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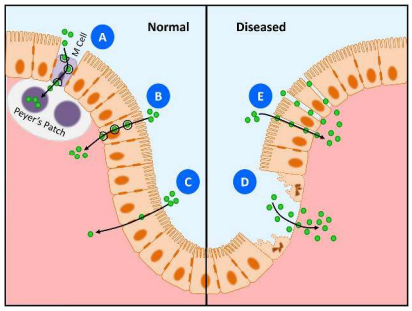

Review of the properties of food-relevant nanoparticles including gastrointestinal tract exposure, transport, and immunogenicity.

Nanoparticles (NP) are being incorporated into consumer products, and the growing list of products containing NP components leads to increasing probability of human exposure and environmental release. However, our understanding of the biological interactions of such materials and their potential impact upon human health lag far behind the technology. Human exposure can occur via three routes, inhalation, dermal exposure and gastrointestinal exposure. This critical review focuses on toxicity induced by food-relevant NP (nanoscale silica (SiO_2), titanium dioxide (TiO_2), zinc oxide (ZnO), and silver (Ag)), in epithelial cells of the gastrointestinal (GI) tract, and pathophysiological responses to oral NP administration *in vivo.* A series of recommendations for modifications of the experimental approaches toward greater consistency, and for future research are suggested.

Critical assessment of toxicological effects of ingested nanoparticles

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ABSTRACT

Nanoparticles (NP) are being increasingly incorporated into foods and food packaging, however, our understanding of the biological interactions of such materials and their potential impact upon human health and disease lag far behind the technology itself. In response to this knowledge gap, many investigations are being directed toward assessment of toxicity induced by food-relevant NP in epithelial cells of the gastrointestinal (GI) tract, and pathophysiological responses to oral NP administration *in vivo*. Studies have focused upon various aspects of toxicity including cell death, inhibition of cell proliferation, oxidative stress, and organ injury and dysfunction, as well as other responses. While data generated by these studies have provided useful information, results have been at times inconsistent and in some cases contradictory. Herein we review the properties of food-relevant NP, including GI tract exposure, transport of NP, and immunogenicity. Then we critically review the major findings of a cross-section of studies of GI-related biological interactions of nanoscale silica (SiO_2), titanium dioxide (TiO_2), zinc oxide (ZnO), and silver (Ag), with particular focus upon the variation in results in the context of administered NP dosage, specific assay methods employed, uncertainties in NP physicochemical properties, and other factors that might influence experimental outcomes. We conclude with a series of recommendations for modification of the experimental approach toward greater consistency, and for addressing several important issues that have thus far been understudied.

1. Introduction

Nanoparticles (NP) are defined as particles with at least one dimension less than 100 nm. Naturally occurring organic NP include proteins and some viruses. Humans have utilized the optical properties of NP dating back to Roman times. Copper and gold NP were used by Romans to color glass.[1] Beginning in the Middle Ages, glazes used to make lusterware contained copper and silver NP for their iridescent properties.[2, 3] Michael Faraday's experiments on the interaction of light with gold NP dispersions are considered the beginning of modern colloid science and thus nanoscience.[4]

There is currently considerable interest in the use of NP for technological applications. NP have a greater surface area per unit mass than the bulk material, which means that there are many more reactive exposed atoms.[5] Thus, nanoparticles, e.g., nanosized gold, are effective catalysts.[6] The quantum confinement effect also contributes to the unique electronic and optical properties of semiconductor NP, such as quantum dots, which fluoresce differently based on size.[6]

NP are being increasingly incorporated into consumer products, however, our understanding of the biological interactions of such materials and their potential impact upon human health and disease lag far behind the technology itself. This lack of knowledge has been noted by the media in recent science news articles.[7, 8] The growing list of products containing NP components increases the probability of human exposure, which can occur via three routes. Inhalation is the primary exposure mechanism for airborne NP such as the nanoscale fraction of particulate air pollution or factory dusts. Inhaled NP can accumulate in the lungs and possibly enter the circulation through inter-alveolar septal capillaries. There is also evidence that inhaled NP can reach the brain by traversing the olfactory bulbs in the nasal cavity.[9] Dermal exposure is the primary route for NP in cosmetics, sunscreens, and other products applied to or in contact with the skin. It is currently not known whether such NP are able to penetrate the stratum corneum and reach the epidermis and underlying tissues in undamaged skin. The third major route, and the focus of this review, is gastrointestinal exposure through NP ingestion. Ingestion may be intentional, as is the case for nanoscale food ingredients, or unintentional/accidental, as in the case of NP in food packaging leaching into package contents or for infants and young children for whom placing objects in their mouths is a major mechanism of exploration of their environment.

1.1 Nanoparticles in food

Nanotechnology is currently of interest to both the food and food packaging industries. NP applications are being considered to enhance flavor and texture, improve nutrient bioavailability, control flavor, color, or nutrient release in consumer-activated products, and reduce the fat needed to create the same taste, texture, and consistency.[10] In food packaging, nanomaterials are of interest to improve the stability, flexibility, and gas barrier properties of packaging, to actively utilize antimicrobial or oxygen scavenging NP to keep food fresh longer, to add nanosensors which can detect and respond to the freshness of the package contents (i.e., color change), and to make packaging biodegradable. Although there is great interest by the food industry in utilizing nanotechnology, many of the aforementioned products are not yet on the market. The Project on Emerging Nanotechnologies has attempted to inventory consumer products using NP.[11] This inventory currently identifies 117 food and beverage products incorporating nanotechnology including food, food storage products, dietary supplements, and products used for cooking.[11]

A 2012 study which analyzed 89 consumer food products found titanium in many of these products, especially sweet foods such as candies, gums, icing, and powdered sugars.[12] They also showed that although food-grade TiO_2 particles, E171, had an average size of 110 nm, the size distribution was broad and 36% of these particles were less than 100 nm and thus classified as NP. Figure 1c shows an electron micrograph of the E171 titania particles, and Figure 1d shows the particles extracted from chewing gum. They estimated a daily exposure to TiO_2 in the US to be 0.2-0.7 mg TiO_2/kg body weight/day for adults and 1-2 mg TiO_2/kg body weight/day for children under the age of 10 due to greater consumption of sweets. A study that isolated TiO_2 from chewing gum found that >93% of TiO_2 particles in the gum were less than 200 nm and 18-44% were less than 100 nm. It was also shown that approximately 95% of the TiO_2 in the gum is swallowed when it is chewed, so consumers are being exposed to much of this TiO_2.[13] Food-grade silica, E551, is used as an anti-caking agent and is present in many powdered food mixes,[14] with 4-43% of the silica being less than 200 nm. Figure 1a shows the electron micrograph of the E551 particles, and Figure 1b shows the particles after extraction from powdered sugar. This study estimated exposure to nanosilica to be 1.8 mg/kg body weight/day for the average adult.[14] Silver NP have applications as antimicrobial agents and are of particular interest to the food packaging industry. Figure 2b shows an electron micrograph of typical Ag NP. Ag has been incorporated into food storage containers and used as antimicrobial coatings on utensils, cookware, and appliances,[11] and there is the potential for Ag NP in food contact materials to migrate into food. Other NP of interest include zinc oxide (ZnO) and magnesium oxide (MgO) for their antimicrobial properties, and nanoclay in films to limit gas permeation.[10] Figure 2a shows an electron micrograph of commercial ZnO NP, and appears in different size and shapes.

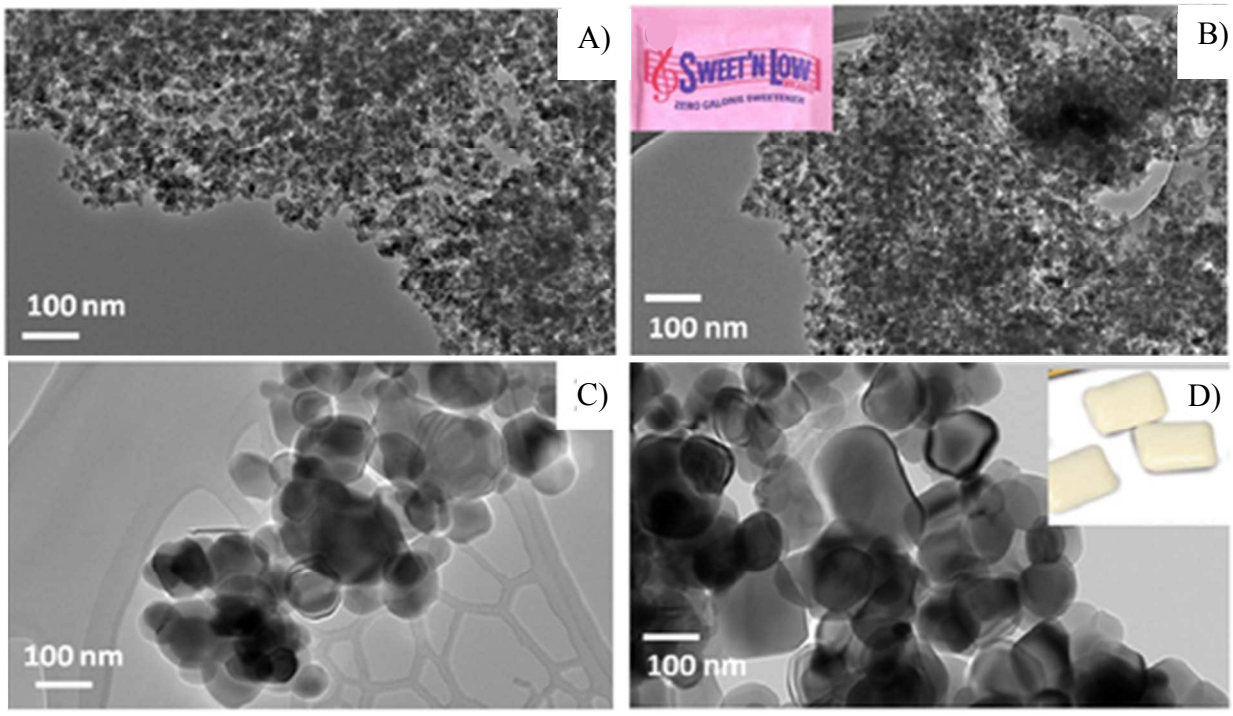

Figure 1: Transmission electron micrographs of A) E551, commercial food-grade silica, B) silica isolated from Sweet'N Low® (inset), C) E171, commercial food-grade TiO_2, and D) TiO_2 isolated from Wrigley's Extra® gum (inset).[15] Reprinted with permission from John Wiley and Sons.

An analysis of 86 wheat breads and 49 wheat biscuits from 14 different countries (the majority were from Italy) by Environmental Scanning Electron Microscopy revealed that 40% of the samples contained microparticulate and nanoparticulate contaminants, involving iron, lead, tungsten, titanium, aluminum, silicon, and silver.[16] This study suggests that consumers may be exposed to NP arising as environmental contaminants in foods even when they are not being intentionally added to food products.

Considerable effort and resources have been invested in studies of the biological interactions and potential hazards of ingested NP. While many of these studies have been very informative, reported results (summarized in this review) have been somewhat inconsistent and, in some cases, contradictory, likely at least partially as a consequence of the variety of methodologies employed in the investigations. In addition, the relevance of results to actual human post-ingestion NP exposure has, in many studies, been limited by the immensity of the NP doses administered in *in vitro* and *in vivo* experiments as compared to estimates of actual human exposure.

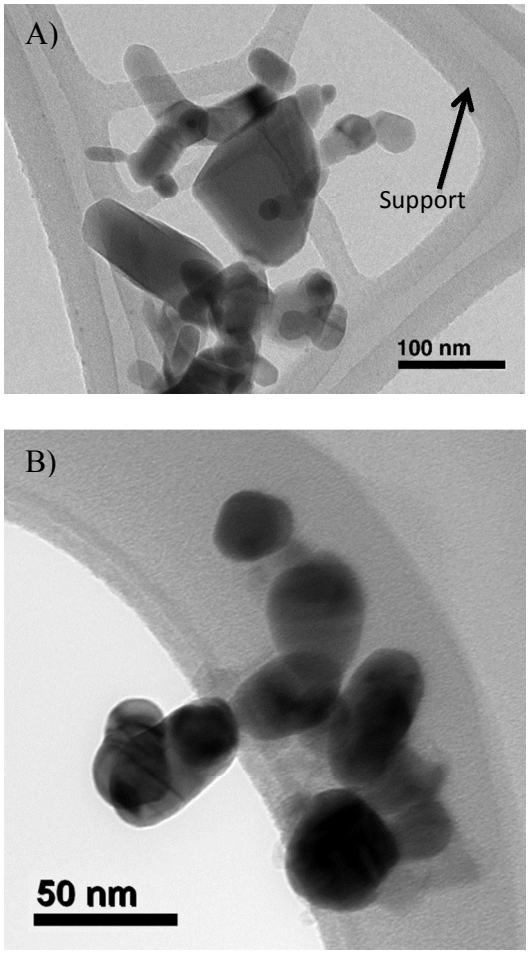

Figure 2: Transmission electron microscopy micrographs of A) ZnO NP[17] (reprinted with permission; Copyright 2013 American Chemical Society) and B) Ag NP[18] (reprinted with permission from Elsevier).

1.2 NP characteristics relevant to toxicity

In health and safety studies with NP to date, it has been recognized that characteristics including size, shape, material, surface charge, solubility, and surface chemistry are all important in determining the toxicity of NP, as shown in Figure 3. The NanoRelease Food Additive project recently published an article outlining some of the NP properties important in the GI tract including particle size, shape, and surface properties.[19] Not only are characteristic NP properties important to consider for their biological effects, but they may also be modified by the conditions of the GI tract.[19] Thus, it is important to not only investigate the role of NP characteristics *in vitro*, but also to confirm the importance of these characteristics using *in vivo* studies where the NP will be interacting with the entire GI tract and the body as a whole.

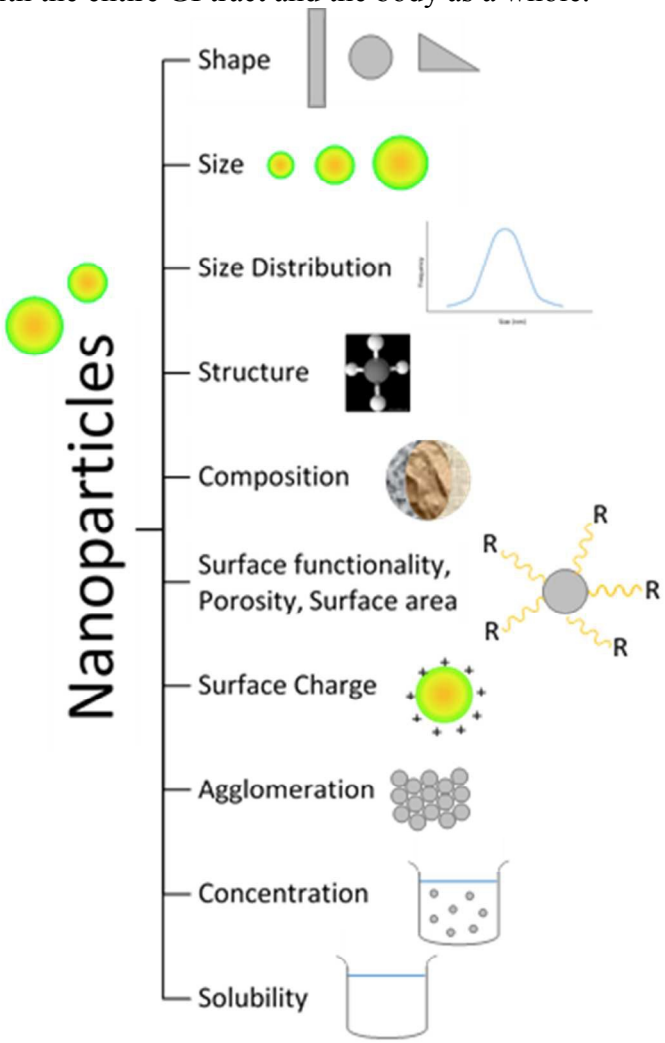

Figure 3: The nanoparticle characteristics outlined here are some of the properties that determine nanoparticle behavior and toxicity.

1.2.1 Solubility

In vivo, the solubility of NP determines their biopersistence. Soluble or degradable NP may not remain long enough to accumulate during chronic exposure. The solubility of amorphous silica in water is approximately 115 ppm at 25°C,[20] which is greater than other forms of silica including quartz.[21] It has been suggested that solubility of silica will play a large role in

determining its toxicity due to its limited persistence.[21] *In vitro*, NP may dissolve in cell culture media. Soluble NP are also likely to begin dissolving in the storage solution. One study showed that ~14% of citrate-stabilized Ag NP in water will dissolve at 25°C after 3000 hours while 70% dissolves at 37°C.[22] Thus, it is important to be aware of the possible dissolved species present before *in vitro* or *in vivo* NP exposure. *In vivo*, NP will quickly come into contact with digestive enzymes and fluids. The low pH environment of the stomach may accelerate dissolution of NP. One study with various Ag NP showed that exposure to simulated stomach fluid for 15 minutes caused aggregation and partial dissolution of Ag NP along with precipitation of AgCl on the surface of the particles.[23] Ag NP toxicity is mediated through Ag ions and similar toxicity has been observed using Ag NP and Ag ions at comparable doses (based on dissolved Ag).[24] Dissolved ions, particularly Ag ions, may form complexes with other species within the culture media or in cells, but are still largely available to interact with cells.

Studies have shown that intracellular as well as extracellular ZnO NP dissolution can mediate toxicity and the role of these pathways may vary by cell type.[25] Thus, dissolution occurring both within and outside of cells may be important to provide toxic ions.

NP are internalized by endocytosis and transported through the endocytic pathway to lysosomes. In lysosomes, the pH is lowered to degrade cellular debris as well as extracellular materials and this acidic environment may increase the rate of NP dissolution within cells. It has been shown that silica NP solubility in artificial lysosomal fluid (at pH 5.5) was 5.0%, ZnO NP solubility was 100%, and TiO_2 and Ag NP solubility were both 0%.[26] The lack of Ag NP solubility in lysosomal fluid may be due to formation of precipitates that remain associated with the Ag NP surface,[23] which is the reason free Ag ions were not detected in artificial lysosomal fluid.[27, 28] Studies in alveolar macrophages have shown that silica NP can lead to disruption of lysosomal membranes. This is likely a result of the inability of silica NP to be degraded in the lysosomes rather than a consequence of lysosomal NP dissolution. This increased lysosomal permeability may be involved in the induction of apoptosis.[29, 30] A study in THP-1 macrophages showed that treatment with 10.7 nm ZnO led to lysosomal destabilization and eventually cell death.[31] This was proposed to be an effect of dissolution of ZnO within the lysosomes, creating Zn^{2+} which damages the lysosomal membrane and is released into the cell where these ions can damage organelles and potentially lead to cell death.[31]

It is known that Ag NP dissolution intracellularly is important and may provide a continual source of Ag ions,[32] and there is evidence that Ag NP dissolution also occurs within lysosomes. Ag NP have been localized to lysosomes after cell internalization in spermatogonial stem cells[33] and BEAS-2B lung epithelial cells.[28] In earthworms, citrate-coated Ag NP were shown to decrease lysosomal stability.[34] One study showed that lysosomal degradation of GSH-Ag NP released Ag ions when incubated in a lyososmal-mimic mixture for 12 hours and lysosomal degradation of Ag NP was necessary in human fibroblasts to induce oxidative stress.[35] Collectively, it is clear that NP solubility both extracellularly and intracellularly will play a role in the toxicity of the NP. Thus, toxicity of soluble NP may depend on whether the ions produced upon dissolution are toxic.

1.2.2 Size

Size will determine the movement of NP through the mucus layer and the proportion of NP internalized by intestinal epithelial cells. Optimal particle diameters for the most efficient endocytosis of NP have been estimated to be 54-60 nm for spherical particles,[36] 27.4-30.6 nm for cylindrical particles,[36] and, calculated using a different model, 50-60 nm for spherical particles.[37] NP size-dependent uptake efficiency was confirmed by an *in vitro* study in human cervical

cancer cells which found that 50 nm spherical gold particles were internalized more efficiently than 14, 30, 74, or 100 nm particles.[38] However, a study in human colon adenocarcinoma cells found that 100 nm polystyrene particles were taken up better than 50, 200, 500, or 1000 nm particles.[39] This difference may indicate that NP material type or other characteristics such as surface coating can alter the optimal size for cellular uptake.

In vivo, when gold NP of 1.4, 5, 18, 80, and 200 nm were administered by intraesophageal instillation, significantly more 1.4 nm NP entered the circulation and accumulated in blood, kidney, and urine reticulendothelial system after 24 hours, suggesting that smaller particles are distributed most readily. However, the 18 nm particles accumulated the most in the heart and brain,[40] suggesting that accumulation may depend on other factors that regulate exocytosis. In agreement with this conclusion, the study with gold NP showed that although 50 nm particles were internalized most efficiently, the smallest NP were exocytosed more efficiently such that 14 nm particles were exocytosed more than 30, 50, 74, and 100 nm particles.[38] This difference in efficiency of endocytosis and exocytosis of NP will be important for NP accumulation and induction of toxicity.

1.2.3 Shape

Shape can play a role in the efficiency of NP uptake. Internalization efficiency based on NP shape may also be dependent on cell type. A study in HeLa cells showed that transferrin-coated gold spherical NP were found to be internalized more (14 and 50 nm sizes) than 20×30, 14×50, or 7×42 nm transferrin-coated gold nanorods, but the nanorods were exocytosed more efficiently than the spheres.[41] Similarly, 14×40 and 14×74 nm gold nanorods were taken up less efficiently by HeLa cells than 14 and 74 nm gold spherical NP.[38] The cell internalization of nanorods with a lower aspect ratio was higher in both of these studies. This was confirmed in another study showing that shorter rods (with lower aspect ratios) were internalized more by HeLa cells than longer rods.[42] However, once NP were internalized, cytotoxicity was independent of the aspect ratio or length of nanorods.[42] Contrary to these studies, internalization of 15×50 nm cetyltrimethylammonium bromide-coated gold nanorods was found to be more efficient than that of 15 or 50 nm gold spheres in monocytes and macrophages isolated from human blood.[43] It is possible that these differences are due to the professional phagocytic character of monocytes and macrophages, which may internalize NP differently than HeLa cells. Regardless, NP shape may play a role in the bioavailability of NP to cells and the body.

1.2.4 Surface charge

Administration of negative and positive 2.8 nm gold particles by intraesophageal instillation to rats revealed greater entry of the negatively-charged particles into the circulation and a trend of greater accumulation of negatively-charged particles in the examined tissues including the liver and urine.[40] Neutral iron oxide NP (dextran-coated) were poorly internalized by cells. Either negatively-charged (heparin or DMSA-coated) or positively-charged (aminodextran-coated) iron oxide NP were better internalized by cells, but the positively charged particles accumulated more in cells.[44] Another study showed differences in the interaction of positive versus negative quantum dots with cell surface scavenger receptors on murine macrophages in serum-free media and negatively-charged quantum dots were internalized more slowly by cells, suggesting different methods of internalization.[45]

Surface charge of particles may dictate how they interact with other biological fluids such as the mucus lining the intestinal tract. Net neutral or near neutral but highly charged polyelectrolyte NP composed of polyacrylic acid and chitosan (mimicking the densely charged but net neutral surface of many viruses) can migrate better through mucus than similar particles

with different ratios of polyacrylic acid and chitosan with a net positive or negative charge.[46] Negatively-charged particles can bind to mucin strands through hydrophobic adhesive interactions and positively-charged particles can bind strongly to the negatively-charged glycosylated mucins.[47] Thus, charge will be an important factor in determining the fraction of NP able to reach the intestinal epithelial surface.

1.2.5 Surface coatings/corona

It is known that proteins and other biomolecules will adsorb to the surface of NP in biological systems.[48] The protein corona is composed of two layers, a tightly bound hard corona covered by a more transient, loosely-associated soft corona layer.[48] Some studies have observed that NP will induce cell damage only in the absence of a corona. Silica NP were found to be internalized more by HeLa and A549 cells treated with serum-free media to prevent formation of a corona.[49] Silica NP in serum-free media were also shown to have a stronger adhesion to the cell membrane and caused greater cell damage and toxicity in A549 cells. After one-hour exposure of silica NP in serum-free medium to cells, coronas composed of cytosolic, cytoskeletal, and cell membrane proteins had formed on NP, which may be the cause of the cell damage and toxicity observed.[49] Agglomeration of NP in the presence of serum may also play a role in decreased toxicity of these particles to cells, as was observed with silica NP in mouse fibroblast cells.[50] Protein adsorption to NP and the composition of the corona can alter how NP are recognized and internalized by cells[51] as well as NP interactions with intracellular components. Thus, the protein corona and the exact nature of the surface of NP are likely to play an important role in the response to NP. The presence of serum in cell culture media used for *in vitro* studies may be an important factor in the subsequent NP toxicity.

2. *In vitro* models of digestion

The digestive tract creates a barrier that prevents most unwanted food components from penetrating further into the body. The main purpose of the digestive tract is to break food into its more basic components, specifically sugars, lipids, amino acids, vitamins, minerals, which can then be absorbed and used by our bodies. Enzymatic digestion is then continued in the stomach, largely by pepsin, which digests proteins and is active in the acidic environment ranging from pH 1 to pH 5 depending on whether the stomach is empty or full.

After 1-2 hours in the stomach, the resulting chyme is released into the duodenum where it mixes with pancreatic digestive enzymes, bicarbonate, and bile from the liver, which neutralize the acidic chyme.[52] Bile salts form micelles with hydrophobic cores where lipids and solubilized fats can be digested by pancreatic lipase and other enzymes.

Various researchers have developed *in vitro* digestion models and there is variation in what treatment has been considered “digestion”. Despite this variation, digestion is shown to affect nanoparticle dissolution and aggregation and may alter NP toxicity. A study that added silica NP to sample food matrices and subjected these NP to an *in vitro* digestion procedure showed that both the food matrix and the stage of digestion likely play large roles in the bioavailability of silica NP. The digestion consisted of saliva (pH 6.8), gastric juice (pH 1.3), duodenal juice (pH 8.1), and bile juice (pH 8.2) components, all of which were composed of many representative salts, sugars, enzymes, and mucins. They added food-grade E551 silica to model food matrices (hot coffee, instant soup, and pancake). Before digestion, 30% of E551 in coffee was nanosized (5-200 nm) while 13% and 5% were nanosized in soup and pancakes, respectively. Large agglomeration of silica was observed in the gastric digestion stage due to the low pH (the isoelectric point where silica is neutrally charged occurs at pH 2-3) and the high

electrolyte concentration. However, after full digestion, 80% of E551 in coffee was in the nano-size range (5-200 nm) while approximately 15% of E551 added to soup and pancake was nano-sized.[53] Despite the differences in available NP depending on the food matrix, this study did detect some E551 available as NP in the intestinal environment in all foods tested.

Several studies have analyzed NP toxicity after *in vitro* digestion and found it to be slightly different from the toxicity induced by NP that did not undergo digestion. One study simulated gastric and intestinal digestion of 14 nm silica and < 10 nm ZnO NP in solutions consisting of representative ionic and pH conditions but no digestive enzymes. They compared NP, which were not subjected to this digestion, to the digested NP in Caco-2 intestinal epithelial cells. Analysis of the NP suspensions before and after digestion revealed that less than 10% of both silica and ZnO NP dissolved, and the soluble fraction changed very little after digestion. Both silica and ZnO NP were found to be taken up by cells and induced reactive oxygen species (ROS) formation in cells, particularly at doses of 80 $\mu g/cm^2$, but simulated digestion of the NP largely inhibited ROS production. However, digested NP induced similar toxicity to cells based on the reduction of the tetrazolium salt WST-1 by cellular enzymes (at doses of 5-20 $\mu g/cm^2$) and were able to induce similar induction of interleukin-8 (IL-8) secretion at 20 $\mu g/cm^2$ as the undigested NP.[54] Thus, digestion of NP seems to be able to change NP toxicity mildly, but not inhibit its toxicity altogether. Another study subjected 7 nm Ag NP to an *in vitro* digestion in saliva for 5 minutes at pH 6.4, gastric juice for 2 hours at pH 2, and intestinal juice for 2 hours at pH 7.5. These digestive solutions contained ionic components as well as mucins, enzymes, salts, and other digestive components. They observed a slight reduction (requiring 10 µg/mL vs. 5 µg/mL) and 12-24 hour delay in Ag NP cytotoxicity after the digestion as detected by impedance measurements, but this difference was not observed using the CellTiter Blue assay.[55] They suggest this is due to an increase in particle aggregation that hinders Ag ion release, once again showing that digestion is able to alter NP toxicity. Based on these studies, it is clear that both the food matrix and the digestive process could modify the toxicity of a given NP.

3. GI tract exposure to NP

While absorption of orally ingested materials occurs to some degree along the entire alimentary canal (GI tract), the bulk of absorption takes place in the small and large intestines with the large intestine mostly dedicated to absorption of water. There are several specific mechanisms by which ingested NP traverse the epithelial barrier and reach the portal circulation in healthy small intestine (Figure 4) including regulated internalization and active transport through intestinal epithelial cells (transcytosis), strictly regulated paracellular transport through inter-epithelial tight junctions, and transcytosis through intestinal M cells in the process of immunologic sampling of intestinal contents by lymphoid follicles of Peyer's patches. Under conditions of intestinal disease in which permeability is pathologically enhanced, paracellular transport may be increased as a consequence of dysregulation of intercellular tight junctions, and NP in the intestinal lumen may pass unimpeded through regions of epithelial damage or denudation.

Since inorganic NP are not digestible (although ZnO has been observed to dissolve in the low pH environment of the stomach), once ingested they may pass through the entire length of the GI tract and be eliminated in feces. Alternatively, they may follow the path of digested nutrients and traverse the intestinal epithelium to enter the capillaries of the villi. From there the NP would proceed through the intestinal veins to the hepatic portal vein and to the liver. NP that remain in the vasculature can pass through the liver, ascend the inferior vena cava to the right heart, traverse the lungs via the pulmonary circulation and return to the left heart. From the left

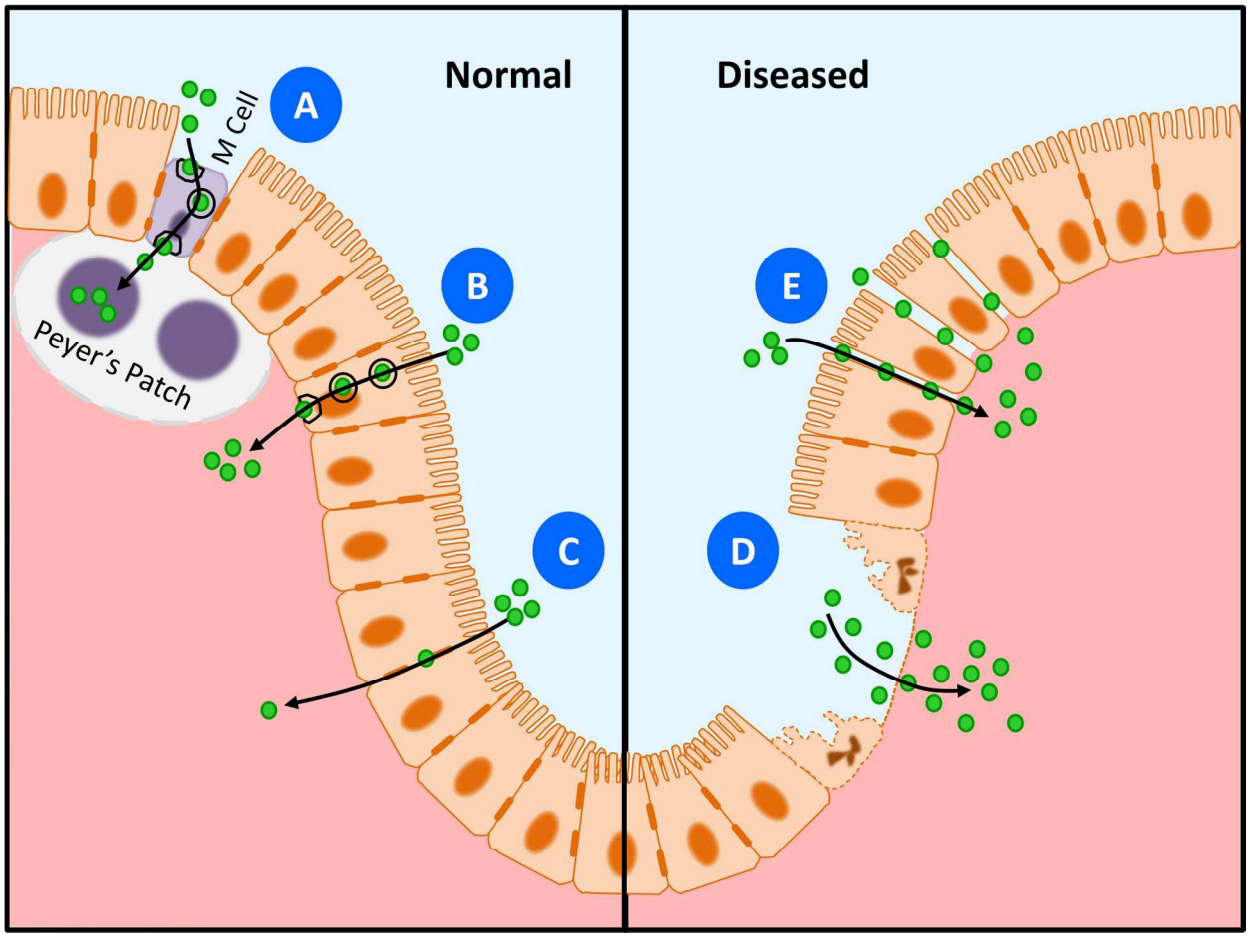

Figure 4: Cross section of the intestinal epithelium illustrating potential mechanisms by which ingested nanoparticles traverse the epithelial barrier in healthy and diseased intestine. Internalization and active transport to Peyer's patch lymphoid follicles by M cells (A); active transcellular transport through epithelial cells (transcytosis, B); limited paracellular transport through tightly regulated intercellular tight junctions (C); unrestricted migration through foci of damaged or denuded epithelium (D); dysregulated transcellular migration through intercellular tight junctions of abnormal permeability (E).

heart, the NP would enter the systemic circulation with the potential of reaching all other organs of the body.

Each step of this journey provides the opportunity for the NP to interact with tissues they contact. The NP may be acutely toxic to the intestinal epithelial cells or may disrupt their barrier function. NP that traverse the epithelial barrier and reach the portal circulation may accumulate in the liver and induce an inflammatory response or otherwise disrupt hepatocyte function through induction of oxidative stress or other responses. NP that pass through the liver and reach the systemic circulation may accumulate in other organs and similarly induce inflammation or otherwise disrupt organ function. Note that even a small fraction of ingested NP crossing the epithelial barrier to reach the circulation could potentially accumulate in tissues to significant levels over a lifetime of consumption.

3.1 Estimated dose, potential distribution and potential biological interactions

The length and diameter of the small intestine vary considerably among individuals, making the mucosal surface area, the interface through which absorption occurs, difficult to estimate. Adding to this difficulty is the presence of circular mucosal folds (plica circularis) and intestinal villi, finger-like projections of mucosa into the lumen, as well as the presence of microvilli on the luminal surface of intestinal epithelial cells (enterocytes). Hence, estimates of the small intestinal mucosal surface area span a broad range from 30 m^2 to 200 m^2.[56, 57] Dekkers et al.[14] have estimated daily ingestion of SiO_2 NP in foods at 1.8 mg/kg body weight, and Weir et al.[12] have estimated foodborne TiO_2 NP ingestion at 0.2 – 0.7 mg/kg body weight per day. Assumption of a 70 kg adult and even distribution of the ingested NP over the entire mucosal surface of the small intestine generates the mucosal exposure estimates shown in Table 1.

Nanoparticle	Estimated daily ingestion	Estimated mucosal surface area 30 m^2	200 m^2
SiO_2	1.8 mg/kg	0.42 μg/cm^2	0.063 μg/cm^2
TiO_2	0.2 – 0.7 mg/kg	0.047 – 0.163 μg/cm^2	0.007 – 0.024 μg/cm^2

Table 1: Estimated small intestinal mucosal epithelium NP exposure based upon estimated daily NP ingestion and mucosal surface area.

The exposure levels shown in Table 1 must be qualified by several factors. First, the assumption that ingested NP are distributed evenly over the entire intestinal epithelial surface is obviously an oversimplification. The contents of the GI tract are not static, but rather propelled forward by peristalsis. Hence, the residence time of materials in a given location will affect the opportunity for their absorption. In addition, the epithelium is covered by a layer of mucus which may impede NP from reaching the underlying enterocytes. Alternatively, NP that become trapped in the mucus may accumulate to higher levels in a given location, and if immobilized adjacent to the enterocyte membrane, NP may be more likely to be absorbed. Still, the intestinal epithelial dosages estimated in actual human NP exposure (as described above) are far lower than those applied in many experiments, which have been as high as 100 μg/cm^2 *in vitro* and 2000 mg/kg/day *in vivo*.

4. Transport of nanoparticles

Because of the importance of nutrient absorption within the small intestine, intestinal architecture is specialized for maximal surface area. The epithelial layer is composed of villous projections and crypts of Lieberkühn between villi (Figure 4). The villi are vascularized for quick delivery of nutrients to the bloodstream.[58]

4.1 *In vitro* intestinal epithelial models

Several continuous cell lines are commonly used to study the intestinal epithelium, including Caco-2, HT-29, and T84 cells. If cultured under appropriate conditions, these cells form tight junctions, produce mucus, form microvilli on the apical side of the cells, and generally exhibit the characteristics of *in vivo* intestinal epithelial cells.[59-61] Caco-2 cells were isolated from a patient with colon adenocarcinoma and observed to form a brush border of microvilli at confluency that expressed some small intestinal hydrolases including sucrase-isomaltase, alkalkine phosphatase, and aminopeptidase.[62] However, this cell line is made up of morphologically heterogeneous cells, not all of which formed the brush border structure. Thus, brush border-expressing Caco-2 cells were cloned to form the C2BBe1 cell line.[63] These cells

have been extensively characterized and used to study brush border formation. When grown on filter supports, confluent C2BBe1 cell monolayers become polarized with distinct apical and basolateral sides. The brush borders of confluent C2BBe1 cells are very similar to those *in vivo* and express many of the same microvillar proteins including villin, fimbrin, sucrase-isomaltase, myosin I, fodrin, and myosin II.[63, 64]

There are at least two drawbacks with using these *in vitro* models. The first issue is that the paracellular permeability of the Caco-2 cell line is limited, as reflected in the high transepithelial electrical resistance (TEER) values (230-1000 Ω cm^2). Such high values are not reflective of the entire intestinal tract, since the small intestine (duodenum, jejenum and ileum) has TEER values as low as 30-100 Ω cm^2. Thus, the Caco-2 model will have lower permeability than expected in the small intestine *in vivo*.[65] This has led to the development of cell culture models, including the intestinal epithelial cell line 2/4/A1, which display permeabilities closer to that of the small intestine.[66] The second drawback of these *in vitro* models is that they do not contain the full diversity of epithelial cell types found *in vivo*. Although there is some mucus production by these cell lines, the mucus layer is often patchy and not as dense as found *in vivo*. This lack of cell diversity also contributes to the increased permeability of *in vitro* cell models, as epithelial permeability is likely to be underestimated in cell models without M cells, even though these cells make up only a small proportion of the epithelium. To address some of these issues, models that are more sophisticated have been developed to better represent the full complement of epithelial cells.

One study used a tri-culture of Caco-2 and HT29-MTX cells cultured above Raji B cells to create monolayers containing M cells and Goblet cells.[67] This study investigated the effects of polystyrene NP on iron absorption and found that treatment of cells with 50 or 200 nm NP at doses of 2×10^{13} ~50 nm particles/mL or 1.25×10^{12} ~200 nm particles/mL (estimated as equivalent to potential pharmaceutical doses) increased iron uptake and transport across the epithelial layer in this tri-culture model. This was accompanied by decreased TEER values, suggesting disruption of the epithelial barrier, which is likely allowing the increased iron transport. This increase in iron transport was confirmed *in vivo* in broiler chickens fed polystyrene NP for 2 weeks, indicating that this *in vitro* model may be representative for what will occur *in vivo*.[67] Thus, these models provide a way to more closely represent relevant features of the intestinal epithelium in an *in vitro* system.

4.2 Possible routes of transport of NP

In order for ingested NP to penetrate into the body beyond the GI tract, NP will have to be transported across the intestinal epithelium. NPs can be endocytosed by cells and transported transcellularly. A study that treated Caco-2 cells with < 40 nm TiO_2 NP found NP both within and below cells of the monolayer without detecting changes in intercellular junction structure. Based on the intact cellular junctions, lack of cell death to create gaps in the epithelium, and the presence of NP within cells, the authors concluded that TiO_2 NP transport was most likely transcellular.[68] Many other studies have reported internalization of NP by Caco-2 cells, suggesting that transcellular transport is possible across the intestinal epithelium.[69-74] Ingested NP can also undergo paracellular transport between cells when there is a gap in the epithelium or increased permeability of the tight junctions. This is likely to play a role in NP transport in diseased intestines, which may have greater epithelial permeability, and, depending on disease severity, more gaps in the epithelium. Finally, NP can be phagocytosed by M cells. Because of their function to sample luminal contents, an *in vitro* co-culture model of M cells was able to transport five times as many chitosan-DNA NP across the epithelial layer as Caco-2 cells

alone.[75] This suggests that M cells may be an important means of NP transport across the epithelium. However, since there are vastly more enterocytes than M cells, enterocytes will still play a large role in NP transport. It has been observed for many years that Peyer's patches contained pigmented macrophages and this pigment was determined to consist of granular aluminum, silicon, and titanium, which was presumably taken up from the diet, and was generally in the size range of a few hundred nanometers in diameter.[76] This further suggests that NP transport by M cells will be important and this accumulation of inorganic food additives in macrophages and potentially other cells of the intestinal epithelium will likely only amplify as NP are more frequently used and these particles are more readily transported across the epithelium.

5. Immunogenicity of ingested nanoparticles

Upon introduction of foreign material such as NP into the body, the question that arises is whether that material will be immunogenic. This has been explored for NP used in foods that come into contact with the intestinal immune system. Immune cells such as lymphocytes, dendritic cells, and macrophages are found in the lamina propria beneath the intestinal epithelium in addition to those that reside within lymphoid follicles. These cells can respond to antigens transported across the epithelium. There is also evidence that dendritic cells of the lamina propria extend processes between cells of the epithelium to sample antigens in the lumen while preserving the tight junctions between the cells.[77] Luminal IgA plays a large regulatory and defensive role in the intestinal immune system. When these cells recognize pathogenic bacteria or pro-inflammatory disease states, intestinal epithelial cells promote an inflammatory response by secreting the chemokine IL-8 and the pathogen-elicited epithelial chemoattractant to recruit neutrophils.[78]

Since one of the major chemokines released by intestinal epithelial cells is IL-8, many *in vitro* studies have evaluated IL-8 secretion as an indication of an immune response elicited by food-relevant NP treatment. Multiple studies have shown that NP do induce IL-8 secretion and some of these papers are summarized in Table 2. A 3-fold induction in IL-8 secretion was observed in undifferentiated Caco-2 cells treated with 15 nm but not 55 nm silica NP at 32 μg/mL in serum-free media.[72] In another study, both 14 nm silica NP and 10 nm ZnO NP induced IL-8 secretion in Caco-2 cells at a dose of 20 μg/cm^2. IL-8 expression and secretion was similar using NP that had undergone *in vitro* digestion and those that had not undergone digestion. Silica NP increased IL-8 mRNA expression much more strongly in undifferentiated cells than differentiated cells and this trend held for IL-8 protein secretion as well. For ZnO NP, there was only slight induction of IL-8 mRNA expression in either differentiated or undifferentiated cells, but IL-8 secretion was strongly induced in differentiated cells. The fact that there was no induction of IL-8 secretion in undifferentiated cells is likely because ZnO NP treatment at this dose induces significant cytotoxicity to cells.[54] Another study showed that TiO_2 (21 nm), Ag (20-30 nm), and ZnO NP (20 nm) all induced IL-8 secretion in Caco-2 cells at doses of 10 and 100 μg/mL after 48-hour treatment. Ag NP induced the most IL-8 production, followed by ZnO NP and finally TiO_2 NP, which induced only slightly increased IL-8 production.[79] In SW480 cells (another colon carcinoma-derived cell line), only TiO_2 NP slightly increased IL-8 generation while almost no IL-8 was detected in ZnO-treated cells, likely due to toxicity of the ZnO to cells.[79] Treatment of Caco-2 cells with 50-70 nm ZnO NP increased IL-8 production after 6 and 24 hours at doses of 1 and 2.5 μg/cm^2. Similarly, IL-8 production increased after 24-hour treatment of cells with <25 nm TiO_2 NP at doses of 1 and 2.5 μg/cm^2.

Nanoparticles	Dose	Cells	IL-8	Ref.
15 nm silica	32 µg/mL	Undifferentiated Caco-2	3-fold induction in IL-8 secretion	72
55 nm silica	32 µg/mL	Undifferentiated Caco-2	No induction in IL-8 secretion	72
14 nm silica, digested and undigested	20 µg/cm^2	Caco-2	IL-8 secretion, greater increase in IL-8 mRNA and IL-8 secretion in undifferentiated cells	54
19 nm silica	27 µg/mL	Caco-2	Little IL-8 release	69
21 nm TiO_2	10 and 100 µg/mL	Caco-2 SW480	Slight increase in IL-8 production Slight increase in IL-8 generation	79
< 25 nm TiO_2	1 and 2.5 µg/cm^2	Caco-2	Increased IL-8 production	80
15 nm TiO_2	27 µg/mL	Caco-2	Little IL-8 release	69
20 nm ZnO	10 and 100 µg/ml	Caco-2 SW480	IL-8 production No IL-8 production (likely due to toxicity)	79
50-70 nm ZnO	1 and 2.5 µg/cm^2	Caco-2	Increased IL-8 production	80
50-70 nm ZnO	3 and 5 µg/cm^2	LoVo	~ Double the amount of IL-8 release in untreated cells	81
20-30 nm Ag	10 and 100 µg/mL	Caco-2 SW480	Significant IL-8 production No IL-8 production	79
PVP-capped < 20 nm Ag	39 µg/cm^2	Caco-2/THP-1/MUTZ-3 co-culture	Induced IL-8 release in co-culture but not Caco-2 monoculture. Less IL-8 induction was observed after pretreating cells with IL-1β to induce an inflammatory state prior to Ag NP treatment	82
25 nm Ag spheres and 80-90 nm Ag rods	27 µg/mL	Caco-2	Significant IL-8 release	69

Table 2: Summary of literature showing an immune response to NP treatment in intestinal epithelial cells.

However, there was no release of IL-6 or tumor necrosis factor-α (TNF-α) observed after treatment with ZnO or TiO_2 NP.[80] Polyvinylpyrrolidine (PVP)-capped Ag NP (< 20 nm) induced IL-8 release beginning at doses of 39 µg/cm^2 in a co-culture model containing Caco-2 cells grown on top of human macrophages (THP-1) and dendritic cells (MUTZ-3) embedded in collagen, but did not induce IL-8 release in monocultures of Caco-2 cells until a dose of 312.5 µg/cm^2 Ag NP.[82] Co-cultures treated with IL-1β for two days to induce an inflammatory state also increased IL-8 production after treatment with Ag NP, but to a lesser extent than in the non-inflamed co-cultures.[82] A study interested in NP use in paints found that 19 nm SiO_2 and 15 nm TiO_2 NP induced only slight IL-8 release in Caco-2 cells, but Ag NP (25 nm spheres and 80-90

nm rods) induced a more significant IL-8 release at 27 μg/mL after 48-hour treatment.[69] Treatment of LoVo cells (colon carcinoma-derived epithelium) with 50-70 nm ZnO NP at 3 and 5 μg/cm^2 for 24 or 48 hours increased IL-8 release to approximately double the amount released in untreated cells.[81] These studies suggest that ingested NP may be able to induce some immune response although this likely depends on the NP properties.

Several studies outside of the intestines have linked induction of inflammation by NP to oxidative stress. A study in J774 murine macrophages showed that IL-1α mRNA expression (which leads to TNF-α release) after treatment of cells with PM_{10} was dependent on both calcium signaling and ROS production.[83] Work with silica NP has suggested that ROS induced by silica NP treatment can trigger proinflammatory responses.[84] Treatment of RAW264.7 macrophages with 12 nm silica NP showed that silica induced ROS generation and depletion of glutathione (GSH) beginning at 5 ppm silica NP and these macrophages also generated NO, which plays a role in signaling related to inflammation. Administering silica NP intraperitoneally to mice at 50 mg/kg caused an increase in NO as well as inflammatory cytokines in blood, suggesting that this inflammatory reaction could be related to ROS generation.[84] From these and similar studies, it is not clear whether oxidative stress induced by NP is solely responsible for induction of inflammatory responses or if NP can also directly induce inflammation, but these responses do seem to be related and likely feed into one another.

Several of the previously reported studies that observed IL-8 induction in intestinal models also observed oxidative stress in these cells, suggesting that the immunogenicity may be connected to oxidative stress.[54, 72, 80, 81] However, other studies did not observe oxidative stress in cells,[69, 79] suggesting that an inflammatory response is not necessarily dependent on oxidative stress.

Although studies investigating immunogenicity *in vivo* are more limited, there is some evidence that NP administered orally may cause inflammatory responses. TiO_2 microparticles (Kronos® 1171, 260 nm) and NP (66 nm) administered to mice by gavage at a dose of 100 mg/kg body weight/day for 10 days all increased inflammatory cytokines in the ileum including interferon-γ (IFN-γ), TNF-α, IL-4, IL-23, and TGF-β, and increased $CD4^+$ T cells in duodenum, jejunum, and ileum of treated mice.[85] However, other studies have suggested that some NP may not stimulate an immune response or may even induce a tolerogenic response. Administration of 10, 75, and 110 nm Ag NP to rats at doses of 9, 18, and 36 mg/kg body weight/day for 13 weeks resulted in decreased expression of mucins, Toll-like receptors, and the T cell regulation genes FOXP3, GPR43, IL-10, and TGF-β in the ileum, suggesting that Ag NP may be leading to a greater tolerance of bacteria by the intestines.[86] Based on these studies, further investigation is required to determine the ability of specific NP to induce an immune response when ingested.

6. Critical review of the toxicological effects of the most commonly used inorganic nanoparticles

We now focus on the toxicity of the food-relevant NP SiO_2, TiO_2, ZnO, and Ag in more detail based primarily upon studies reported in the last five years. Key details of each study are listed in **Tables 1S-8S (Supplementary Section)** and the following text summarizes this work, followed by critical assessments of these bodies of work.

6.1 Silica nanoparticles

E551, the common food-grade silica additive, is a form of synthetic amorphous silica (SAS, Figure 2a,b shows the electron micrographs). SAS exists in four forms including

pyrogenic silica, precipitated silica, silica gel, and colloidal silica.[87] E551 has not been used in the majority of published *in vitro* experiments. Rather, most studies have used amorphous silica NP, in many cases without specification of the particular form. While these NP may represent the nano-scale fraction of E551, as previously discussed, NP size, charge, surface chemistry, and other properties all play significant roles in determining NP toxicity, and the specific characteristics of the NP used in experiments are not always clearly described in publications. This may at least partially explain why results of studies of NP biological interactions taken collectively have been fraught with inconsistencies and, in some cases, contradictions.

6.1.1 Acute *in vitro* cellular toxicity (Table 1S)

A number of investigations have focused on the assessment of direct acute toxicity of silica NP to intestinal epithelial cells and other cell lines *in vitro*, as detailed in Table 1S. Among such conditions are the specific compositions/properties of the silica NP including naked, pristine SiO_2 NP and NP that have been coated with various materials. Likewise, administered NP dosage and exposure times have varied among *in vitro* and *in vivo* studies over a very broad range.

A study of 25-30 nm colloidal silica NP in six cell lines (both mouse and human lung, colon, and macrophage cell lines) demonstrated that macrophages were most sensitive to silica NP-induced toxicity, showing 20% reduction in viability in response to 1.9 μg/mL and approximately 50% reduction at 12.5 μg/mL, while the colon cells were the least sensitive, showing 20% reduction in viability in response to 59.1 μg/mL and approximately 50% reduction at 100 μg/mL.[88] This suggests that silica NP toxicity likely differs between cell and tissue types and silica NP may be less toxic in the intestines than in the lungs.

Silica NP have been shown to be able to mediate toxicity through oxidative stress generation resulting in DNA damage and induction of apoptosis.[89] This mechanism of toxicity has been observed in multiple intestinal epithelial cell studies to date. A study in which Caco-2 cells were exposed to 15 nm silica NP showed decreased cell viability at a dose of 32 μg/mL and an increase in caspase-3 activation in cells at 64 μg/mL, indicating initiation of apoptosis.[72] The silica NP were genotoxic to cells and increased the frequency of micronucleus formation. In addition, a slight increase in ROS production was observed in response to 15 nm silica NP treatment at 32 μg/mL.[72] Thus, the authors concluded that oxidative stress is likely involved in induction of cell death and DNA damage by silica NP. A study in Caco-2 and human gastric epithelial cells (GES-1) demonstrated that 10-50 nm silica NP induced LDH release, a decrease in cell viability based on the Cell Counting Kit-8 (CCK-8) assay, a partial inhibition of cell proliferation, a slight S phase cell cycle arrest in GES-1 cells and S and G_2/M phase arrest in Caco-2 cells, and a slight increase in ROS generation. Neither induction of apoptosis nor necrosis was observed in this study, but ROS generation may be contributing to the silica NP-induced decrease in viability and cell cycle arrest. Since these effects were observed at high silica NP doses (often 200 μg/mL), the authors conclude that silica NP are safe below doses of 100 μg/mL.[90]

A study in differentiated and undifferentiated Caco-2 cells showed that 14 nm silica NP were internalized by both types of cells and induced ROS formation. Silica NP induced toxicity in undifferentiated cells based on the reduction of the tetrazolium salt WST-1 by cellular enzymes after 4- and 24-hour treatments at doses of 20 μg/cm^2 and 5 μg/cm^2, respectively, but no toxicity was apparent in differentiated cells.[54] Results of these experiments clearly indicate that cellular differentiation state is an important factor in cellular sensitivity to NP-mediated cytotoxicity.

A study with various types of NP in serum-starved Caco-2 cells showed that 14 nm silica NP were toxic to cells at doses of 20 and 80 μg/cm^2 based upon both LDH release after 4 and 24 hours and a decrease in metabolic activity (WST-1 assay). The silica NP also induced DNA damage and glutathione depletion in cells, implying a role for oxidative stress.[91] A study measuring HT-29 cell proliferation by impedance demonstrated similar proliferation between untreated control cells and cells treated with 10 μg/mL 25 nm Rhodamine B-embedded silica particles, but slight inhibition of cell proliferation in response to treatment with 100 nm *meso*-tetra(N-methyl-4-pyridyl) porphine tetratosylate salt (TMPyP)-embedded silica particles. The investigators observed an increase in cell death, especially after treatment with 10 μg/mL ~100 nm silica particles (up to 40%). There was a slight increase in double-strand DNA breaks as determined by phosphorylated histone H2Ax foci, particularly with 100 nm silica.[92] Although the authors did not evaluate ROS production or initiation of oxidative stress, their observations are also consistent with a ROS-mediated mechanism of toxicity, which may lead to DNA damage and cell death.

As alternatives to oxidative stress/ROS, data generated by several investigations have supported other mechanisms of cellular toxicity induced by silica NP. One such mechanism is silica NP-induced increase in lysosomal permeability and destabilization of cellular lysosomes. The inability of the lysosome to degrade silica NP causes a loss of lysosomal membrane integrity and can lead to ROS-independent initiation of apoptosis in silica NP-exposed cells.[89] A study performed in HT-29 cells with 12 and 40 nm silica particles reported an increase in cell proliferation (significant but relatively small) after treatment with 93.8 μg/cm^2 12 nm silica NP for 24, 48, or 72 hours as measured by the sulforhodamine B assay (measures sulforhodamine B-bound protein to determine cell number). Increased LDH release and decreased mitochondrial activity was observed and effects were more pronounced in proliferating cells than confluent cells. ROS production was not observed and little DNA damage was detected after silica treatment, indicating that toxicity was likely not mediated through oxidative stress. Treatment with 12 nm silica NP increased total cellular glutathione and γ-glutamylcysteine-ligase needed to synthesize glutathione at a dose of 31.3 μg/cm^2 and above. This increase in GSH seems to be due to an upregulation in the ERK1/2 (extracellular signal-regulated kinases) pathway rather than oxidative stress. The MAPK (mitogen-activated protein kinases)/ERK1/2 pathway can help regulate the cell cycle and thus may also be responsible for the increased cell proliferation.[93]

In contrast to the investigations summarized above, other studies have demonstrated no toxicity of silica NP in intestinal epithelial cells. A study of Caco-2 and colon carcinoma RKO cells showed minimal toxicity induction by 10 nm silica NP at doses of up to 100 μg/cm^2 based upon formazan assays. Cells exposed to 50 μg/cm^2 silica NP for 4 hours also showed minimal changes in gene expression as determined by whole genome microarray analysis.[94] Another study of undifferentiated Caco-2 cells in serum-containing culture medium (20%) treated with 32 and 83 nm fluorescent silica NP demonstrated no cellular toxicity. These NP were internalized by cells and agglomerated near the cell nucleus, but no cytotoxicity (WST-1 assay) or genotoxicity (comet assay) was observed in cells treated with up to 200 μg/mL NP.[73] These different outcomes (compared to the NP-mediated toxicities summarized above) may suggest a protective effect of serum in the culture medium, which has been shown to ameliorate silica NP toxicity.[95]

Our own studies of silica NP-mediated acute toxicity in intestinal epithelial cells likewise demonstrated minimal effect. C2BBe1 cells (a Caco-2 sub-clone) were exposed for 24 hours to well-characterized 12 nm SiO_2 NP, either pristine or following treatment with digestive enzymes,

at a concentration of 10 μg/cm^2. Necrosis, apoptosis, membrane damage, and mitochondrial activity were measured by Sytox Red stain, Annexin V stain, LDH assay, and MTT assay, respectively. Data generated by these assays and shown in Figure 5 demonstrated no decrease in viability or mitochondrial activity. Repeated weekly 24-hour NP treatments likewise induced no decrease in cell viability.[17] However, continuous exposure of actively proliferating cells to silica NP did moderately attenuate proliferation.[18]

6.1.2 *In vivo* studies of ingested silica NP (Table 2S)

While *in vitro* experiments have provided useful data regarding acute direct effects of NP upon intestinal epithelial cells, the biological interactions of ingested NP potentially extend far beyond the intestinal epithelium. In this regard, *in vitro* studies are extremely limited in their ability to model interactions that might follow absorption of NP across the intestinal epithelial barrier. Hence, a number of studies of the impact of orally administered silica NP, conducted primarily in rodents, are reviewed here.

Repeated oral administration (20 mg SAS/kg body weight/day) for up to 5 days in rats resulted in very little accumulation of silicon in any organ as detected by inductively coupled plasma-dynamic reaction cell-mass spectrometry (ICP-DRC-MS).[96] Although there seemed to be slightly more accumulation of silicon in liver and spleen of females over males, the authors concluded that there is very little absorption of silica from the GI tract. A long-term study in which 20 and 100 nm negatively-charged colloidal silica NP were administered to Sprague-Dawley rats by gavage at doses of 500, 1000, and 2000 mg/kg body weight/day for 90 days revealed no clinical signs of toxicity over the treatment period. Necropsy results did show differences in organ weights of lung and liver in the 20 nm NP treatment groups and in kidney, lung, submandibular gland, and ovary in the 100 nm NP treatment groups. However, these

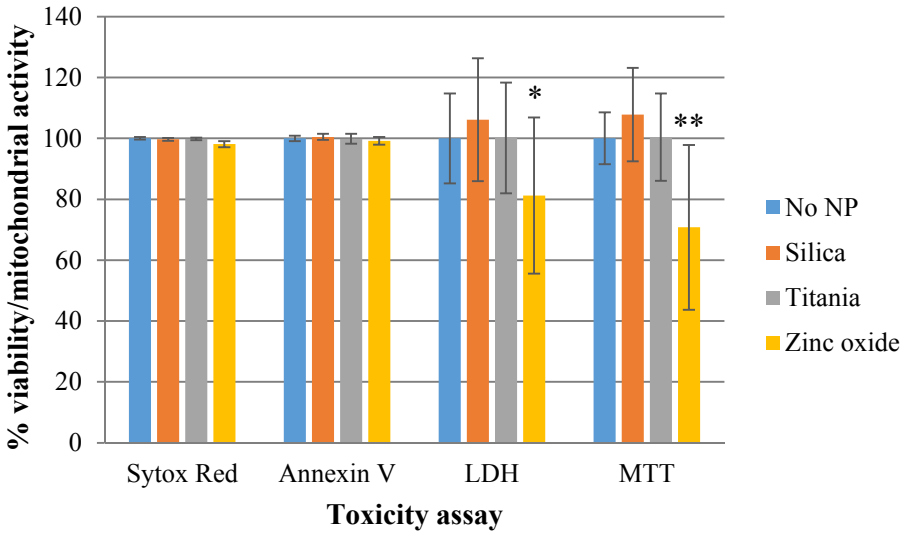

Figure 5: Toxicity of 24-hour treatment of C2BBe1 cells with 10 μg/cm^2 SiO_2, TiO_2, and ZnO NP based on the Sytox Red assay (measuring necrotic cell death), the Annexin V assay (measuring apoptosis), the LDH assay (measuring membrane damage), and the MTT assay (measuring metabolic activity). Significance as compared to untreated controls was determined using Student's *t*-test (* indicates $p < 0.01$, ** indicates $p < 0.001$).[17] Reprinted (adapted) with permission. Copyright 2013 American Chemical Society.

changes did not seem to be dose-dependent or correspond to histopathological observations, leading the investigators to conclude that these effects were not related to silica ingestion.[97]

Several studies have provided evidence that ingested silica NP traverse the intestinal epithelium. One such study of orally administered 70, 300, and 1000 nm silica particles in mice for 28 days at 2.5 mg/day provided no indication of NP toxicity or tissue injury following 28-day silica administration and concluded that silica NP induced no toxicity in these animals. However, absorption studies using an everted gut sac method did demonstrate silica NP absorption across the epithelium. The everted intestinal segment was incubated for 45 minutes in a suspension of 70 nm pristine silica NP, or silica NP functionalized with either carboxyl or amine groups to analyze the impact of NP surface charge, and found greater absorption of the carboxyl-functionalized silica, although absorption of the amine-functionalized particles was more efficient than the unlabeled 70 nm silica particles. Surprisingly, there was no significant difference in the absorption of 70 nm particles over 300 or 1000 nm particles, but there was some degree of absorption of all particles over background levels.[98] These experiments suggest that silica NP are indeed absorbed across the intestinal epithelium. This observation was confirmed by a study utilizing silica NP with incorporated Rhodamine B isothiocyanate to allow *in vivo* imaging of the NP distribution in mice, which showed significant localization of fluorescent signal to kidneys as well as lungs and liver of treated mice beginning as early as two hours after administration.[99]

We have likewise demonstrated that orally administered silica NP traverse the intestinal epithelium. In order to insure that the presence of a fluorescent label on the surface of the NP does not modify their interaction at biological interfaces, we synthesized 20 – 30 nm silica NP with fluorescent cores of either quantum dots or Rhodamine 6G. Extensive analysis of NP demonstrated surface characteristics quite similar to commercial silica particles. Following administration to mice by gavage, fluorescent silica NP were localized by confocal fluorescence microscopy in kidney, lung, spleen, and brain, in addition to multiple segments of the GI tract, as shown in Figure 6.[100]

Some studies have also demonstrated sufficient GI absorption of silica NP to induce toxicity. In one such investigation, 30 nm and 30 μm silica particles (silica obtained from rice husk) were administered to mice in their diet for 10 weeks (total silica intake was 140 g silica/kg body weight). Apparent liver toxicity was observed in the nano-fed group based upon increased alanine aminotransferase (ALT) and evidence of fatty liver in H&E staining.[101] Another study in which two silica NP materials, SAS (7 nm particles) and NM-202 (10-25 nm particles), were orally administered to mice for 28 days at NM-202 doses of 100, 500, or 1000 mg/kg body weight/day, or SAS doses of 100, 1000, or 2500 mg/kg body weight/day, noted minimal to modest silica accumulation in several tissues, increased liver fibrosis after 84-day exposure, and moderately increased expression of fibrosis-related genes.[102]

Several *in vivo* and *in vitro* studies of silica interactions have reported that lower doses of NP allow greater absorption and lead to greater toxicity. Van der Zande et al.[102] showed that more silica NP seemed to be absorbed through the GI tract at lower doses and found that in simulated *in vitro* intestinal conditions, the silica had stronger gel-like properties at higher concentrations, which may decrease its bioaccessibility and thus absorption through the GI epithelium. The investigators proposed that the relatively high pH and salt concentrations present in the intestine would enhance the gelating behavior of silica. Thus, this may be an important

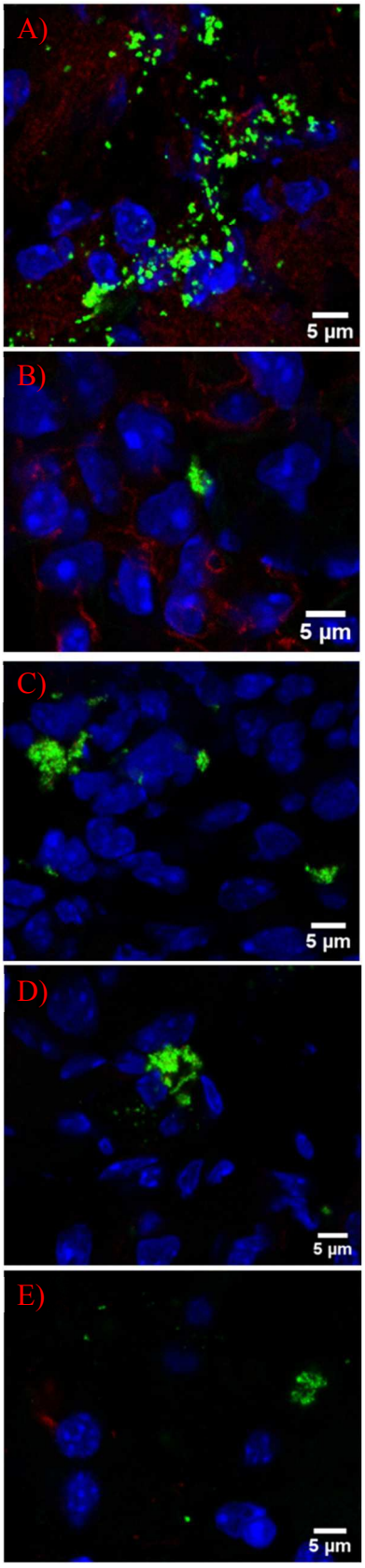

Figure 6: Confocal microscopy of tissue sections from mice treated daily for four days with Rhodamine 6G/silica nanoparticles. Nanoparticles (green) were observed in GI tract tissues including stomach (A) as well as non-GI tract tissues including lung (B), spleen (C), kidney (D), and brain (E). Sections were stained with DAPI (cell nuclei; blue). Red staining is E-cadherin (junctions between epithelial cells) in stomach, lung, spleen, and kidney, and GFAP in brain.[100] Reprinted with permission from Dove Medical Press.

factor for studies performed with high doses of silica NP, but will be less of an issue at actual human exposure levels of silica.[102] In another study, nanoscale SAS administered orally to rats induced a slight increase in micronucleated cells in colon crypts at only the lowest dose, 5 mg/kg body weight/day of NM-200 and NM-201.[103] Based upon van der Zande's proposal, the absence of this effect at higher doses could be attributed to gelation of the silica, leading to lower absorption. However, the suspension of silica used to administer NP to rats in this study (6 mg/mL) was a lower concentration than what was tested by van der Zande et al. (9 mg/mL), so it seems unlikely that there would be much gelation of silica at the administered doses.[102, 103] An *in vitro* study using 100 nm TMPyP-embedded silica particles demonstrated greater toxicity at lower doses (10 μg/mL) than at the highest doses (150 μg/mL). The authors suggest that this could be a protection response by cells that is initiated at a certain level of toxicity.[92] However, this could also be due to gelation of the silica NP at the higher doses reducing both internalization by cells and toxicity.

6.2 Titania nanoparticles

TiO_2 is the naturally occurring oxide of titanium and can be purified from ilmenite ore ($FeTiO_3$). TiO_2 exists in multiple crystal structures including rutile, anatase, and brookite. Anatase and rutile TiO_2 phases are the most commonly synthesized and used.[104] Anatase TiO_2 has been reported to be more reactive than rutile TiO_2.[105, 106] TiO_2 NP can consist of a mixture of rutile and anatase phases,[107] and the proportions of rutile and anatase is relevant when assessing NP toxicity. Food-grade TiO_2, E171, has a broad size distribution with average particle size of 110 nm[12] (electron micrograph shown in Figure 2c,d). As noted above in the discussion of silica, the specific compositions/properties of the TiO_2 NP used in experiments have varied and have not been consistently thoroughly described. Likewise, administered NP dosage and exposure times have varied over a very broad range.

6.2.1 Acute *in vitro* cellular toxicity (Table 3S)

TiO_2 NP have been shown to cause cytotoxicity via oxidative stress-dependent pathways leading to DNA damage, cell cycle arrest or delay, and mitochondrial dysfunction, particularly in pulmonary and inhalation models.[107] However, experiments in intestinal epithelial cells are generally in agreement that TiO_2 NP are nontoxic. A study in Caco-2 cells treated with TiO_2 NP (<40 nm) showed a decrease in epithelial monolayer integrity by decreased TEER measurements and a loss of localization of γ-catenin to cell adherens junctions beginning at 6 days after continuous TiO_2 NP treatment and continuing to 10 days at an acute dose of 1000 μg/mL. No decrease in TEER was observed after acute exposure and no induction of cell death was observed after acute or chronic exposure.[68] These investigators also observed cellular internalization of TiO_2 and apparent transport of TiO_2 across the epithelium as determined by confocal microscopy of the cell monolayer at different depths. Studies of transport across the epithelium in a transwell system revealed that a similar total mass of TiO_2 was transported through the epithelial layer regardless of dose. Cells responded to TiO_2 treatment by increasing

intracellular-free calcium which can regulate calcium-dependent enzymes, but this response was attenuated over a period of 14 days.[68]

In another study, P25 TiO_2 (25% rutile and 75% anatase) treatment (100 μg/mL) of the intestinal epithelial cell lines Caco-2 and SW480 demonstrated slight toxicity as measured by MTT assay in SW480 cells with TiO_2 suspended in buffered synthetic freshwater (to mimic NP contamination in drinking water) but no toxicity in cell culture media or in Caco-2 cells.[79] The differences in toxicity observed between the cell culture media and synthetic freshwater could be due to the absence of or differences in composition of the protein corona on NP in the freshwater versus the culture medium. However, minimal toxicity was observed in either cell type under all conditions. No induction of ROS production was observed by TiO_2 in either cell type, but there was a slight increase in IL-8 production after TiO_2 treatment in both cell lines at doses of 10 and 100 μg/mL.[79]

A study in Caco-2 cells investigated differences between cellular treatment with bulk TiO_2, P25, nano-anatase, and nano-rutile forms of TiO_2. There was no change in LDH release after treatment with any of the TiO_2 particles at a dose of 1 mg/L (1 μg/mL). The investigators observed cellular accumulation of TiO_2 that seemed to be due to active cellular internalization, with anatase internalized more rapidly than rutile forms of TiO_2, suggesting that the specific physical characteristics of TiO_2 affect its biological interactions. The authors also observed TiO_2-mediated changes in cellular electrolytes. All forms of TiO_2 induced depletion in potassium and elevation of magnesium, and P25 or anatase TiO_2 induced elevation of calcium.[108]

Another study comparing anatase (4, 7, or 215 nm) to anatase/rutile TiO_2 NP mixtures (22 or 25 nm) in Caco-2 cells showed that anatase/rutile but not anatase-only samples induced LDH release, decreased metabolic activity (to ~70%) at 80 μg/cm^2, and induced DNA damage at 20 μg/cm^2. No decrease in total cellular glutathione was observed, suggesting that these responses may not be mediated by oxidative stress.[106] Although the toxicity induced by the anatase/rutile TiO_2 NP was mild, this study suggests that rutile TiO_2 NP may be the toxic component for these cells. However, this conflicts with results of research performed in other cell types including fibroblasts and lung epithelial cells, which showed that anatase TiO_2 was 100 times more toxic than rutile TiO_2.[105] Thus, some other difference between these NP formulations may be contributing to the differences observed in cytotoxic potential.

Treatment of Caco-2 cells with 20-60 nm TiO_2 NP did not induce cytotoxicity up to a dose of 20 μg/cm^2 even though TiO_2 NP did increase cellular ROS. There was a decrease in cell proliferation to ~80% of the control as measured by the colony forming assay at 20 μg/cm^2, but overall toxicity was considered to be negligible.[80] However, the increased ROS production indicates that these NP may be capable of inducing toxicity at higher doses. Another study used a 3D intestinal model in which human macrophages (THP-1) and dendritic cells (MUTZ-3) were embedded in collagen on a transwell insert and Caco-2 cells were then grown on top of this layer. TiO_2 NP (7-10 nm) did not induce toxicity or inflammation in the co-culture model under inflammatory or noninflammatory conditions,[82] confirming the safety of TiO_2 NP in a more complex *in vitro* model incorporating immune cells. Nano-TiO_2 isolated from chewing gum elicited minimal toxicity in Caco-2 and GES-1 (gastric epithelium) cells based upon LDH release and WST-8 assay up to a dose of 200 μg/mL, although pure P25 TiO_2 decreased cell viability by approximately 20% in GES-1 cells.[13] While a slight increase in ROS production was observed in response to exposure to chewing gum-extracted nano-TiO_2, the authors concluded that the NP were relatively safe. Thus, although TiO_2 NP seemed minimally toxic in these studies, there is some indication that mild toxicity induced by TiO_2 NP may be mediated by oxidative stress. This

may be of particular significance considering that consumers are being exposed to this form of TiO_2.

We have demonstrated minimal toxic effect of TiO_2 NP in intestinal epithelial cells. Similar to our experiments with silica, C2BBe1 cells were exposed for 24 hours to well-characterized 20-25 nm TiO_2 NP, either pristine or following treatment with digestive enzymes, at a concentration of 10 μg/cm^2. Necrosis, apoptosis, membrane damage, and mitochondrial activity were measured by Sytox Red stain, Annexin V stain, LDH assay, and MTT assay, respectively. Data generated by these assays demonstrated no decrease in viability or mitochondrial activity, as shown in Figure 5. Repeated weekly 24-hour NP treatments likewise induced no decrease in cell viability.[17] However, continuous exposure of actively proliferating cells to TiO_2 NP did moderately attenuate proliferation.[18]

Several studies have shown that TiO_2 NP can disrupt normal microvilli structure in intestinal epithelial cells. Even if TiO_2 NP do not induce acute toxicity, disruption of important structures such as microvilli may affect normal cellular functions, particularly nutrient absorption across the intestinal epithelium. A study in which C2BBe1 cells were treated with food-grade E171 TiO_2 and TiO_2 particles isolated from the candy coating of chewing gum demonstrated that both forms of TiO_2 could disrupt the brush border (microvilli) in these cells at a dose of 0.1 μg/cm^2 and that this effect is not simply due to sedimentation of aggregated particles.[74] This disruption was characterized by limp rather than erect microvilli and fewer total microvilli. The investigators also observed cellular internalization of TiO_2 24 hours after treatment with this dose, which could require remodeling of the actin cytoskeleton and further disruption of the brush border.[74] Further, the low dose of TiO_2 used in these experiments is more representative of actual intestinal epithelial cell exposure estimated in human consumption. In another study, scanning electron microscopy of Caco-2 cells treated with <40 nm TiO_2 NP revealed disruption of cell microvilli leading to decreased numbers of microvilli and changes in shape. Microvilli no longer stood erect and seemed to be absorbed into cells. Unlike toxicity (decrease in TEER was observed at 1000 μg/mL), this effect was observed at doses as low as 10 μg/mL, which may again represent more physiologically relevant exposure levels.[68] These studies necessitate continued *in vivo* studies to further investigate the effects of TiO_2 NP exposure on microvilli.

6.2.2 *In vivo* studies of ingested titania NP (Table 4S)

Despite minimal direct acute *in vitro* toxicity of TiO_2 NP in intestinal epithelial cells, the *in vivo* impact of TiO_2 NP ingestion depends greatly on NP absorption across the GI epithelium and into the circulation. A study estimating the ability of NP to cross a Caco-2 monolayer using a transwell system and 18 nm TiO_2 NP demonstrated that an amount just above the detection limit of 0.4% of the applied concentration of NP (100 μg/mL) was able to cross the Caco-2 monolayer.[109] When 100 mg/kg body weight of TiO_2 NP was orally administered to mice, the authors observed no significant increases in TiO_2 in tissues 24 hours following administration,[109] which is in agreement with the low-level transport of TiO_2 NP across the epithelial layer observed *in vitro*. However, they did observe significant TiO_2 uptake in at least one isolated Peyer's patch by TEM analysis.

In a separate study, investigators orally administered 21 nm TiO_2 NP (80% anatase, 20% rutile) to rats daily for 13 weeks at 260.4, 520.8, and 1041.5 mg/kg body weight/day. A slight increase in the titanium content of blood in treated animals was observed, which was only significant in male rats. There was no increase in TiO_2 content of liver, kidney, spleen, or brain, as measured by ICP-MS. The authors also did not detect increased titanium in the urine, but there

was considerable titanium in feces, suggesting that little TiO_2 was absorbed through the GI tract.[110]

In contrast, other investigators have reported the presence of TiO_2 NP in tissues after oral administration *in vivo*, suggesting greater intestinal absorption than described above. Mice administered a single oral dose of 5 g/kg body weight of 25, 80, or 155 nm TiO_2 particles showed evidence of liver injury at 2 weeks post-NP administration as indicated by increased ALT, aspartate transaminase (AST) and LDH serum levels, hydropic degeneration around the central hepatic vein, and spotty necrosis in hepatocytes. Also, increases in blood urea nitrogen and pathological changes in the kidney (swelling in renal glomeruli, proteinic liquid-filled renal tubules) indicated renal injury.[111] A study of toxicity induced by ingestion of TiO_2 also showed liver and kidney damage 7 days after a 5 g/kg body weight administration of TiO_2 NP, as well as slight increases in ROS production and decreases in glutathione peroxidase and superoxide dismutase (SOD) levels in various tissues.[112] These findings indicate that potential toxicity or other biological interactions of ingested nanoscale TiO_2 NP may extend beyond the GI tract. Indeed, NP that traverse the GI epithelium and reach the circulation may distribute to, and have an impact upon, nearly any organ/tissue of the body.

6.3 Zinc oxide nanoparticles

Zinc oxide (ZnO) NP are used in biosensors, light emitting diodes, photo-detectors, and in sunscreens to absorb UV radiation,[113] and are of interest to the food industry. ZnO also has antimicrobial properties. ZnO particles have a wurtzite crystal structure and NP are often synthesized using the sol-gel method or hydrothermal method, which allows for control of NP shape and size (Figure 2a shows an electron micrograph of commercial ZnO NP).[113] As with other NP reviewed above, the specific physicochemical characteristics of ZnO can have profound impact upon their interactions at biological interfaces.

6.3.1 Acute *in vitro* cellular toxicity (Table 5S)

Various studies have suggested that ZnO NP toxicity is due to dissolution of the NP either outside cells or within cells leading to increased availability of zinc ions able to interact with enzymes and other cell components. It has been demonstrated in various models that ZnO NP mediate toxicity through oxidative stress, and lysosomal destabilization and mitochondrial dysfunction contribute to the cytotoxic response.[25] These mechanisms appear to apply to intestinal epithelial cells as well. In one study, 90 nm ZnO NP at 10 µg/mL decreased Caco-2 cell viability and inhibited cell proliferation based on live/dead cell staining, and CCK assay demonstrated a decrease in cell activity (decreased proliferation + increased cell death) to a level less than 30% of control cells after 24-hour treatment. ZnO NP increased ROS production up to 2.5 times that of control cells, increased cellular glutathione, and decreased SOD levels, suggesting an oxidative stress response.[114] In another study, MTT and LDH assays revealed dose-dependent toxicity of 26, 62, and 90 nm ZnO NP in Caco-2 cells (in serum-free media) from 6.25 to 100 µg/mL. All ZnO NP increased ROS production in cells and decreased intracellular glutathione levels. ZnO NP treatment also increased cell death. Propidium iodide staining to assess DNA content of cells revealed a slight S and G_2/M phase cell cycle arrest at 50 µg/mL doses of all ZnO NP (although most obvious with 26 nm ZnO NP) which may allow time for DNA damage repair.[115] These findings again suggest that ZnO NP toxicity is mediated through oxidative stress. A study in Caco-2 cells demonstrated a dose-dependent increase in LDH release and decrease in metabolic activity (WST-1 assay) following 24-hour treatment with 5, 20, and 80 µg/cm^2 of ZnO NP.[91] ZnO NP treatment also increased oxidative DNA damage and decreased total cellular glutathione content, again consistent with an oxidative stress-dependent

mechanism of toxicity. Based upon differences between data generated by the WST-1 assay and those generated by the LDH assay, the authors suggest the importance of the application of multiple toxicity assays in measurement of NP cytotoxicity.[91]

In a separate study, 50-70 nm ZnO NP-mediated toxicity in Caco-2 cells was detected as decreased viability by Neutral Red uptake assay at 10 μg/cm^2 and decreased cell proliferation by colony formation assay at 5 μg/cm^2, both in serum-free medium, and at 20 μg/cm^2 for both assays in serum-supplemented medium.[80] Since the Neutral Red assay measures ability of cells to retain dye in lysosomes requiring ATP to maintain an appropriate pH gradient, this suggests that ZnO NP may interfere with lysosomal integrity. ZnO NP treatment was also found to increase ROS production after 6 hours and increase IL-8 production after 6 and 24 hours at a dose of 1 μg/cm^2, suggesting that ZnO cytotoxicity is being induced through oxidative stress.[80]

In a study comparing responses of differentiated and undifferentiated Caco-2 cells to ZnO exposure, the investigators observed less than 10% dissolved Zn in the ZnO suspension. They observed no change in the soluble fraction of ZnO NP following *in vitro* simulation of digestion in pH and salt-based gastric and intestinal solutions.[54] ROS production was found to be induced by 20 μg/cm^2 ZnO NP but not following simulated digestion. The WST-1 assay revealed significant ZnO NP-induced toxicity in response to both 4- and 24-hour treatments at 5 μg/cm^2 in undifferentiated cells, but only after 24 hours and at 20 μg/cm^2 in differentiated cells.[54] This suggests a ROS-dependent mechanism of toxicity, but also demonstrates the importance of cellular differentiation state as well as the importance of NP surface chemistry, which was likely altered by *in vitro* digestion.

Not all investigations of ZnO interaction with intestinal epithelial cells have demonstrated the induction of oxidative stress. A study of toxicity of NP in drinking water demonstrated ZnO NP-mediated toxicity in Caco-2 cells at a dose of 100 μg/mL and in SW480 cells at 10 and 100 μg/mL in cell culture medium, but toxicity at lower doses of ZnO NP in synthetic freshwater (10 μg/mL in Caco-2 cells and 1 μg/mL in SW480 cells). ZnO NP treatment did not induce production of ROS in Caco-2 or SW480 cells, suggesting that toxicity may not be due to oxidative stress, but ZnO NP treatment did increase IL-8 production in Caco-2 cells.[79] Treatment of Caco-2 and RKO cells with ZnO NP revealed toxicity based on a formazan viability assay and the lethal concentration for 50% of cells (LC50) for ZnO NP was determined to be 27 μg/cm^2 in RKO cells and 28 μg/cm^2 in Caco-2 cells.[94] Pretreating with TNF-α to mimic inflammation did not significantly alter ZnO effects on cell viability. ZnO was localized within cells by TEM, but it was difficult to find and seemed to be associated with autophagic responses. This is consistent with lysosomal dissolution of ZnO. Gene expression studies revealed changes in genes responsible for metal metabolism, cellular stress responses, chaperonin proteins, and protein folding, but no indication of a proinflammatory or oxidative stress response.[94] Thus, although oxidative stress seems to often be responsible for ZnO NP-induced toxicity, this is likely in conjunction with other mechanisms. Our own experiments, for example, have demonstrated attenuation of mitochondrial activity in C2BBe1 cells following 24-hour exposure to 10 μg/cm^2 ZnO NP, as shown in Figure 5.[17] Regardless, it is clear that ZnO NP induce at least some level of toxicity in intestinal epithelial cells and thus this potential toxicity demands further study, particularly following ingestion *in vivo*.

ZnO incorporated into food products will be ingested as a component of the food matrix. Treatment of cells with ZnO NP in combination with fatty acids, common food components, was examined in Caco-2 cells.[116] ZnO NP alone decreased cell proliferation based on incorporation of the thymidine analog bromodeoxyuridine (BrdU) into DNA and decreased cell viability as

determined by MTT and WST-1 assays in a threshold-like pattern at a dose of 32 μg/mL. Addition of palmitic acid (PA) or a free fatty acid (FFA) mixture increased the potency of ZnO in the MTT and WST-1 assays but not the BrdU assay. Also at a dose of 32 μg/mL ZnO, most cells were observed to have detached from the cell culture plate. ZnO NP and PA together induced mitochondrial ROS production at 16 μg/cm^2, while ZnO NP + FFA decreased intracellular ROS production but had no effect on mitochondrial ROS. ZnO NP or FFA were able to destabilize lysosomes based on a decrease in LysoTracker fluorescence but this effect was slightly more pronounced with ZnO + PA or FFA at 4 μg/cm^2. These findings suggest that the interactions of ZnO NP with other food components will add to the complexity of NP-induced cellular responses and necessitates studies of NP in specific food matrices.

6.3.2 *In vivo* studies of ingested ZnO NP (Table 6S)

The studies of oral administration in rodents that have been performed thus far suggest that the proportion of ZnO NP absorbed from the intestines may be sufficient to cause toxic responses in various tissues. However, as with most NP that are administered orally, the fraction of the administered dose that is actually absorbed from the intestines is likely relatively small. A study in which hexagonal 40 nm ZnO NP were orally administered to rats for 13 weeks at doses of 134.2, 268.4, and 536.8 mg/kg body weight/day demonstrated decreased body weight in male but not female rats at the highest administration dose after 13 weeks. A dose-dependent increase in zinc concentrations in serum was also observed. Inductively coupled plasma-mass spectrometry (ICP-MS) analysis showed increased Zn concentration in liver and kidneys at the highest dose, but not in spleen or brain. The authors also observed a dose-dependent increase in Zn concentration in urine as well as feces, with significantly more Zn in feces than in any tissue or bodily fluid, suggesting that most of the ingested ZnO is not being absorbed in the GI tract.[110] Still, there are indications that ZnO NP do accumulate in tissues upon repeated oral administration.

A separate investigation in which 20 nm ZnO NP were administered to Sprague-Dawley rats at doses of 125, 250, and 500 mg/kg daily by oral gavage for 90 days demonstrated increased concentrations of zinc in the plasma in a dose-dependent manner after prolonged NP exposure.[117] Although accumulation of ZnO NP in solid tissues was not addressed in this study, this finding suggests the potential for progressive *in vivo* accumulation of orally administered ZnO NP.

While the previous studies have been directed primarily toward documentation of the *in vivo* distribution of ingested ZnO NP, other investigations have focused upon post-ingestion toxicity. In one such investigation, ZnO nano- and micron-sized particles were administered by oral gavage to Sprague-Dawley rats in a single bolus of 5, 50, 300, 1000, or 2000 mg/kg body weight. Analysis of animals 14 days post-administration demonstrated ZnO NP-mediated elevation of ALT and AST values, indicating liver damage.[118] Elevation of liver enzymes was not induced by micron-scale ZnO and this response was inversely related to dose, meaning that the greatest toxicity was observed at the lowest ZnO dose. The authors explain this as a possible effect of greater NP aggregation at higher doses resulting in decreased intestinal absorption and hence decreased dose to the liver. All treated animals showed greater incidence of microlesions in the liver and pancreas and the animals administered nano-ZnO also had lesions in the stomach and heart.[118]

In a separate study, Wistar rats were administered 20 nm ZnO NP by gavage daily at a dose of 100, 200, or 400 mg/kg body weight/day for 14 days. The investigators found that oral administration of ZnO NP increased serum inflammatory markers TNF-α and IL-6 as well as

LDH levels in a dose-dependent manner. Serum immunoglobulin G (IgG) was increased at all doses. They found reduced GSH in lung homogenates and also observed lung damage by histology and a dose-dependent increase in lung congestion.[119] This study supports the previously summarized reports that ingested nano-scale ZnO is being absorbed in the GI tract in sufficient quantities to reach other tissues and, in this case, cause significant lung damage. Although this investigation did not include other tissues, the findings provide a strong rationale for assessment of injury in other tissues as well.

Two additional investigations provide additional evidence for toxicity of ingested ZnO NP. Kim et al.[120] administered 100 nm negatively- (citrate-coated) or positively- (L-serine-coated) charged ZnO NP to Sprague-Dawley rats by daily oral gavage at doses of 31.25 mg/kg, 125 mg/kg, or 500 mg/kg for 90 days and observed decreases in blood biochemical parameters, including mean cell volume, mean cell hemoglobin, and mean cell hemoglobin concentration, as well as total protein and albumin levels, which may be due to ZnO-induced anemia. Histopathological changes were observed in the stomach, eye, pancreas, and prostate gland, again showing the potential for ZnO NP absorption across the GI epithelium and multiple-tissue toxicity *in vivo*.[120] In a separate study, oral administration of 50 nm ZnO NP to mice at a dose of 2.5 g/kg body weight induced increases in serum zinc concentration beginning 30 minutes post-gavage and increased levels of zinc in liver, spleen, and kidney. Increased levels of AST, ALT, and LDH in serum and histopathological lesions in the liver indicative of hepatic swelling and vacuolization in treated mice support ZnO NP-induced liver damage.[121] While these findings demonstrate that ingested ZnO NP have the potential to induce toxicity in multiple tissues, it should be noted that the doses administered in these experiments were almost certainly far in excess of what might be expected in normal human daily consumption.

6.4 Silver nanoparticles

Ag NP are increasingly incorporated in a multitude of food-related applications. When Ag NP are synthesized by chemical reduction, protective polymers are often used to stabilize the NP and keep the NP from aggregating.[122] This means that different preparations of Ag NP will have different surface properties based on the capping agent used, which may change the behavior of the Ag NP. Thus, thorough characterization of Ag NP is essential when they are used for research purposes.

6.4.1 Acute *in vitro* cellular toxicity (Table 7S)

Although Ag NP have a long history of use in medicine, there is recent evidence showing that Ag NP can be toxic to mammalian cells. With the incorporation of Ag NP into a rapidly increasing number of consumer products, it is necessary to investigate the effects of environmental exposure to Ag NP. Although there is some disagreement in the literature, it is thought that Ag NP dissolution is required for toxicity which is then directly mediated by Ag ions.[123] The discrepancies observed among various biological investigations may stem from the difference between dissolution of Ag NP outside of cells versus inside cells.

In many cell models, Ag NP have been shown to mediate toxicity through an oxidative stress-dependent mechanism, although Ag NP likely also mediate oxidative stress-independent intracellular effects. Exposure of human lung fibroblasts and glioblastoma cells to 6-20 nm Ag NP increased ROS production at a concentration of 25 μg/mL, induced mitochondrial injury at 25-100 μg/mL and DNA damage at 25-50 μg/mL, and induced G_2/M phase cell cycle arrest at 50-100 μg/mL.[124]

Similar toxicity has been observed in intestinal epithelial cells, although fewer studies have been conducted in these cells. Peptide-coated (L-cysteine L-lysine L-lysine) 20 nm Ag NP

decreased cell viability at concentrations of 5 μg/mL in undifferentiated cells and 25 μg/mL in differentiated cells, as determined by the CellTiter-Blue assay, and increased LDH release at 5 μg/mL in undifferentiated Caco-2 cells.[125] Impedance measurements showed an initial increase in proliferation over the first 6 hours of Ag NP treatment and then a decrease to a cell index near 0. Proliferating cells were much more sensitive to Ag NP than differentiated cells based on viability and cell enumeration. There was also a dose-dependent increase in ROS production at 3 μg/mL of 20 nm NP and 10 μg/mL of 40 nm NP, but no detectable micronucleus formation.[125] Thus, the observed toxicity may be due to induction of oxidative stress.

A study in which 7 nm Ag NP stabilized with Tagat TO and Tween 20 were subjected to simulated *in vitro* digestion in saliva for 5 minutes at pH 6.4, gastric juice for 2 hours at pH 2, and intestinal juice for 2 hours at pH 7.5 demonstrated a slight reduction in cytotoxicity induced by digested NP compared to undigested particles (toxicity induced by 5 μg/mL undigested NP and 10 μg/mL digested NP), as detected by impedance measurements. The authors suggest this is due to an increase in particle aggregation which is hindering Ag ion release. This study once again shows greater sensitivity of proliferating cells to Ag NP than differentiated cells.[55] This also highlights the importance of studying Ag NP toxicity in models representative of *in vivo* conditions such as digestion.

In a more mechanistic study in Caco-2 cells, <100 nm Ag NPs were shown to be internalized by cells and a dose-dependent decrease in cell viability starting at 10 μg/mL was demonstrated by MTT and trypan blue exclusion. No increase in ROS production was detected by 2',7'-dichlorofluorescein or Mitotracker assays, however, a decrease in total cellular glutathione levels and depolarization of the mitochondrial membrane potential are suggestive of oxidative stress. Ag NP treatment also induced activation of the stress-responsive gene Nrf2 and the expression of heme oxygenase-1 (HO-1), which is cytoprotective and controlled by Nrf2.[70] Pretreatment of cells with the antioxidant N-acetylcysteine attenuated Nrf2 and HO-1 mRNA expression, implying that these genes are regulated through an oxidative stress-dependent pathway. The investigators observed a baseline Ag ion concentration of 2.18% in cell culture medium, which increased to 3.47% 24 hours following addition of Ag NP, and suggested that Ag ions mediate much of the Ag NP toxicity through Ag ion release either outside of or within cells after internalization of Ag NP.[70]

Our own experiments have also demonstrated Ag NP-induced oxidative stress responses in intestinal epithelial cells. C2BBe1 cells were exposed for 24 hours to 23 nm citrate-stabilized Ag NP at a concentration of 10 μg/cm^2. Sytox Red and Annexin V-stained cells assayed by fluorescence flow cytometry demonstrated no evidence of necrosis or apoptosis, respectively. Mitochondrial activity as assessed by MTT assay was slightly attenuated. Importantly, continuous Ag NP exposure of cells plated at low density at concentrations as low as 0.25 μg/cm^2 completely inhibited proliferation, as shown in Figure 7. Analysis of cell cycle phase distribution of NP-treated cells by assay of DNA content demonstrated an accumulation of cells in G_2/M phase, indicating a block or delay in mitosis. A reduction in GSH/GSSG ratio in Ag NP-treated cultures implies an oxidative stress response, both of which are suggestive of DNA damage.[18]

Not all studies have demonstrated oxidative stress in response to Ag NP exposure. An investigation related to NP incorporation into paints demonstrated dose-dependent toxicity of nanosilver (25 nm spheres and 80-90 nm rods) in Caco-2 cells resulting in decreased viability and enzymatic/metabolic activity of cells starting at a Ag concentration of 27 μg/mL as measured by a tetrazolium-based assay, and increased cell necrosis. However, no induction of

ROS production was observed in response to 4-hour Ag NP exposure.[69] This may be due to limited sensitivity of the 2',7'-dichlorodihydrofluorescein diacetate assay to detect oxidative stress or may suggest that the observed toxicity is due to an oxidative stress-independent mechanism.

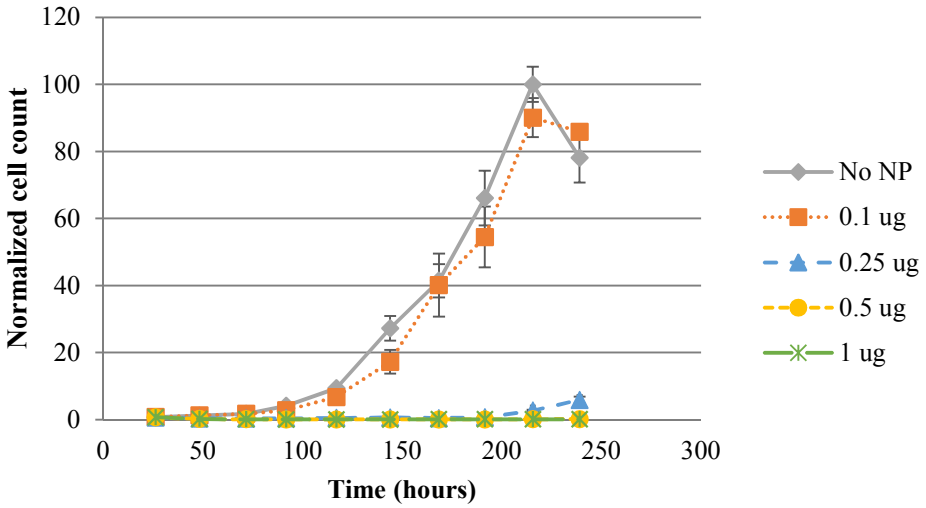

Figure 7: Growth curves compiled by counting proliferating C2BBe1 cells treated continuously with different doses (in µg/cm^2) of Ag NP daily for 10 days.[18] Reprinted with permission from Elsevier.

In contrast, several studies have shown limited Ag NP toxicity which may be due to differences in the Ag NP formulations or doses applied. An investigation into the biological impact of NP in drinking water demonstrated a trend of decreased viability of Caco-2 cells after 24-hour treatment with 100 µg/mL 20-30 nm Ag NP in cell culture medium and buffered synthetic freshwater, but the observed decrease was not significantly different than controls. That trend was not apparent in SW480 cells treated with Ag NP in cell culture medium, but there was significant toxicity of Ag NP in buffered synthetic freshwater.[79] Ag NP treatment did not induce ROS production in either cell type, but did induce significant IL-8 production in Caco-2 cells only. This may again indicate an oxidative stress-independent mechanism of toxicity.

Another study showed Ag NP (35 nm) internalization by Caco-2 cells and transport of these NP across the epithelial monolayer using confocal microscopy (detecting Ag particles by their reflectance) with no evidence of toxicity of Ag NP at 31.25 µg/cm^2 (50 µg/mL).[126] A study in the M cell co-culture model demonstrated minimal decrease in viability of cells at doses up to 50 µg/mL using 20, 34, 61, and 113 nm Ag NP. The investigators then treated cells with 5 and 25 µg/mL doses of the different sized Ag NP for 4 hours and analyzed whole-genome mRNA expression, demonstrating changes in expression of genes involved in the oxidative stress response, endoplasmic stress response, and apoptosis. Following treatment of cells with Ag ions only, they saw a similar gene expression response and thus concluded that Ag NP toxicity is likely mediated through ion release.[24]

Another study of Caco-2 cells exposed to 10 µg/mL of 90 nm Ag NP demonstrated Ag NP-mediated decrease in cell activity as measured by the CCK-8 assay (to about 60-70% of control with multiple doses of Ag NP), but no accompanying induction of cell death. The authors also found no ROS production or change in SOD levels, but they did observe a slight increase in

total GSH and thus antioxidant capacity in cells. Hence, the induction of oxidative stress in cells by Ag NP was, in this case, minor.[114] Yet another study in Caco-2 cells detected a decrease in cell viability by Alamar Blue reduction assay upon treatment with 20 nm citrate-stabilized Ag NP at a concentration of 10 μg/mL. Decreased dsDNA content was also observed, indicating attenuated cell proliferation and increased DNA damage induced by Ag NP treatment. No ROS production was detected, but the investigators did observe a decrease in mitochondrial membrane potential indicating mitochondrial injury.[71] They conclude that the toxicity is a result of mitochondrial membrane damage leading to mitochondrial dysfunction.

A study in the M cell co-culture model in which Raji B cells are cultured under a Caco-2 monolayer investigated toxicity of Ag NP administered in combination with several phenolic compounds that may be found in food matrices. Ag NP of < 20 nm alone were found to be cytotoxic at 30 μg/mL by MTT assay and to induce oxidative stress at 45 μg/mL. Decreased TEER measurements following Ag NP treatment and increased transport of Lucifer Yellow across the epithelial monolayer indicated disruption of the epithelial barrier. Tight junction disruption was indicated by changes in occluden and ZO-1 localization. Co-administration of the phenolic compounds quercetin and kaempferol were partially protective against Ag NP toxicity but resveratrol was not.[127] This once again suggests that assessment of NP toxicity will be more complex in the presence of the food matrix. A study using a 3D transwell co-culture model with Caco-2 cells cultured above THP-1 macrophages and MUTZ-3 dendritic cells demonstrated dose-dependent PVP-capped < 20 nm Ag NP-mediated toxicity based upon LDH release and decreased TEER values as well as IL-8 production at NP concentrations ranging from ~40 - >300 μg/cm^2.[82] The Caco-2 monolayer was more sensitive to Ag NP-mediated perturbation than Caco-2 cells in the co-culture system. However, co-cultures treated with IL-1β to simulate an inflammatory environment were more sensitive to Ag NP than quiescent cells.[82] This study suggests that the cell microenvironment is a major factor in Ag NP toxicity and highlights the need for *in vivo* studies to more thoroughly address this issue.

6.4.2 *In vivo* studies of ingested Ag NP (Table 8S)

Multiple *in vivo* studies have been conducted in which Ag NP have been orally administered to rodents or other animals to further investigate Ag NP toxicity. It has been shown that some Ag NP are absorbed across the intestinal epithelial barrier and disseminated to tissues and organs throughout the body.[128-132] It was determined from a case study in a woman suffering from argyria (prolonged contact with or ingestion of Ag), that 18% of an orally administered dose of Ag was retained,[133] i.e., not excreted in urine or feces, apparently remaining in her body for 30 weeks, and this has been used to estimate intestinal Ag NP absorption in humans.[134] Some studies have also shown Ag NP-induced intestinal epithelial damage following oral administration. In one such study, mice were administered 3-20 nm Ag NP at daily doses of 5, 10, 15, and 20 mg/kg body weight for 21 days and Ag NP were shown to decrease body weight at all doses. The Ag NP also damaged intestinal epithelial cell microvilli and intestinal glands, and increased numbers of inflammatory cells were observed in the lamina propria underlying the epithelium.[135] The investigators suggest that the damage to microvilli may have impaired nutrient absorption, leading to the decreased body weight.[135] Another study administering 60 nm Ag NP to Sprague-Dawley rats at doses of 30, 300, and 1000 mg/kg/day for 28 days demonstrated a dose-dependent accumulation of Ag NP in several regions throughout the intestines including the lamina propria of the small and large intestines. Ag NP treatment also caused greater numbers of goblet cells to release their mucus and there was a change in mucus

composition. Greater cell shedding from the tips of the villi was also observed.[136] Thus, damage to the intestines may be of particular concern in response to Ag NP ingestion.

Other studies have demonstrated damage to organs, including liver, after oral administration of Ag NP. Oral administration of 56 nm Ag NP to F344 rats at doses of 30, 125, and 500 mg/kg/day for 13 weeks induced a mild decrease in body weight of male rats over controls after 4 weeks and dose-dependent accumulation of Ag in testes, liver, kidneys, brain, lungs, and blood (all tissues examined). Dose-dependent increases in alkaline phosphatase and cholesterol levels for male and female rats at the highest dose and a greater incidence of bile-duct hyperplasia were observed following Ag NP administration,[137] suggestive of liver toxicity.

A separate investigation in which 22, 42, and 71 nm Ag NP were orally administered to mice at a dose of 1 mg/kg/day for 14 days demonstrated distribution of Ag NP to the brain, lung, liver, kidney, and testis. The authors also found increased levels of TGF-β in serum and increased B cell distribution after Ag NP treatment, indicating an inflammatory response. Mild liver and kidney toxicity as determined by increases in ALP and AST in the serum and mild inflammatory cell infiltration in the kidney cortex were observed after 28-day oral administration at 1 mg/kg Ag NP. This was accompanied by increases in IL-1, IL-6, IL-4, IL-10, IL-12, and TGF-β levels.[132] Thus, Ag NP oral administration may lead to liver and kidney toxicity as well as inflammatory responses.

Another concern is whether Ag NP will be transferred across the placenta and could be toxic to the growing fetus. This was examined in a study of pregnant rats. Females were orally administered 7.9 nm citrate-coated Ag NP at 250 mg/kg beginning 14 days before mating through the mating and gestation period until post-partum day 4. Ag concentration measured at day 4 post-partum demonstrated significant accumulation in neonatal liver, kidney, lung, and brain tissue.[138] Thus, the impact of Ag accumulation upon fetal and neonatal development clearly deserves further investigation.

Since Ag NP possess antimicrobial activity, ingestion of Ag NP may modify the composition of intestinal microflora. To address this issue, 2-7 nm Ag NP were added to drinking water at doses of 5, 15, and 25 mg/kg given to quail as a model animal for poultry over 12 days. The investigators found no major changes in quail gut microflora, but the highest dose of Ag NP did increase the population of lactic acid bacteria in the cecum.[139] Further experiments need to be done to confirm whether Ag NP can alter gut microflora, especially over longer exposure periods.

7. Post-ingestion nanoparticle interactions in intestinal disease

Increased intestinal permeability found in diseased intestines may have significant implications for the ability of ingested NP to traverse the intestinal epithelium and gain access to the circulation. NP may also have different effects when the intestine is already inflamed. Several *in vitro* models have been developed to investigate NP toxicity in an inflamed environment by treating cells with an inflammatory cytokine prior to treatment with NP.[82, 140] One study employing a co-culture model with THP-1 macrophages and MUTZ-3 dendritic cells embedded in a collagen scaffold beneath Caco-2 cells demonstrated that co-cultures treated with IL-1β to induce an inflammtory state were more sensitive to < 20 nm PVP-capped Ag NP-induced toxicity at a dose of 312.5 μg/cm^2 based upon LDH release and at 78.125 μg/cm^2 based on TEER measurements.[82] However, Ag NP-mediated IL-8 release was slightly decreased in the inflamed co-culture model compared to the non-inflamed co-culture.[82] TiO_2 NP (7-10 nm) did

not induce toxicity in this model.[82] Hence, these models may be helpful in determining differences in NP toxicity in the presence of inflammation.

However, these issues also need to be addressed *in vivo*. In addition to greater transport across an inflamed intestine with increased permeability, ingested NP may also exacerbate inflammation and disease. Clinical studies have been performed to assess whether Crohn's disease patient symptoms were alleviated by a low microparticle diet (which eliminated any sources of TiO_2 and silica among other natural contaminants or additives). An initial study with 18 participants showed efficacy of a low microparticle diet in decreasing Crohn's disease activity index,[141] but a larger study with 70 participants did not replicate these findings.[142] In the larger study, patients in the control group were supplemented with 5 mg/day TiO_2 to establish some baseline intake. This is well below the intake estimated by Weir et al.[12] (0.2-0.7 mg/kg body weight) and thus with increasing NP use in foods, it will be necessary to ensure that normal dietary NP intake is not harmful, particularly to populations with diseased intestines in whom it is still unclear whether NP may play a role in disease triggering or progression.

8. Biological interactions of ingested nanoparticles: Critical assessment of collective research

8.1 Variability in experimental outcomes

As mentioned earlier in this review and as documented in the summaries above, taken collectively, recent investigations of biological interactions of ingested nanoparticles have generated often inconsistent, and in some cases conflicting results. As an example, Figure 8 presents data generated by 17 separate studies of the impact of Ag NP upon *in vitro* cellular metabolic activity, as measured by tetrazolium or resazurin-bassed assays, organized in a dose-response format. Although the slope indicates a slight dose dependence in the responses, there is clearly wide variation in the data as quantitated by an R^2 value of 0.0646.

There are multiple potential sources of inconsistencies between studies including primarily NP dosage, physicochemical properties of NP used in experiments, specific assay methods and experimental protocols employed to generate data, and the type, differentiation state, and proliferative activity of the target cells. We begin with considerations of NP dosage.

Also, as previously noted, NP doses administered in experiments have varied over an extremely broad range, but nearly all doses greatly exceed those estimates of actual human consumption which are currently available. An estimated daily human consumption of silica NP, for example, was reported as 1.8 mg/kg.[14] Averaged over the epithelial surface of the small intestine (clearly an oversimplification) this translates to epithelial exposure levels of 0.42 – 0.063 μg/cm^2 as a function epithelial surface area estimate (Table 1). In contrast, silica NP doses administered to cells *in vitro* have been as high as 100 μg/cm^2 (or up to 1800 μg/mL), with adverse cellular effects reportedly beginning to appear at doses between 0.1 and 200 μg/mL. Silica NP doses administered in animal experiments have been as great as up to 2500 mg/kg/day × 84 days, with very few studies reporting doses ≤ 20 mg/kg/day, and adverse effects reportedly beginning to appear at doses between 5 and 1000 mg/kg.

Human TiO_2 NP consumption has been estimated at 0.2-0.7 mg/kg/day,[12] translating to intestinal epithelial exposure levels of 0.007-0.163 μg/cm^2 averaged over the epithelial surface of the small intestine. In contrast, TiO_2 NP doses administered to cells *in vitro* have been as high as 1000 μg/mL, with few studies reporting doses as low as 10 μg/cm^2, and adverse cellular effects reportedly beginning to appear at doses between 0.35 and 1000 μg/mL. Likewise, TiO_2 NP doses administered in animal experiments have been as great as 1000 mg/kg/day for 13 weeks, with

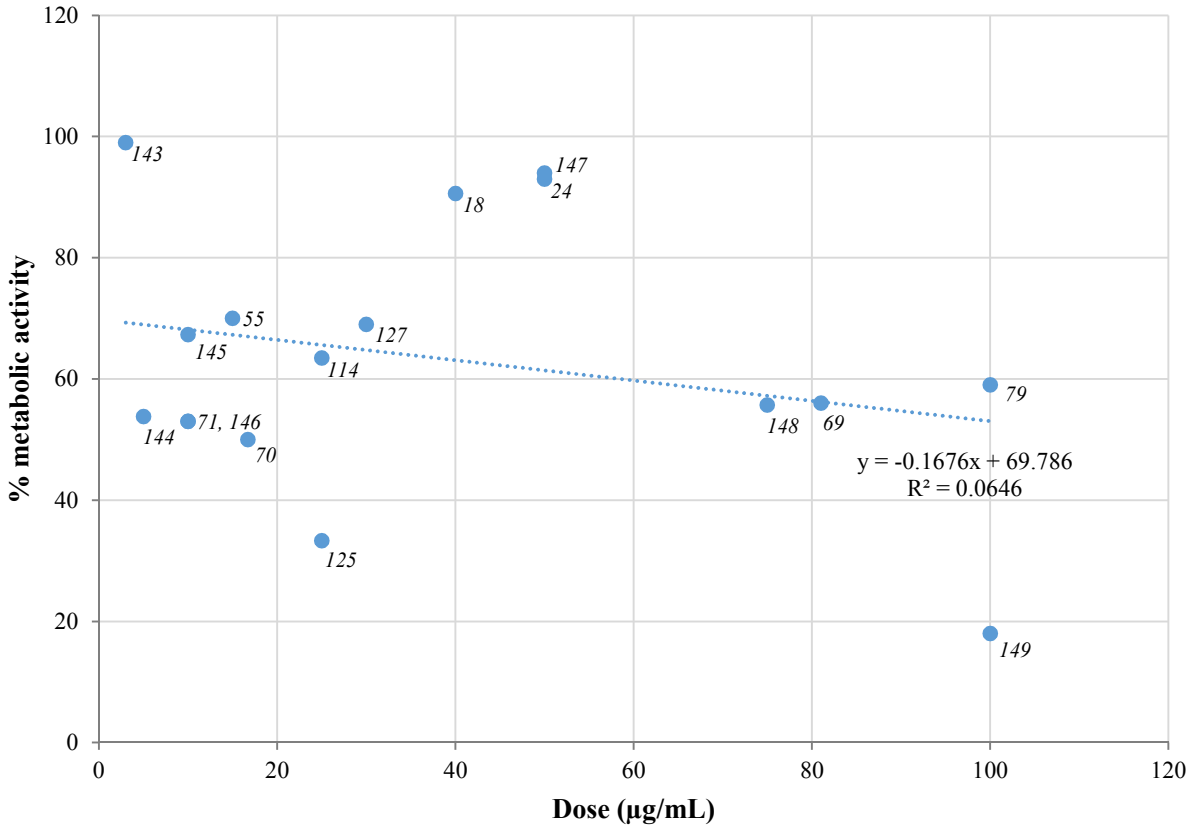

Figure 8: Data from 17 studies that investigated the effects of Ag NP treatment on metabolic activity of intestinal epithelial cell models *in vitro* were used to plot the relationship between Ag NP dose (in µg/mL) and percentage cellular metabolic activity as communicated in the literature. Data used were from tetrazolium or resazurin-based assays. Each data point is labeled with the reference from which the data was obtained.

one study reporting administration of a single dose of 5 g/kg. Adverse *in vivo* effects have been reported beginning at doses between 1 and 600 mg/kg.

It must be noted here that NP doses administered to cultured cells *in vitro* are often difficult to compare among different investigations. This owes to the fact that for many experiments NP dose is expressed in units of µg/mL and for others in units of µg/cm^2 of monolayer surface area. Without knowing the volume of culture medium in which the NP were incubated with cells and the monolayer surface area, it is not possible to compare doses expressed in µg/mL to those expressed as µg/cm^2, and that essential information is not often reported.

Although no estimates of human consumption of ZnO or Ag NP are currently available, considering that food-related ZnO and Ag NP use is for the most part currently limited to food packaging and the amount of NP leaching from the packaging into the food product is probably quite low, levels of GI tract exposure to these NP are likely to be lower than for silica and TiO_2. However, dosage in *in vitro* and *in vivo* experiments conducted with Ag and ZnO NP has generally been of similar magnitude as those conducted with silica and TiO_2. There are a few studies with lower doses with Ag NP, including our own, conducted at doses less than 1 µg/cm^2. Adverse cellular effects induced by ZnO NP have reportedly begun to appear at doses between

0.125 and 1000 µg/mL *in vitro* and at doses between 10 and 750 mg/kg *in vivo*, while Ag NP-induced adverse effects have been reported beginning at doses between 1.25 and 100 µg/mL *in vitro* and between 0.037 and 5000 mg/kg *in vivo*.

As with NP dosage, the specific type of NP used in experiments has varied widely. As described earlier in this review, NP characteristics such as material composition, shape, surface charge, solubility, and surface chemistry are all essential factors determining the nature of biological interactions of NP (Figure 3), in particular the manner in which NP interact with body fluids, cell and organelle membranes, cytoplasmic components, and biomolecules. In designing experiments and interpreting data generated by those experiments, it is likewise essential to recognize that ingested NP may be modified by the environment of the GI tract. A number of analytical protocols are available to provide a complete physicochemical profile of NP, including dynamic light scattering and transmission electron microscopy (particle size and shape), measurement of zeta potential (surface charge), X-ray diffraction (crystal structure), infrared spectroscopy (particle surface chemistry), and atomic absorption spectroscopy (particle dissolution), among others. Unfortunately, investigations of biological interactions of NP have not consistently thoroughly characterized NP, making comparisons among results of different studies and resolution of conflicting outcomes difficult.

Another potential source of variability of results among similarly designed experiments is the specific assay method employed to measure a given effect. For example, multiple techniques are available to measure cellular toxicity and/or cell death including Annexin V staining, LDH release assay, MTT assay, and several others. However, while all of these assays are directed towards quantitation of the same general outcome, they *directly* measure different cellular responses. Annexin V binds to phosphatidylserine which is exposed upon the outside of the cell membrane only during the process of apoptosis, while release of LDH from cells is an indication of membrane rupture, and the MTT assay directly measures mitochondrial activity. While all of these cellular responses are symptomatic of toxicity, the specific nature of the toxicity may not manifest equally in all assays. Several of the publications cited herein in which multiple assay methods were used concurrently, including our own work, demonstrate this point.[17, 18, 80, 125]

Finally, the specific type of cell used in *in vitro* experiments, its differentiation state, and its proliferative activity at the time of NP exposure can all profoundly affect the outcome. For example, we and others have demonstrated that rapidly proliferating, undifferentiated cells are more sensitive to NP-induced toxicity than their differentiated, non-proliferating counterparts.[18, 54, 55, 125] This is, in fact, a common theme in biology and a fundamental principle which guides many modes of cancer therapy. However, this information is often not included in reports of cellular responses to NP exposure.

8.2 Mechanisms of NP-mediated toxicity

For the purpose of this discussion, *toxicity* is defined *in vitro* as any adverse effect upon cell function or structure, and *in vivo* as any adverse histological or physiological changes. Of the many *in vitro* and *in vivo* studies reviewed herein that have documented some form of NP-induced adverse biological effect, some (but not all) have addressed specific mechanisms of NP-mediated toxicity.

Several studies of intestinal epithelial cell exposure to silica NP *in vitro* have demonstrated oxidative stress responses, including increases in intracellular ROS levels, DNA damage, and apoptosis. In at least one study, a decrease in mitochondrial activity was observed in the absence of evidence of oxidative stress, and in still other investigations, alternative

mechanisms have been documented in the absence of oxidative stress such as destabilization of lysosomes.

TiO_2 NP-induced oxidative stress and associated DNA damage and mitochondrial dysfunction have been well-documented in inhalation studies. However, these responses have been less consistently observed in intestinal models. Only a few of the many *in vitro* investigations of intestinal epithelial cell exposure to TiO_2 NP have provided evidence of intracellular increase in ROS levels. Alternative documented toxicological mechanisms associated with TiO_2 NP exposure include changes in intracellular electrolytes and increased free calcium levels. In addition, several *in vivo* studies of TiO_2 NP ingestion have demonstrated structural disruption of intestinal epithelial cell microvilli.

In contrast to silica and TiO_2, nearly all investigations of cellular exposure to ZnO and Ag NP have demonstrated or implicated oxidative stress responses including increased intracellular ROS levels, DNA damage, and mitochondrial dysfunction. In addition, a number of adverse effects have been observed in animal studies of responses to oral administration of ZnO NP or Ag NP, including indications of liver, kidney, and lung damage, widespread histological lesions in many organs, increases in serum inflammatory mediators, adverse changes in blood biochemical parameters, and anemia, among others. It must be noted, however, that the effects summarized in the preceding paragraphs have been induced by unrealistically massive doses of NP far in excess of those estimated in actual human consumption.

A final consideration in the assessment of the biological impact of ingested NP relates to their potential interactions beyond the limits of the GI tract. Several studies have demonstrated that ingested NP are able to traverse the intestinal epithelium, enter the circulation, and accumulate in liver, kidneys, and other organs. Our own experiments, summarized earlier in this review, demonstrated accumulation of ingested silica NP in liver, kidney, lung, spleen, and brain, indicating that ingested NP are able to not only enter the portal circulation, but are also able to traverse the liver and enter the systemic circulation, gaining access to any organ in the body. This potential broad distribution explains the findings reported in several studies of ingested NP-induced liver, kidney, and lung injury, as well as histologically observed lesions in pancreas, stomach, and heart, and other systemic adverse effects. It must be noted, however, such responses were induced by extremely large NP doses, exceeding estimated human exposures by as much as three orders of magnitude.

9. Summary

Despite dosage issues, as well as uncertainties and inconsistencies in experimental outcomes, it seems unlikely that either ingested silica or TiO_2 NP at estimated quantities of human consumption impose significant acute toxicity upon tissues of the GI tract or other organs. However, considering evidence that both of these NP are able to traverse the intestinal epithelial barrier and enter the circulation, NP accumulation in various organs over the long term may be cause for concern. While currently available data on the balance of post-ingestion NP retention and elimination in feces and urine are unclear, even a small fraction of ingested NP entering the circulation over decades of low-level consumption could potentially accumulate in tissues to levels that may disrupt organ function.

In contrast to silica and TiO_2 NP, in the context of acute toxicity ZnO and Ag NP may demand a greater level of caution. Data generated by the many investigations reviewed herein collectively indicate that Ag NP in particular are more acutely toxic at lower concentrations. For example, as described earlier in this review, we demonstrated that Ag NP completely inhibited

intestinal epithelial cell proliferation at concentrations as low as 0.25 μg/cm^2. If such inhibition of proliferation should occur in the stem cell populations in the intestinal crypts, disruption of the constant regeneration of villus epithelium could compromise the barrier function of the intestinal epithelium. In addition, if ingested Ag NP reach the umbilical circulation in pregnancy and cross the placenta, inhibition of fetal cellular proliferation could have serious consequences upon fetal development. A final source of concern with regard to Ag NP safety is the commercial availability of Ag NP suspension marketed as an unregulated dietary supplement promoting antimicrobial and/or immune-strengthening properties (as mentioned earlier in this review). Overuse of such a product may pose potential hazards.

We conclude this review with several recommendations for the path forward to improve our understanding of the biological interactions of ingested NP, particularly in the context of dose-dependent adverse effects, as a foundation for the establishment of rational regulation of NP incorporation into foods and food packaging. As pointed out earlier, many of the parameters which can dramatically affect the outcome of experiments, as well as the assay methods employed, are often not considered or not reported by investigators. Hence, standardization of experimental protocols including NP characterization, specific assay methods and dose ranges, as well as cell types and animal models would promote consistent collective interpretation of experimental results and conclusions among investigations. Recommendations for such protocol standardization have been generated by the NanoRelease Food Additive Project of the International Life Sciences Institute (ILSI) and can be found here: http://www.nanotechia.org/activities/nanorelease-food-additive-developing-methods-measure-release-nanoparticles-food

As mentioned earlier, the impact of ingested NP upon individuals with intestinal diseases, particularly inflammatory bowel diseases (IBD) in which the intestinal epithelial barrier function is compromised and absorption across the epithelium is dysregulated and frequently nonselectively enhanced, has been understudied and hence, deserves greater attention. Several animal models of IBD and dysregulated epithelial barrier function are available to support such work. Similarly, the potential for ingested NP to cross the placenta in pregnancy and, particularly in the case of Ag NP, to inhibit fetal cell proliferation, provides strong incentive for investigation of the impact of ingested NP upon fetal and neonatal development in pregnant animals. In addition, valuable information would be provided by a comprehensive investigation of the specific molecular interactions between NP and intestinal epithelial cells as well as cells of other organs in which ingested NP may accumulate. Data generated by such studies would have the potential to predict the specific NP properties that promote (or inhibit) their traversing the intestinal epithelial barrier and entry into the circulation, as well as the dose-dependent level of toxicity and specific nature of injury induced by ingested NP in cells in which they may accumulate (hepatocytes, for example). Such information would be highly valuable in the formulation of regulatory guidelines for NP incorporation into foods.

While the studies proposed above would provide valuable information, the experiments would necessarily be conducted in non-human animals (most likely rodents). It is well-established that there are significant differences between rodent and human GI tracts and other organs. Hence, data generated by a large, long-term, prospective human epidemiologic investigation correlating dietary intake (vegan versus normal American diet, for example) with health status could potentially provide definitive answers to questions regarding the safety/hazards of NP ingestion in foods. Until such information is available, common sense will have to suffice as a guide to dietary consumption.

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