconsequential. For ingested NPs, even particles that are not absorbed may have toxic effects if they induce alterations of the normal microbiome. Additionally, there is the possibility that a pre-existing altered microbial state, such as gram negative bacterial overgrowth, can affect NP absorption, perhaps by adherence of NP to LPS. This may result in enhanced delivery of either LPS or the NP themselves. Finally, lumenal NP may affect gut microbial metabolism, potentially influencing nutrient absorption or xenobiotic metabolism (Bu et al., 2010; Cattani et al., 2010). Targeted studies using single and co-administration of NP in combination with either a bacterial toxin (e.g., LPS) or a xenobiotic would be informative in this area. The limited studies that have been done concerning microbes and NP

ingestion predominantly relate to agricultural animals and use indirect endpoints of gut microbial alteration (e.g., growth rate or feed conversion) (Ahmadi, 2009; Ahmadi et al., 2009; Fondevila et al., 2009; Ahmadi and Kurdestany, 2010; Ahmadi and Kordestany, 2011; Ahmadi and Rahimi, 2011). It would be informative to assess the effects of administered NP on gut microbes with a DNA-based technique like pyrosequencing that eliminates culture-bias and allows more sensitive detection of rare members of the microbiome (Zoetendal et al., 2004; Young and Schmidt, 2008; Hadrup et al., 2012; Young, 2012).

7.3 Relationship of observed toxicities to likely health risk

A major barrier to assessing relevance of experimental NP toxicity and 'real world' human health risk is the difficulty in relating experimental exposures to likely realistic human exposures. For many materials, accurate information about environmental exposures is lacking. It is also difficult to extrapolate findings from the higher, shorter-term doses typical of *in vivo* toxicity studies with the likely outcome of chronic, minimal dose exposures.

Overall, for the *in vivo* studies reviewed here, ingested NP appeared to have low toxicity. For AgNP, no adverse effects were reported at doses lower than 125 mg/kg (Kim et al., 2010a). For TiO_2NPs, up to 5,000 mg/kg were tolerated with no adverse effects (Warheit et al., 2007) and no effects were noted at up to 1,000 mg/kg for oral administration of SWCNTs (Kolosnjaj-Tabi et al., 2010). Gottschalk et al. (2006) predicted environmental concentrations for AgNP, CNTs, and TiO_2NPs in surface water in Switzerland, considering current manufacturing and production volumes and likely environmental fates (Gottschalk et al., 2006). They predicted concentrations of 30–80 ng/L for AgNP, 0.5–0.8 mg/L for CNTs, and 0.7–16 ug/L for TiO_2. Considering a daily water intake of 3 L of water per day (0.04 L/kg/d for a 70 kg individual), this would correlate to a maximal intake of 1×10^{-6} mg/kg/d for AgNP, 0.024 mg/kg/d for CNTs, and 6×10^{-4} mg/kg/d for TiO_2. Clearly environmental levels will vary and there are likely to be other sources of exposure or even intentional ingestion (e.g., AgNP). Nevertheless, considering that daily doses in the range of 10^2 to 10^3 mg/kg range were well tolerated for these materials in the experimental studies, these substances are unlikely to be highly toxic at current levels of environmental exposure. Factors such as the apparent higher absorption of low dose, shorter aspect ratio CNTs (Philbrook et al., 2011) bear scrutiny with respect to potential accumulation in tissue with low level environmental or occupational exposures, particularly in sensitive periods of life (e.g., pregnancy).

Finally, a striking difference in reported toxicity is evident between the *in vivo* and *in vitro* studies, with the majority of *in vitro* studies reporting effects by at least some parameters (cytotoxicity, altered membrane permeability) while the majority of *in vivo* studies reported no effects except at very high dose levels. Better methods of determining the delivered dose at the cellular or organ level for *in vivo* studies would assist in rational comparison with *in vitro* results [Deering et al., (2008), #245; Novak et al., (2012), #265]. Additionally, improved *in vivo* methods of measuring more subtle functional parameters with respect to metabolism, immune function, oxidative stress, or other physiological aspects would be helpful.

Based on these findings, ingested NPs appear unlikely to have acute or severe toxic effects at typical levels of exposure, however more subtle or chronic effects bear further investigation. This is particularly true with respect to intestinal permeability or oxidative

stress, and host-gut microbial balance, which have not been adequately explored. With increasing recognition of the importance of adequate materials characterisation and adequate metadata, future investigations of these and other areas may be more easily applied to risk assessment and human health.

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